RESEARCH ARTICLE

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

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The present work evaluates the effect of antimicrobial treatment of acute *Chlamydia psittaci* infection after inoculation in calves. Treatment of tetracycline alone was compared to treatment of tetracycline and rifampicin and no treatment. Reisolation of the pathogen was not possible in calves with tetracycline and rifampicin treatment, but pathogens could be detected by PCR in all animals, regardless of treatment. This study showed that elimination of *Chlamydia* spp. by antimicrobial treatment remains difficult and further research is necessary in this field.

ABSTRACT

Antimicrobial treatment of chlamydial infections is known to be of limited efficacy. In this study, effects of doxycycline (D), usually the drug of choice, were compared with the combined therapy of doxycycline and rifampicin (R) in a bovine model of respiratory *Chlamydia psittaci* infection. After intrabronchial inoculation of the pathogen, 30 animals were assigned to five groups (n = 6 per group): untreated controls, monotherapy with D (5 mg kg⁻¹ day⁻¹ or 10 mg kg⁻¹ day⁻¹), and combination therapy of D and R (600 mg day⁻¹). Treatment continued until day 14 post inoculation (d.p.i.). Clinical signs, inflammatory markers, and pathological findings confirmed successful infection in all animals. Reisolation of the pathogen was possible in 4/6 untreated animals and in 4/12 animals treated with D alone until 4 d.p.i., but in none of the calves of the two D + R groups. Pathogen detection was possible in all animals without significant differences among groups. Severity of disease and time course of its resolution, assessed by clinical and pathological findings as well as inflammatory parameters, were not significantly different between untreated controls and calves receiving D alone or in combination with R. Regardless of the treatment regimen, all groups recovered clinically and cleared the infection within 2 weeks.

Key words: *Chlamydia psittaci*; bovine model of respiratory infection; antimicrobial treatment; tetracycline; doxycycline; rifampicin

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INTRODUCTION

Chlamydiae are obligate intracellular bacteria causing a variety of diseases in hosts of different species, including humans.

The zoonotic bacterium Chlamydia (C.) psittaci (formerly: Chlamydiophila psittaci) is widespread in domestic poultry and psittacine birds, where it causes respiratory symptoms and general disease. C. psittaci is also present in the respiratory, intestinal, and genital tracts of cattle (Kauffold et al., 2007; Kemmerling et al., 2009) and has been shown to cause respiratory disease in an intrabronchial infection model in calves (Reinhold et al., 2012). When transmitted to humans, it causes or-nithosis or psittacosis, which is associated with respiratory and flu-like symptoms and can, as in birds, have any course from asymptomatic to lethal. Psittacosis is a zoonosis frequently acquired by individuals working with birds, for example 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).

Efficacious drug treatment of affected patients is still an unresolved issue. The same applies to other important human chlamydial infections, that is C. trachomatis (urogenital disease, ocular trachoma) and C. pneumoniae (persistent upper respiratory tract infections and community acquired pneumonia).

In both human and veterinary medicine, the treatment of choice for chlamydial infections was based on tetracyclines, followed by macrodilides and quinolones. All these antimicrobial substances reach high intracellular concentrations and were therefore considered as suitable drugs against the intracellularly located chlamydiae. Nevertheless, the success of antimicrobial treatment of chlamydial infections remains questionable. There are numerous clinical studies addressing this issue, but many of them were based on serological diagnosis only, and the success of treatment was associated to the improvement of clinical signs rather than elimination of the pathogen (Lipsky et al., 1990; Whatley et al., 1991; Block et al., 1995; File et al., 1997; Harris et al., 1998; Moola et al., 1999; Hammerschlag and Roblin, 2000; Patel et al., 2000; Finch et al., 2002; Carter et al., 2010). Another fact limiting the validity of these clinical studies is that they were usually not placebo controlled. Studies that included 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). There is also evidence of recurring chlamydial infections after the end of antibiotic treatment in humans (Bragina et al., 2001; de Vries et al., 2009) and animals (Reinhold et al., 2011).

It was recently shown in a cell culture model of persistent chlamydial infection that combined treatment with doxycycline and rifampicin was superior in reducing the number of chlamydial inclusions compared to doxycycline alone (Wolf et al., 2010). Based on these in vitro findings, effects of the traditional (i.e. doxycycline alone) and the newly proposed treatment regimen (i.e. combination of doxycycline and rifampicin) should be compared in a biologically relevant large animal model. Using a well-defined model of an experimentally induced acute respiratory C. psittaci infection in calves (Reinhold et al., 2012; Ostermann et al., 2013a, b, 2014), the present controlled and partially blinded study evaluated whether antimicrobial treatment with doxycycline and rifampicin in combination exceeds the effects of doxycycline administered at two doses appropriate for both humans and cattle. To assess success and pathophysi- logical effects of antimicrobial treatment regimens, clinical outcome, pathogen shedding and dissemination, reisolation, and pathogen detection in affected tissue, as well as host response in terms of local and systemic inflammation were used as readout parameters.

