

The Effect of Two Different Oxytetracycline Treatments in Experimental *Ehrlichia phagocytophila* Infected Lambs

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Stuen S, Bergström K: The effect of two different oxytetracycline treatments in experimental *Ehrlichia phagocytophila* infected lambs: Acta vet. scand. 2001, 42, 339-346. – The effect of 2 different oxytetracycline treatments in acute *E. phagocytophila* infected lambs was investigated. Twenty 5-month-old lambs of the Dala and Ryggia breeds were used. Ten lambs were inoculated intravenously with a stabilate of an ovine *E. phagocytophila* strain. On the third day of fever, 4 lambs were given long-acting oxytetracycline (Terramycin prolongatum vet[®], Pfizer) (20 mg/kg) intramuscularly and another 4 lambs were given short-acting oxytetracycline (Terramycin vet[®], Pfizer) (10 mg/kg) intravenously for 5 consecutive days. The lambs were examined for the presence of *Ehrlichia* infection by blood smear evaluation, polymerase chain reaction (PCR) and antibody titre against *E. equi*. One month after the last antibiotic treatment, 250 ml citrate blood from each of these lambs were inoculated into each of 10 susceptible lambs, which were observed during the following 6 weeks. The results indicate that oxytetracycline given in the acute stage of the infection may effectively terminate the development of fever, rickettsemia and weight reduction in *E. phagocytophila* infected lambs. No difference was observed between the 2 treatment groups. However, at least 3 of 8 antibiotic treated lambs (37.5%) were still infected with granulocytic *Ehrlichia* 3 months after treatment.

sheep; antibodies; Ehrlichia equi; persistence; tick-borne fever.

Introduction

Granulocytic *Ehrlichia* infections are observed in an increasing number of species of animals in Europe (Brouqui 1999). The tetracycline group has so far been the recommended antibiotics in treatment of granulocytic ehrlichiosis in both animals and man (Woldehiwet & Scott 1993, Dumler 1996). Tetracycline has been administered to cattle and sheep with *Ehrlichia phagocytophila* infection and has resulted in a rapid resolution of the fever (Venn & Woodford 1956, Foggie & Allison 1960, Brodie et al. 1988). In addition, successful field use of long-acting tetracycline as a prophylactic measure

against tick-borne fever (TBF) and tick pyaemia in lambs has been reported (Brodie et al. 1986).

One dose of short-acting oxytetracycline results in an abrupt fall in the temperature in TBF infected lambs and relapses are common (Scott 1976). However, a report by Brodie et al. (1988) indicates that relapses do not occur after long-acting oxytetracycline treatment. The purpose of this study was to investigate the effect of 2 different oxytetracycline treatments to clear the experimentally infected lambs from *E. phagocytophila* infection.

Materials and methods

Twenty 5 months old lambs of the Dala and Rygja breeds were used. The mean bodyweight of the lambs was approximately 40 kg at the start of the study. None of the animals had previously been on *Ixodes ricinus* infested pasture and all animals were kept indoors during the experiment. Ten lambs were inoculated intravenously on day 0 with 0.5 ml (containing approximately 1.3×10^6 infected cells pr. ml) of a whole blood dimethyl sulphoxide stabilate of an *E. phagocytophila* strain originally isolated from a sheep (Stuen *et al.* 1992). On the third day of fever, day 6 post inoculation, 4 lambs (LAT-group) were given long-acting oxytetracycline (Terramycin prolongatum vet[®], Pfizer) (20 mg/kg) intramuscularly and another 4 lambs (T-group) were given short-acting oxytetracycline (Terramycin vet[®], Pfizer) (10 mg/kg) intravenously for 5 consecutive days. Six weeks after the primary inoculation, the infected lambs were treated intramuscularly with 2 mg dexamethasone (Vorenvet vet[®], Boehringer Ingelheim) daily for 4 days. On the first day post treatment, each of 10 susceptible lambs was inoculated intravenously with 250 ml citrate-blood taken directly from the previously inoculated animals; each of the 10 lambs receiving blood from only one donor lamb. The clinical, haematological and serological reactions of the recipient lambs were observed during the following 6 weeks.