MATERIALS AND METHODS

Legal conformity and ethics statement

This study was carried out in strict accordance with European and National Law for the Care and Use of Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments and the Protection of Animals of the State of Thuringia, Germany (Permit Number: 04-004/11). All experiments were conducted in a containment of biosafety level2 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

In this prospective and controlled study, 30 conventionally raised calves (Holstein-Friesian, male) were included. Animals originated from one farm without any history of Chlamydia-associated health problems. In advance, the herd of origin was randomly checked for chlamydial antigen and antibodies by Chlamydiaceae-specific PCR and indirect ELISA, respectively, by the National Reference Laboratory for Chlamydioides. Calves were purchased at the age of 14–29 days weighing between 48.8 and 72.0 kg (60.5 ± 6.0; mean ± SD). After a quarantine period of at least 21 days and confirmation of a clinically healthy status, animals were included in the study.

Throughout the entire study, animals were reared under standardized conditions (room climate: 18–20 °C, rel. humidity: 60–65%) and in accordance with international guidelines for animal welfare. Nutrition included commercial milk replacers and coarse meal. Water and hay were supplied ad libitum. None of the given feed contained antibiotics.

Study design

At the age of 38–52 days, each calf was inoculated with 10^5 inclusion forming units (ifu) of C. psittaci strain DC15. Preparation of the challenge strain was described elsewhere (Reinhold et al., 2012). Bronchoscopic and intranasal inoculation was performed with 8 mL of the chlamydia-containing inoculum as described previously (Prohl et al., 2014). Groups were age matched, and weight (72.9 ± 6.7 kg, mean ± SD) of the animals did not differ statistically significantly between the groups at the time of inoculation (Kruskal–Wallis test, P > 0.98).

Twenty four calves (four groups of six calves each) underwent antimicrobial treatment, whereas six calves served as untreated controls (C). The four treatment groups were treated according to the following regimens: Group D1: 5 mg kg^-1 body weight (bw) day^-1 doxycycline (Centidox 100%, Eurovet Animal Health B.V., Bladel, Netherlands); Group D2: 10 mg kg^-1 bw day^-1 doxycycline; Group D3: 5 mg kg^-1 bw day^-1 doxycycline plus 600 mg rifampicin day^-1 (Eremfut i.v. 600 mg, Riemser Arzneimittel AG, Greifswald, Germany); and Group D4: 10 mg g^-1 bw day^-1 doxycycline plus 600 mg rifampicin day^-1. Doxycycline was applied orally with the milk replacer twice daily starting 36 h post inoculation (p.i.). Rifampicin was administered intravenously in 500 mL isotonic saline solution once daily over 30 min starting 48 h p.i. Treatment continued until 13 days p.i. (d.p.i.), and all animals were euthanized and necropsied 14 d.p.i.
Venous blood samples were drawn three times before and seven times after inoculation of the animals (see scheme in Fig. 1). After the beginning of antimicrobial treatment, blood was always drawn 18 h after treatment with doxycycline and 21 h after treatment with rifampicin.

Bronchoscopy was performed on all calves on 4 and 9 d.p.i. to collect bronchoalveolar lavage fluid (BALF) and bronchial brushings. One hour before inoculation, nasal and fecal swabs were taken. Fecal and pharyngeal swabs were obtained 4 and 9 d.p.i. Immediately before necropsy at 14 d.p.i., nasal and fecal swabs were taken.

Only the two veterinarians who performed the treatments and monitored the animals knew the assignment of the animals to the different treatment groups. Variables assessed in vivo (body temperature, respiratory rate, and heart rate) were measured values and can therefore be regarded as ‘objective’. All specimens examined ex vivo were given an individual ID according to the rules of our institute’s quality management system, thus disabling lab investigators to identify individual animals or treatment regimens.

Clinical scoring

All animals were clinically examined twice daily, starting 4 days before challenge. Observations were summarized using a scoring system as described previously (Reinhold et al., 2012).

Collection of swabs

Swabs were sampled at time points mentioned above. Fecal and nasal swabs (Heinz Herenz Medizinalbedarf GmbH, Hamburg, Germany) were obtained from the conscious animal, whereas pharyngeal swabs (Erich Eydam KG, Kiel, Germany) were sampled when the animal was under general anesthesia for bronchoscopy.

Blood collection and blood analyses

Venous blood was sampled from the jugular vein of all animals 7 days, 4 days and 1 h prior to inoculation and 1, 2, 3, 5, 7, 10, and 14 d.p.i. (Fig. 1).

Blood collection, white blood cell count, white blood cell differentiation, serum preparation, and quantitative detection of lipopolysaccharide binding protein (LBP) using an enzyme-linked immunosorbent assay (ELISA) were described previously (Ostermann et al., 2013a, b).

Necropsy, pathological evaluation and collection of tissue samples

Euthanasia of the calves and exenteration of the lung have been described in detail (Reinhold et al., 2012). Distribution, quality and extent of pulmonary lesions were recorded. Tissue samples collected from lung with and without macroscopic alterations as well as from mediastinal lymph node were fixed in neutral buffered formalin for histological and immunohistological examination or stored at −20 °C for PCR analysis. Aliquots of pulmonary lesions were collected for reisolation of C. psittaci and for bacterial culture to exclude other pulmonary pathogens. Samples of healthy lung tissue, muscle, and liver were stored at −80 °C to determine the tissue levels of the antibiotics. Then, a complete necropsy was performed.

Histology and immunohistology

Tissues collected at necropsy were fixed in 3.5% neutral buffered formalin for 24 h and embedded in paraffin. Hematoxylin and eosin-stained paraffin sections were evaluated for lesions. Chlamydiae were labeled in paraffin sections by indirect immunoperoxidase method using the antichlamydial-LPS antibody ACI-P500 (Progen, Heidelberg, Germany) as primary antibody and peroxidase-labeled sheep anti-mouse IgG (NA 931, GE Healthcare Europe GmbH, Freiburg, Germany) as secondary antibody.

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

Collection of bronchoalveolar lavage fluid

Isotonic, sterile, body-warm saline was used as flushing liquid. The BALF obtained by aspiration was immediately collected in siliconized glass bottles and stored on ice until further use. The BALF was sampled at 4 and 9 d.p.i. in the living animal (in vivo) and 14 d.p.i. in the exenterated lung (post mortem).

In vivo

Endoscopic sampling of BALF in the anesthetized animal was performed as described previously (Prohl et al., 2014). The BALF recovery in vivo was 83.1 ± 4.6% (mean ± SD) and did not differ significantly between the treatment groups.

Post mortem

BALF was obtained from freshly exenterated lungs immediately after exsanguination. At two different locations (Lobus medius,
Lobus caudalis sinister), three subsequent washes using 20 mL of body-warm isotonic saline solution for each instillation (in total 120 mL; 60 mL per lung lobe) were installed using glass syringes and a catheter inserted through the trachea. The BALF recovery post mortem was 60.8 ± 10.5 % (mean ± SD) and was not significantly different among treatment groups.

BALF cytology
Absolute number of leukocytes in BALF was determined by cell counting using improved ‘NEUBAUER chambers’. To quantify leukocyte populations, 400 mL of native BALF were placed on glass slides. The cellular sediments were stained according to PAPPENHEIM (HemaDiff, bioanalytic GmbH, Umkirch/Freiburg, Germany), and the percentages of leukocyte populations (lymphocytes, macrophages, giant cells, eosinophil granulocytes, segmented and banded forms of neutrophil granulocytes) were determined by counting a total of 200 cells. The absolute cell numbers of the leukocyte subsets in BALF were calculated based on the absolute number of leukocytes and the percentages of leukocyte populations.

Cells and supernatant of BALF were separated by centrifugation (300 g; 20 min). Analysis of total protein in BALF supernatant was described elsewhere (Reinhold et al., 2012).

Collection of bronchial brushings
On 4 and 9 d.p.i., bronchial brushings were obtained during bronchoscopy from the right lung, just caudal of the Bifurcatio tracheae as described previously (Prohl et al., 2014). The brush was then rinsed in stabilizing SPGA medium [containing sucrose, phosphatide substances, glucose, and bovine albumin (Bovarnick et al., 1950)]. Samples obtained at 4 d.p.i. were processed immediately; samples obtained at 9 d.p.i. were stored at −20 °C until further use.

Reisolation
Isolation of C. psittaci in buffalo green monkey (BGM) cells was performed from bronchial brushings (4 and 9 d.p.i.) and from altered lung tissue collected at necropsy (14 d.p.i.) according to standard procedures.

Detection of chlamydial DNA using quantitative real-time PCR (qrt-PCR)
Samples of macroscopically altered and macroscopically normal lung tissue, mediastinal lymph node, swabs (pharyngeal, nasal, and fecal), and venous blood were subjected to DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. Two aliquots of 1.0 μL of the final eluate were used as templates in qrt-PCR testing for the family Chlamydiaceae (Ehrlich et al., 2006). Ct values of less than 39.0 in both replicates measured were considered positive. When one aliquot showed a Ct value of < 39.0 and the other one was > 39.0, the sample was considered as doubtfully positive.

For the swabs and blood samples, additional testing for the species of C. psittaci was performed (Pantchev et al., 2009).

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

**Plasma preparation**
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/milliQ water 20/80 and then filtered on polyvinylidene fluoride (PVDF) 0.45 μm.

**Tissue preparation**
Pieces of 0.5 g of each sample were shaken with 250 μL milliQ water and 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 ammonium acetate 0.2 M and filtered on PVDF 0.45 μm.

**LC-MS-MS analysis**
The samples were analyzed using an Agilent1290 HPLC (Agilent Technologies) coupled with MS-MS detector 6460 (Agilent Technologies). The chromatographic column was Agilent Zorbax Eclipse Plus C18 (50 mm × 2.1 mm × 1.8 μm). Twenty microlitre of each sample were injected and separated using a gradient from 95% water 0.1% FFPA, and 5% acetonitrile to 100% acetonitrile and a flow rate of 0.4 mL min⁻¹ for 15 min.

For plasma quantification, the calibration was prepared in matrix with spiked plasma from 5 to 200 ng/mL⁻¹ with sulfaphenazole as internal standard.

For tissue quantification, the calibration was prepared in matrix with spiked tissue from 50 to 500 ng/mL⁻¹ with sulfaphenazole as internal standard.

The detection was performed using positive electrospray ionization (ESI+) with the MRM transitions: doxycycline: 445/428 and 445/154, rifampicin: 823/791 and 823/399, and sulfaphenazole (internal standard): 315/158 and 315/92.

In some tissues, only traces of rifampicin could be detected, for example muscle of two animals of the D₉₀R group and one animal of the D₀R group; lung of one animal of the D₉₀R group and one animal of the D₀R group. In these cases, the rifampicin level was assumed to be 50 ng·g⁻¹ for statistical calculations.