Rectal temperatures were measured daily in all lambs at the same hour in the morning during the whole experimental period of 3 months. In addition, the temperatures were also measured 2 h and 6 h after oxytetracycline treatment in the infected lambs. The incubation period was defined as the period between inoculation and the first day of fever ($\geq 40.0^\circ\text{C}$), and the duration of fever was recorded as the number of days with a body temperature of at least 40.0°C . EDTA-blood samples were collected on days 0,

2-10, 14 and thereafter weekly for over 2 months. In addition, EDTA-blood samples were collected 2 h and 6 h after oxytetracycline treatment in the infected lambs and also if the rectal temperature in any individual lamb was above 40°C . Hematological values including total and differential leucocyte counts were determined electronically (Technicon H1[®], Miles Inc., USA) and blood smears were prepared and stained with May-Grünwald Giemsa. Four hundred neutrophils were examined on each smear by microscopy and the number of these cells containing *Ehrlichia* inclusions was recorded. In addition, these blood samples were also tested for granulocytic *Ehrlichia* infection by a polymerase chain reaction (PCR) technique according to Stuen & Olsson Engvall (1999).

Serum samples from all lambs were collected each week during the experimental period. An indirect immunofluorescence antibody assay (IFA) was used to determine the antibody titre to *E. equi* (Artursson *et al.* 1999). Briefly, 2-fold dilutions of sera were added to slides pre-coated with *E. equi* antigen (Protatek International and Organon Teknika). Bound antibodies were visualized by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-sheep immunoglobulin (Cappel, Organon Teknika). Sera were screened for antibodies at dilution 1:40. If positive, the serum was further diluted and retested. A titre of 1.6 (\log_{10} reciprocal of 1:40) or more was regarded as positive.

All experimental lambs were weighed weekly. Statistical calculations were done by Statistix[®], version 4.0 (Analytical Software).

Results

The *E. phagocytophila* inoculated lambs showed infected neutrophils on day 3 (Table 1) and fever on day 4. On the day after antibiotic treatment (day 7) the temperature had dropped to preinfected levels (Table 2). Three of the oxytetracycline treated lambs showed neutrope-

Table 1. The mean percentage of infected neutrophils in 10 *E. phagocytophila* infected lambs. Eight of the lambs were treated with antibiotics on day 6 after the primary inoculation; 4 lambs (LAT-group) were treated with long-acting oxytetracycline (20 mg/kg), and 4 lambs (T-group) were treated with short-acting oxytetracycline (10 mg/kg) for 5 consecutive days. Two lambs were untreated controls.

	Day 0	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9*
LAT-group	0	<1	3.1	67.7	54.7	12.7	1.5	<1 #
T-group	0	<1	11.4	65.7	58.5	13.0	1.8	<1 #
Controls	0	<1	4.8	55.5	49.0	51.0	46.5	33.5

one lamb was found infected.

* one lamb in each of the treated groups was found positive by PCR analysis on day 10.

nia ($<0.7 \times 10^6$ cells/l) for 2 days, i.e. on days 9 and 10; one lamb in the LAT-group and 2 in the T-group, respectively. In contrast, the 2 infected control lambs were neutropenic for 8 days, i.e. on days 12-20.

No difference in clinical reaction was observed between the lambs treated once with long-acting or for 5 days with short-acting oxytetracycline. The mean body temperature was reduced significantly 6 h after oxytetracycline treatment (Student's paired *t*-test, $p < 0.03$) (Table 2). No relapse of fever was observed in the treated lambs during the rest of the experimental period, except in one lamb at day 53.

The number of infected neutrophils (rickettsemia) in the antibiotic treated lambs was reduced compared with the control lambs one day after oxytetracycline treatment. A significant

reduction (Student's paired *t*-test, $p < 0.0005$) in the absolute number of infected neutrophils was recorded 2 h after oxytetracycline treatment (Table 2). In addition, the morulae in stained blood smears from oxytetracycline treated lambs were more dense and dark blue compared to untreated controls the first days after treatment.

Infected neutrophils were observed in all lambs 2 days after antibiotic treatment, but were absent 4 days after treatment. However, PCR analysis showed that 2 lambs were positive also on that day. *Ehrlichia* infection was not detected in the peripheral blood of the treated lambs during the next month, neither by blood smear examination nor by PCR analysis.