**Exclusion of coinfections**
The herd of origin was known to be free of bovine herpes virus 1 (BHV-1) and bovine virus diarrhea/mucosal disease virus (BVDV). Routine microbiological screening on the day after purchase revealed that all animals were negative for Salmonella infections (fecal swabs) and relevant enteric parasites (fecal smears). Two days after purchase, all animals were treated with a single intramuscular injection of marbofloxacin (Forcyl 160 mg/mL⁻¹, Vétoquinol GmbH, Ravensburg, Germany) at a dose of 10 mg·kg⁻¹ bw. Influence of marbofloxacin on the study could be ruled out as residues in most tissues have been reported to be below the limit of quantification already 192 h after treatment in calves (EMEA, 1999). To verify relevant respiratory copathogens, the presence of Mycoplasma, Pasteurella, or Mannheimia spp. was evaluated in nasal swabs taken immediately before challenge and before necropsy, as well as in lung tissue samples obtained during necropsy.
Neither Mannheimia haemolytica nor Mycoplasma bovis was detected in any sample. Pasteurella multocida was detected once in a nasal swab from 2 of 30 calves (7%), but never in any lung tissue sample. Based on serology, systemic infection with Mycoplasma bovis could be excluded (ELISA Kit for Mycoplasma bovis, Bio-X-Diagnostics, Jemelle, Belgium). Serology was also used to check for viral copathogens, that is bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI-3), adenovirus type 3, BHV-1, and BVDV (Bio-X respiratory penta ELISA Kit, Bio-X-Diagnostics).

During the quarantine period, all 30 calves included were checked serologically for antibodies against Chlamydiaceae (ELISA test; IDEXX GmbH, Ludwigsburg, Germany). All animals were serologically negative prior to inoculation.

### Statistical methods

R (R Development Core Team 2011) has been used for statistical evaluation of the data. In addition, the package Plotrix (Lemon, 2006) was used for computing the graphs. Due to the group size of \( n = 6 \), data was assumed not to be normally distributed and Kruskal–Wallis test was carried out to compare values of more than two different groups. Pairwise comparison with Mann–Whitney U-test with Bonferroni’s adjustment was used for post hoc testing. For comparison of two groups, Mann–Whitney U-test 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.

### RESULTS

#### Antibiotic levels

In plasma collected 1 hr prior to inoculation, no significant concentrations of doxycycline (< 5.0 ng/mL) or rifampicin (< 5.0 ng/mL) were present. After the beginning of treatment, antibiotic levels in plasma reached their maximum at 5 d.p.i. and were minimal at the end of the study (14 d.p.i.) in all treated groups. Interestingly, plasma concentrations of doxycycline were significantly higher in the Group D5R than in the Group D5 and significantly higher in the Group D10R than in the Group D10 (pairwise comparison, Mann–Whitney U-test, Bonferroni’s adjustment, \( P < 0.05 \)). There were no significant differences in rifampicin concentrations in blood between the groups D5R and D10R (Mann–Whitney U-test, \( P > 0.05 \)).

In tissue samples obtained at necropsy 14 d.p.i., doxycycline and rifampicin were detected in lung, liver, and muscle. Concentrations of doxycycline in lung tissue samples were clearly higher in the Group D5R than in the Group D5 and higher in the Group D10R than in the Group D10, but this difference could only be statistically secured for the two groups of calves treated with 10 mg doxycycline kg\(^{-1}\)day\(^{-1}\) (Kruskal–Wallis test, \( P = 0.01 \), and pairwise comparison, Mann–Whitney U-test, Bonferroni’s adjustment, \( P = 0.05 \)). Median and range of antibiotic levels in blood plasma and tissue for the different treatment groups on the same day (D5, D10, D5R, and D10R) are shown in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Time, d.p.i.</th>
<th>Doxycycline [ng mL(^{-1})]</th>
<th>Rifampicin [ng mL(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1425 (982.2, 1869.9)</td>
<td>4210 (1610.0, 5424.0)</td>
</tr>
<tr>
<td>5</td>
<td>1497 (1070.0, 1930.6)</td>
<td>4520 (1240.0, 2700.0)</td>
</tr>
<tr>
<td>7</td>
<td>1625 (1180.0, 2300.0)</td>
<td>4650 (1460.0, 3200.0)</td>
</tr>
<tr>
<td>10</td>
<td>1485 (1020.0, 2150.0)</td>
<td>4520 (1180.0, 1930.0)</td>
</tr>
<tr>
<td>Lung</td>
<td>850.6 (692.7, 1071.0)</td>
<td>4210 (1610.0, 5424.0)</td>
</tr>
<tr>
<td>Muscle</td>
<td>350.0 (263.8, 461.9)</td>
<td>4520 (1240.0, 2700.0)</td>
</tr>
<tr>
<td>Liver</td>
<td>330.0 (263.8, 461.9)</td>
<td>4520 (1240.0, 2700.0)</td>
</tr>
</tbody>
</table>

#### Comparative analysis

For comparison of two groups, Mann–Whitney U-test 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.
Table 2. Recultivation of *C. psittaci* from bronchial brushings. Number of animals in which recultivation was possible/total number of animals. C: untreated controls, D$_{5}$: 5 mg kg$^{-1}$ day$^{-1}$ doxycycline, D$_{10}$: 10 mg kg$^{-1}$ day$^{-1}$ doxycycline, D$_{60}$: 5 mg kg$^{-1}$ day$^{-1}$ doxycycline + 600 mg rifampicin, D$_{10}$R: 10 mg kg$^{-1}$ day$^{-1}$ doxycycline + 600 mg rifampicin.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D$_{5}$</th>
<th>D$_{10}$</th>
<th>D$_{60}$</th>
<th>D$_{10}$R</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 d.p.i.</td>
<td>4/6</td>
<td>2/6</td>
<td>2/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>9 d.p.i.</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>14 d.p.i.</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Detection of the pathogen

Reisolation

Isolation of *C. psittaci* from bronchial brushings was only possible at 4 d.p.i. In specimens of 4/6 untreated animals, *C. psittaci* could be recultivated in BGM cell culture. In both Groups D$_{5}$ and D$_{10}$, reisolation of the inoculated pathogen succeeded for 2/6 animals, whereas it failed for all specimens of animals treated with doxycycline and rifampicin (Table 2).