When blood from previously infected and antibiotic treated lambs was inoculated into recip-

Table 2. Mean temperature and percentage and absolute number of infected neutrophils (\pm SD) in 8 *E. phagocytophila* infected lambs at different hours after oxytetracycline treatment, and in 2 untreated control lambs. The lambs were given either long-acting oxytetracycline or 5 days short-acting oxytetracycline.

	0 hour		2 hours		6 hours		24 hours	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Temperature ($^{\circ}$ C)	41.23 ± 0.198	41.40 ± 0.100	41.33 ± 0.254	41.55 ± 0.150	40.53 ± 0.299	41.60 ± 0.200	39.11 ± 0.417	41.40 ± 0.200
% infected neutrophils	56.6 ± 4.24	52.5 ± 4.50	38.1 ± 3.79	49.5 ± 8.50	34.4 ± 3.50	54.0 ± 2.00	12.9 ± 2.47	46.5 ± 7.00
Number of infected Neutrophils (10^9 cells/l)	1.58 ± 0.451	1.55 ± 0.410	1.04 ± 0.366	1.53 ± 0.280	1.06 ± 0.357	1.71 ± 0.535	0.33 ± 0.131	1.96 ± 0.100

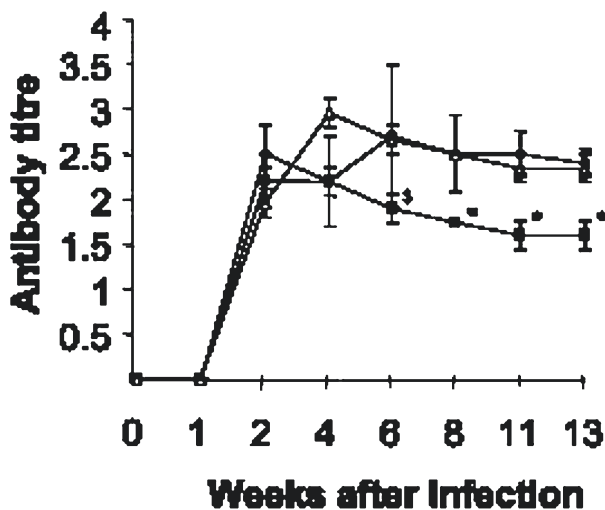


Figure 1. Antibody titre (\log_{10}) to *E. equi* in 10 lambs inoculated with *E. phagocytophila* on day 0. Eight lambs were treated with oxytetracycline on day 6, while 2 lambs were untreated controls. The treated lambs were either given long-acting tetracycline or 5 days short-acting tetracycline. One of the treated lambs did not seroconvert (not shown)

- ◆ Treated and still with rickettsemia 1-3 months after treatment (3 lambs)
- Treated and without rickettsemia 1-3 months after treatment (4 lambs)
 - § only 3 lambs * only 2 lambs
- △ Infected controls (2 lambs)

ient lambs 42 days after primary inoculation and one month after oxytetracycline treatment, 2 of 8 recipient lambs reacted with fever, rickettsemia and seroconversion. One of these lambs had been inoculated with blood from one lamb in the LAT-group and the other with blood from the T-group. The incubation period was 4 and 5 days, respectively, and a positive titre was observed within 14 days. However, rickettsemia or seroconversion was not observed in the other recipient lambs. In contrast, the 2 control lambs were found infected with *E. phagocytophila* on day 42 by blood inoculation.

In addition, one donor lamb in the original T-group that was found *Ehrlichia* negative on the day of blood transfusion by blood smear examination, PCR analysis, and blood inoculation,

reacted with fever and rickettsemia 11 days later (maximum temperature: 41.5°C, duration of fever: 5 days, maximum rickettsemia: 73%, nadir of neutropenia: 0.51 G/l, duration of neutropenia: 3 days). The antibody titre increased from 1:40 to 1:1280 within 14 days, but no weight reduction was observed. None of the other inoculated and antibiotic treated lambs reacted with fever and rickettsemia as a result of cortisone treatment and blood transfusion, nor did the control lambs.

The antibody response to granulocytic *Ehrlichia* is shown in Fig. 1. Apart for one lamb in the LAT-group, all inoculated and oxytetracycline treated lambs reacted with seroconversion. No difference in the antibody titre was observed between the lambs in the 2 antibiotic

treated groups (data not shown). Four weeks after the primary inoculation, the mean reciprocal antibody (\log_{10}) titre was 2.20 ± 0.360 and 2.96 ± 0.151 in the treated and untreated control group, respectively.

One month after antibiotic treatment, 6 out of 8 lambs had a positive antibody titre, while 2 months later only 5 of these lambs had a positive titre. At that time, 3 of these 5 positive lambs and the control lambs had a mean antibody titre of 2.41 ± 0.142 and 2.35 ± 0.151 , respectively, and were also found infected by blood smear examination and PCR analysis. In contrast, the 2 other seropositive lambs had a mean antibody titre of 1.62 ± 0.00 three months after the primary infection, and were, together with the seronegative lambs, not found infected with *E. phagocytophila* at that time (Fig. 1).

The weekly weight gain in the infected and antibiotic treated lambs was reduced by less than 0.8 kg between the first and second week after the primary infection. In contrast, the weekly weight gain in the untreated control lambs was reduced by 5.5 kg in the same period. Three months after the primary infection the total weight gain had been 7.3 ± 2.86 kg and 4.0 ± 0.00 kg in the oxytetracycline treated lambs and untreated controls, respectively.

Discussion

In the acute *E. phagocytophila* infected lambs, the temperature returned to normal within 24 h after oxytetracycline treatment. However, the temperature had already dropped significantly 6 h after treatment. Earlier investigations on *E. phagocytophila* infected cattle have shown that the temperature drops to normal levels 12 h after intravenous oxytetracycline treatment (Venn & Woodford 1956). In TBF-infected dwarf goats the temperature dropped 3 hours after oxytetracycline treatment and was normalised within 6 h (Anika *et al.* 1986).

Pyknotic inclusions were found the first days

after antibiotic treatment. This is in accordance with an earlier study in goats where pyknotic spots were observed within infected cells approximately 8 h after treatment with oxytetracycline (Anika *et al.* 1986).

In the present study, rickettsemia was significantly reduced within 2 h after the treatment. By PCR analysis 2 of the 8 lambs were found infected 4 days after the antibiotic treatment had started. The sensitivity of the PCR technique may have been improved by use of a nested PCR (Barlough *et al.* 1996). However, nested PCR was not available in this study.

No difference in clinical, hematological or rickettsemia reactions were observed between the 2 treatment groups. The mode of action of tetracyclines is the inhibition of bacterial protein synthesis. A serum concentration of $0.5 \mu\text{g/ml}$ has been taken as a minimum inhibitory therapeutic concentration of oxytetracycline for most susceptible pathogens (Davey *et al.* 1985, Escudero *et al.* 1994). According to the manufacturer's recommendation, this plasma concentration is maintained for approximately 84 h in sheep after intramuscular injection with long-acting oxytetracycline (20 mg/kg). Similarly, after intravenous administration of a dose of 10 mg/kg in sheep, this plasma concentration is maintained for 24 h. In the present trial, the inhibitory concentration may therefore have been maintained for at least 6 days in the lambs given short-acting oxytetracycline. The inhibitory therapeutic concentration of the rickettsiae *E. phagocytophila* is, however, not known.

The results demonstrate that oxytetracycline is efficient against *E. phagocytophila* in sheep as previously shown by Brodie *et al.* (1988). However, oxytetracycline could not totally clear the infection from all lambs. At least 3 of 8 lambs were still infected with *E. phagocytophila* one month after oxytetracycline treatment. The efficacy of tetracycline derivatives to eliminate

other *Ehrlichia* infections, for instance an *E. canis* infection, remains controversial. One experimental study indicates that 6 weeks of daily doxycycline treatment was not sufficient to clear *E. canis* from subclinically infected dogs (Harrus *et al.* 1998).

Two hundred and fifty ml blood from one lamb was not infective on the day of blood transfusion, although the lamb showed infection in the circulating blood 11 days later. Earlier study on experimental *E. phagocytophila* infection in lambs has shown that one infected neutrophil is enough to transmit the infection intravenously (Stuen & Artursson 2000). The present result therefore indicates that circulating blood is not always infective in persistently TBF infected lambs, and is in accordance with earlier observations that infection in the peripheral blood varies in *E. phagocytophila* infected animals (Foggie 1951, Stuen *et al.* 1998).