Swabs and tissue

Two weeks after inoculation, chlamydial DNA was detected using qrt-PCR in altered and macroscopically normal lung tissue, as well as in samples of the mediastinal lymph node of all treatment groups. The amount of genome copies per 1,000,000 cells did not differ statistically significantly between the groups (Kruskal–Wallis test, P > 0.05, Fig. 2).

In fecal, nasal, and pharyngeal swabs of animals from all groups, chlamydial DNA was detected sporadically until 14 d.p.i. using qrt-PCR (data not shown).

Blood

Despite antimicrobial treatment, DNA of *C. psittaci* was sporadically detected by qrt-PCR in the blood of animals from all groups from 2 d.p.i. until the end of the study at 14 d.p.i.

For instance, blood samples from one animal of Group D$_{5}$ proved positive at 2 and 10 d.p.i. In Group D$_{10}$, one animal was found positive on 2, 3, and 5 d.p.i. After the beginning of the rifampicin treatment, no animals tested positive for *C. psittaci* in the blood, but there were animals that were doubtfully positive at 3–14 d.p.i. in the D$_{10}$R group and at 5 and 7 d.p.i. in the D$_{5}$R group (Supporting Information, Table S1).

Clinical score

After inoculation with *C. psittaci*, all animals developed clinical signs of illness, such as coughing, swollen lymph nodes, elevated body temperature, forced breathing, elevated heart rate, and hyperemia of mucous membranes. The total clinical score was maximal at 2 and 3 d.p.i. and consisted to approximately 40% of respiratory signs. Health improved continuously until 7 d.p.i. in all animals, when the total clinical score returned to baseline level (Fig. 3). The rectal body temperature was within physiological levels before inoculation (38.7°C; 38.3–39.7, median; range) and reached its maximum at 2 d.p.i. (41.2°C; 40.4–41.7). All animals showed very similar clinical signs, and there was no significant difference in the total clinical score between the groups at any time point during the study (Kruskal–Wallis test, P > 0.05). Also, the daily weight gain during the 2 weeks of the study did not differ significantly among the groups (data not shown).

Systemic inflammation

White blood cells

Within the first 2 days after inoculation with *C. psittaci*, the total number of peripheral blood leukocytes increased 1.5-fold in all calves. At 3 d.p.i., the number of total blood leukocytes dropped to 90% of baseline level, which was reached again at 14 d.p.i. (Fig. 4a). The rise of blood leukocytes was mainly driven by neutrophilic granulocytes (segmented and banded forms), which increased threefold until 2 d.p.i. (Fig. 4b). Monocytes increased 1.5-fold from 1 d.p.i. until 7 d.p.i. and then dropped back to baseline level (data not shown). The changes in the peripheral blood count did not differ significantly between the groups after the beginning of the treatment (Kruskal–Wallis test, P > 0.05).

Acute phase reaction (LBP)

After challenge, LBP concentration in the peripheral blood increased in all animals. At its maximum at 11 d.p.i., the LBP level was 17-fold increased compared to baseline level. The latter was reached at 10 d.p.i. Comparison of LBP concentrations between the groups after the beginning of treatment did not reveal statistically significant differences (Kruskal–Wallis test, P > 0.05) (Fig. 4c).

Figure 2. Chlamydial genome copies in tissue 14 days after inoculation. The number of chlamydial genome copies per one million cells determined with quantitative rt-PCR 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 could be detected in altered lung tissue of all animals, whereas in some animals of all treatment groups no chlamydial DNA could be detected in normal lung tissue and in the mediastinal lymph node. Within the treatment groups, chlamydial genome copy numbers showed a high degree of variation. There were no statistically significant differences between the treatment groups for the amount of chlamydial DNA in the tissues sampled 14 d.p.i. (Kruskal–Wallis test, P > 0.55). Black lines: medians, C: untreated controls, D$_{5}$: 5 mg kg$^{-1}$ day$^{-1}$ doxycycline, D$_{10}$: 10 mg kg$^{-1}$ day$^{-1}$ doxycycline, D$_{60}$: 5 mg kg$^{-1}$ day$^{-1}$ doxycycline + 600 mg rifampicin, D$_{10}$R: 10 mg kg$^{-1}$ day$^{-1}$ doxycycline + 600 mg rifampicin. d.p.i. days post inoculation.
Figure 3. Total clinical score. All animals developed signs of an acute respiratory illness in response to the inoculation with Chlamydia psittaci. Clinical signs were maximal 48h to 3 days after inoculation in all animals. Regardless whether the animals were treated with doxycycline alone or in combination with rifampicin or left untreated, the total clinical score dropped back to baseline level within 8 days. No difference in the clinical score could be observed between the treated and untreated animals and between animals of the different treatment groups (Kruskal–Wallis test, \(P > 0.05\)). C: untreated controls, D\(_5\): 5 mg kg\(^{-1}\) day\(^{-1}\) doxycycline, D\(_{10}\): 10 mg kg\(^{-1}\) day\(^{-1}\) doxycycline, D\(_5\)R: 5 mg kg\(^{-1}\) day\(^{-1}\) doxycycline + 600 mg rifampicin, D\(_{10}\)R: 10 mg kg\(^{-1}\) day\(^{-1}\) doxycycline + 600 mg rifampicin. h, hour. d.p.i., days post inoculation.

Figure 4. Blood parameters. A rise of total blood leukocytes (a) in response to the inoculation with Chlamydia psittaci with maximal numbers on 1 and 2 d.p.i. was followed by a drop slightly below baseline level in all animals. This dynamic was mainly driven by neutrophilic granulocytes (b). The levels of LBP as a marker of inflammation (c) reached maximal values 2 and 3 d.p.i. in all animals, which means a delay of 24h compared to the numbers of blood leukocytes and neutrophilic granulocytes. The dynamic of LBP levels was the same as of the clinical score, and baseline level was reached at 10 d.p.i. Statistically significant differences between the treatment groups could neither be seen in the numbers of blood leukocytes and neutrophilic granulocytes nor in the LBP-levels in the blood serum (Kruskal–Wallis test, \(P > 0.05\)). C: untreated controls, D\(_5\): 5 mg kg\(^{-1}\) day\(^{-1}\) doxycycline, D\(_{10}\): 10 mg kg\(^{-1}\) day\(^{-1}\) doxycycline, D\(_5\)R: 5 mg kg\(^{-1}\) day\(^{-1}\) doxycycline + 600 mg rifampicin, D\(_{10}\)R: 10 mg kg\(^{-1}\) day\(^{-1}\) doxycycline + 600 mg rifampicin. d.p.i., days post inoculation.
Pulmonary inflammation

BALF cytology
Cell count per mL BALF was higher at 9 d.p.i. \((7.4 \times 10^{5} - 3.9 \times 10^{5} - 1.1 \times 10^{6})\) than at 4 d.p.i. \((5.6 \times 10^{5} - 2.3 \times 10^{5} - 1.4 \times 10^{5})\) and it was lowest at 14 d.p.i. \((3.4 \times 10^{5} - 5.5 \times 10^{4} - 7.5 \times 10^{5})\). Neutrophil numbers per mL BALF and percentage of neutrophiles (data not shown) were highest at 4 d.p.i. \((1.8 \times 10^{5} - 8.7 \times 10^{5} - 8.5 \times 10^{5})\) and decreased continually until 14 d.p.i. \((5.0 \times 10^{3} - 0.0 - 7.2 \times 10^{5})\). Neither cell count per mL BALF nor neutrophilic granulocytes per mL BALF and percentage of neutrophiles in the total amount of BALF cells differed significantly between the treatment groups at any time (Kruskal–Wallis test, \(P > 0.12\)) (Fig. 5a and b).

Concentrations of total protein in BALF
The concentration of total protein in the BALF supernatant remained constant from 4 d.p.i. \((179 \mu g/mL^{-1}; 104–495)\) until 14 d.p.i. \((177 \mu g/mL^{-1}; 53–429)\) and did not differ significantly between the treatment groups at any time (Kruskal–Wallis test, \(P > 0.22\)) (Fig. 5c).

Pulmonary lesions
Foci of bronchopneumonia and pleuritis were seen in all calves euthanized 14 d.p.i. after exposition to \(C.\) psittaci corresponding to the sites where the inoculum had been applied, in the caudal part of the left and right apical lobes, the medial lobe and the basal lobes. The circumscribed pulmonary lesions were characterized by necrotic centers and demarcated by fibrous connective tissue. The extent of lesions ranged between 0.9% and 8.9% (median: 4.3%) of pulmonary tissue in all animals without significant differences between the treatment groups (Kruskal–Wallis test; \(P = 0.38\)) (Fig. 6). Histological examination revealed subacute fibrinous bronchopneumonia. The ratio between zones of necrosis and regeneration varied between individual animals, but was not related to treatment groups. Chlamydial inclusions were detected by immunohistochemistry in all calves. Small granular labeling of chlamydia was found in areas with necrosis and few large inclusions in macrophages in zones of regeneration.

DISCUSSION

This study was designed to address the efficacy of antimicrobial treatment under conditions commonly used in human and veterinary medicine. Therefore, doxycycline was administered orally at two different concentrations, that is 5 mg kg\(^{-1}\) day\(^{-1}\) as the dose commonly used for the treatment of human patients and 10 mg kg\(^{-1}\) day\(^{-1}\) as a dose previously described for the use in calves (Papich 2011). The additional application of rifampicin had been shown to increase the antichlamydial effect of doxycycline in a cell culture model (Wolf et al., 2010) and was now to be tested in vivo. Calves at the age of 6–10 weeks have approximately the same weight as adult humans (60-80 kg). For this reason, rifampicin was applied at a dose commonly used for adult humans (600 mg day\(^{-1}\) per animal).

The beginning of treatment was chosen at 36 h after inoculation, when clinical signs became obvious, because this is the time point when human patients affected with psittacosis are most likely to seek medical advice and antibiotic treatment would be initiated.