This result also demonstrates the diagnostic problems in verifying a granulocytic *Ehrlichia* infection in persistently infected lambs, since both stained blood smear investigation, PCR analysis of peripheral blood and blood inoculation trials could be negative. In addition, stress induced by cortisone treatment and heavy blood losses was not enough to cause relapses of fever in 4 of 5 infected lambs, as also observed earlier (Stuen *et al.* 1998). Sheep should therefore be examined several times before they eventually may be declared infected or not with granulocytic *Ehrlichia*.

Except for one lamb, all oxytetracycline treated lambs reacted with a positive antibody titre against *E. equi*. Strong serological cross-reactions between *E. equi*, *E. phagocytophila* and the agent causing human granulocytic ehrlichiosis (HGE) have been reported (Dumler *et al.* 1995, Nicholson *et al.* 1997, Pusterla *et al.* 1997). The sensitivity of the present test may have been increased by use of a more proper antigen (Bjoersdorff *et al.* 1999, Walls *et al.*

1999), but unfortunately *E. phagocytophila* was not available for use as antigen in this study.

Although few lambs are involved in this study, the present results may indicate that the antibody titres are higher in persistently infected lambs than in lambs that have been cleared of the infection. Similar observations have been reported in *E. equi* infection in horses (Nyindo *et al.* 1978) and in *E. canis* infection in dogs (Iqbal & Rikihisa 1994). However, the antibody titre is not a good criterion for assessing recovery from the persistent state of the *E. phagocytophila* infection, both due to individual variation in immune response and persistence of antibody titre in infected lambs (Scott 1981, Paxton & Scott 1989, Stuen *et al.* 1998). Earlier studies also show that the IFA-test can not be used in assessing clearance of *E. canis* after antibiotic treatment, since dogs remain IFA positive for months after clearance of the organism (Iqbal & Rikihisa 1994, Harrus *et al.* 1998).

In the present investigation, the oxytetracycline treated lambs gained more weight than untreated lambs and were 3.3 kg heavier than the inoculated and untreated controls 3 months after the primary infection. No statistical analysis could be done on this material due to a low number of animals. However, this result is in accordance with earlier observations in both calves (Brodie *et al.* 1986, Cranwell 1990) and lambs (Stuen *et al.* 1992).

In conclusion, the present study indicates that oxytetracycline given in the acute stage of the infection could effectively abort the development of fever, rickettsemia and weight reduction in *E. phagocytophila* infected lambs. However, some lambs were still infected with granulocytic *Ehrlichia* after 5 days of oxytetracycline treatment. Further investigations are needed to clarify the appropriate regimen using oxytetracycline to clear lambs from an *E. phagocytophila* infection.

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Sammendrag

Effekten av oxytetracyclin-behandling av lam infisert med Ehrlichia phagocytophila (sjodogg).

I denne undersøkelsen ble oxytetracycliner gitt til lam som var eksperimentelt infisert med *E. phagocytophila* (sjodogg). Tyve 5 måneder gamle Dala- og

Rygja-lam ble brukt. Ti lam ble podet intravenøst med *E. phagocytophila*-infisert blod. På den tredje feberdagen ble 4 lam behandlet intramuskulært med langtidsvirkende tetracycliner (Terramycin prologatum vet[®], Pfizer) (20 mg/kg), mens 4 andre lam ble behandlet intravenøst i 5 påfølgende dager med korttidsvirkende tetracycliner (Terramycin vet[®], Pfizer) (10 mg/kg). To sjodogginfiserte lam ble ikke behandlet. Lammene ble undersøkt med hensyn på en sjodogginfeksjon ved hjelp av blodutstryk, PCR (polymerase chain reaction) og antistoffer mot *E. equi*. En måned etter antibiotikabehandlingen, ble 250 ml citratblod fra hvert av disse lammene podet intravenøst på hvert av 10 mottagelige lam. Resultatet av undersøkelsen tyder på at oxytetracycliner gitt i den akutte fasen av en sjodogginfeksjon effektivt reduserer feber, rickettsemi samt vektapp hos infiserte lam. Ingen forskjell ble observert mellom de 2 behandlede gruppene. Imidlertid var minst 3 av de 8 antibiotika-behandlede lammene (37.5%) fortsatt infisert med *E. phagocytophila* 3 måneder etter behandling.

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