Doxycycline, a tetracycline antibiotic, is known to enter tissues and cells easily (Riond et al., 1989). It inhibits the synthesis of bacterial proteins by binding to the 30S ribosomal messenger RNA and has bactericidal activity against a broad spectrum of gram-positive and gram-negative bacteria, thus rendering it a valuable antimicrobial drug against a number of bacterial infections in both human and veterinary medicine.

Rifampicin, a semi-synthetic antimicrobial drug, is bactericidal against many gram-negative and most gram-positive microorganisms through inhibiting bacterial DNA-dependent RNA polymerase (Thornsberry et al., 1983; Wehrli 1983). Its lipid solubility enables it to enter tissues and leukocytes very well, thus making rifampicin effective against intra- and extracellular microorganisms.

Figure 5. BALF. In the BALF, total cell count (a) was highest 4 and 9 days after inoculation with \(Chlamydia\) psittaci and dropped until 14 d.p.i. in all animals. The proportion of neutrophilic granulocytes (b) as a marker of lung inflammation was highest 4 d.p.i. and dropped continuously until 14 d.p.i. The concentration of total protein in the BALF supernatant (c) stayed within the same limits at all three sampling points. At 4 and 14 d.p.i. levels were above average in some animals of the groups treated with doxycycline and rifampicin, but statistically significant differences between the treatment groups could not be detected, neither for protein levels nor for total cell count and percentage of neutrophils in the BALF (Kruskal–Wallis test, \(P > 0.12\)). C: untreated controls, \(D_{5}\): 5 mg kg\(^{-1}\) day\(^{-1}\) doxycycline, \(D_{10}\): 10 mg kg\(^{-1}\) day\(^{-1}\) doxycycline, \(D_{R}\): 5 mg kg\(^{-1}\) day\(^{-1}\) rifampicin, \(D_{5R}\): 10 mg kg\(^{-1}\) day\(^{-1}\) doxycycline + 600 mg rifampicin, \(D_{10R}\): 600 mg rifampicin. d.p.i. days post inoculation.
extracellular pathogens (Accella 1978, 1983). Rifampicin is commonly used in human patients to treat mycobacterial infections and brucellosis, yet there is a potential of quickly developing resistance. Therefore, rifampicin is always administered in combination with another antimicrobial substance. The use of rifampicin in ruminants for treatment of tuberculosis and paratuberculosis has been proposed (St-Jean and Jernigan 1991), but this would contravene current legislation banning its use in food-producing animals in the European Union.

Concentrations of antimicrobial substances measured here in blood plasma, lung, liver, and muscle of treated calves meet the expectations based on current knowledge about metabolism and pharmacokinetics of doxycycline and rifampicin. Both drugs are metabolized in the liver and are substrates of the same cytochrome P450, CYP3A4 (Preissner et al., 2010). Therefore, the metabolism of doxycycline is limited in animals treated with doxycycline and rifampicin in combination compared to animals treated with doxycycline alone, because of the competition of both drugs for the metabolizing enzyme. This explains plasma levels of doxycycline in animals treated with doxycycline and rifampicin being significantly higher than in animals treated with the same dose of doxycycline alone. Rifampicin is a potent inducer of Cytochrome P450 enzymes (Rodriguez-Antona et al., 2000; Williamson et al., 2013), which explains the decrease in plasma levels of doxycycline and rifampicin toward the end of the study. 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).

Plasmatic concentrations of doxycycline in the Group D10 were in accordance with literature data for the same dose applied orally (Meijer et al., 1993). For doxycycline against C. psittaci strains from turkeys, a minimal inhibitory concentration (MIC) of 50–200 ng mL−1 was reported (Butaye et al., 1997). These values were reached in all analyzed samples of doxycycline-treated calves in this study. The MIC of rifampicin against the strain DC15 used in this study has been reported to be 0.016 μg mL−1 (Wolf et al., 2010), which was by far exceeded by concentrations measured in all tested samples of rifampicin-treated animals.

Despite the fact that our antimicrobial treatment led to doxycycline and rifampicin concentrations above the MIC values against C. psittaci, clinical and pathological findings as well as inflammatory parameters in C. psittaci-inoculated calves were not significantly influenced by treatment with doxycycline alone or in combination with rifampicin. Nevertheless, recrudescence of C. psittaci was not possible in animals treated with doxycycline and rifampicin, indicating an inhibition of chlamydial growth due to the treatment. In contrast, pathogen detection by PCR in lung and mediastinal lymph node at 14 d.p.i. was possible at the same level in all animals. Also, dissemination and shedding of pathogen DNA as detected by qrt-PCR in pharyngeal, nasal, and fecal swabs and venous blood samples took place to a similar extent in all treatment groups.

Elimination of chlamydiae by means of antimicrobial treatment was already reported to fail in both, animals and humans. Experimental infection of mice with C. trachomatis and treatment with doxycycline or azithromycin in combination with rifampicin 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 studies in human patients with C. trachomatis infection, antimicrobial treatment with doxycycline was not capable of eliminating the pathogen from the urinary tract of examined patients (Manhart et al., 2013; Pitt et al., 2013).

The reasons for the incomplete eradication of Chlamydia psittaci by antimicrobial treatment can only be speculated on. It has been hypothesized that chlamydiae develop persistent states characterized by aberrant morphology under antibiotic treatment and become metabolically inactive and are therefore not influenced by the treatment (Hogan et al., 2004). In vivo, this could be demonstrated under experimental conditions in mice (Phillips Campbell et al., 2012). Aberrant bodies were detected in the gut of C. suis infected pigs (Pospischil et al., 2009) and in human atherosclerotic tissue (Borel et al., 2008). So far, we do not have evidence for aberrant bodies of C. psittaci in the doxycycline and rifampicin-treated calves.

The reason for the failure of antimicrobial treatment often is antimicrobial resistance of the bacterium. For chlamydiae, resistance has only been considered as a problem in tetracycline-resistant C. suis strains in pigs (Lenart et al., 2001; Di Francesco et al., 2008; Borel et al., 2012). In other strains, it only occurred sporadically or in vitro (Dessus-Babus et al., 1998; Somani et al., 2000; Binet and Maurelli 2005; Kutlin et al., 2005). The C. psittaci strain DC15 used for inoculation of calves in the present study was confirmed to be sensitive against doxycycline and rifampicin (Wolf et al., 2010), so that antimicrobial resistance can be ruled out as a reason for the absence of a therapeutic effect in this study.

The fact that antibiotic treatment does not necessarily lead to faster remission of pathogen-induced lung lesions was described for subclinical pulmonary abscesses caused by Rhodococcus equi in foals. Two studies revealed the failure to accelerate spontaneous healing through antimicrobial therapy (Venner et al., 2012, 2013). This is in accordance with the findings in the present study: Animals of the different treatment groups neither showed differences in the clinical outcome nor in extension and morphology of the altered lung tissue recorded at 14 d.p.i. The LBP response did not differ between the treatment groups, thus indicating a comparable severity of inflammation in all groups. This suggests that all signs of inflammatory lung disease in the animals, that is clinical signs, elevated LBP levels, increased total cell count and neutrophils in blood and BALF, were due to the healing of Chlamydia-induced lung lesions and could, therefore, not be influenced by antimicrobial treatment, as treatment was initiated when lesions were already present.
The immune response of the animal was sufficient to restore the absence of clinical and inflammatory signs by the end of the study, as clinical score, LBP levels and blood count reached baseline levels at 10 d.p.i. at the latest in treated and untreated animals.

A limitation of the study might be the short observation period of 14 days after inoculation. It does not take into account the fact that kinetics of the clearance of the DNA of intracellular pathogens can take longer than 2 weeks after initial treatment, being a possible reason for the fact that differences in chlamydial genome numbers in blood and tissue could not be detected between treated and untreated animals in the present study. It may be hypothesized that a longer observation period would reveal differences in the amount of detectable DNA, but this remains subject to further studies.

Even though we have not been able to observe an impact on the course of disease in our study, we do not doubt that doxycycline is a useful treatment of C. psittaci infections. Before antimicrobial treatment was widely available, the mortality from psittacosis in humans was reported to be between 30% and 40% (Smith et al., 2010). Nowadays, the disease can be successfully treated with antibiotics if diagnosed correctly. Nevertheless, not all cases of human psittacosis require antibiotic treatment. There are plenty of reports of moderate and subclinical cases that resolved without antibiotic treatment (Heddema et al., 2006; Harkinezhad et al., 2007; Vanrompay et al., 2007; Branley et al., 2008; Verminnen et al., 2008). It is also widely accepted that, although psittacosis in humans is a notifiable disease in many countries, the actual case number is far underestimated, as many cases remain asymptomatic or very mild and, therefore, individuals do not seek medical advice. A study on experimentally C. psittaci-infected birds also showed convincingly that doxycycline was useful in the treatment of avian psittacosis (Guzman et al., 2010). Another very recent study showed that doxycycline treatment was successful in eradicating C. psittaci infections in clinically ill pigeon flocks (Krautwald-Junghanns et al., 2013). Based on our results, we recommend limiting the use of doxycycline treatment to cases where the host alone is not able to cope with the infection. The chosen time point for the beginning of treatment in this study (i.e. 36 h pi for doxycycline and 48 h pi for rifampicin) was too early to make any prediction on the further outcome of disease. A higher efficacy of a combination of doxycycline and rifampicin in treating a C. psittaci infection in vivo could not be detected in this study, but this still needs further evaluation in different models.

This study has again shown that elimination of Chlamydia spp. by means of antimicrobial treatment remains difficult and further research is necessary in this field. Yet it is another example that antibiotic treatment does not always make a difference in terms of clinical outcome, and cases where antibiotic treatment makes sense must be selected critically.

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STATEMENT
Part of the data was presented at the 54th congress of the ‘German Respiratory Society’, Hannover (Germany), March 20-23, 2013, at the ‘World Equine Airway Symposium’, Calgary (Canada), July 15–17, 2013, at the ‘European Respiratory Society Annual Congress’, Barcelona (Spain), September 7–11 and at the ‘Herbsttagung der Sektion Zellbiologie sowie Infektion und Tuberkulose in der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin’, Marburg (Germany), October 25–26, 2013.

Conflict of interest statement. None declared.

SUPPLEMENTARY DATA
Supplementary material is available at Pathogens and Disease online.

Table S1. Detection of C. psittaci with qrt-PCR in blood. Number of animals is given. Samples were analyzed in duplicate, two ct-values < 39.0 were considered positive, one ct-value < 39.0 and the other > 39.0 were considered doubtfully positive and everything else as negative. d.p.i.: days after inoculation.

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