

Persistence of Granulocytic *Ehrlichia* Infection During Wintertime in Two Sheep Flocks in Norway

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Stuen S, Djuve R, Bergström K: Persistence of granulocytic *Ehrlichia* infection during wintertime in two sheep flocks in Norway. Acta vet. scand. 2001, 42, 347-353. – Granulocytic *Ehrlichia* infection in sheep is common in Norway in areas with *Ixodes ricinus*. In this study, 2 sheep flocks that had been grazing on *I. ricinus* infested pastures the previous season, were blood sampled after being housed indoors for nearly 6 months during wintertime. Thirty animals from each flock were examined for granulocytic *Ehrlichia* infection in the peripheral blood by blood inoculation studies, stained blood smear evaluation, polymerase chain reaction (PCR) analysis and serology (IFA-antibodies). The animals were sampled twice within a three-week period, the first time before and the second time after lambing. Two sheep in one flock were found *Ehrlichia* positive by both blood smear evaluation and PCR before lambing, and 3 sheep were found positive after lambing; 2 by blood smear examination and 3 by PCR. In the other flock, no sheep was found infected before lambing, but 2 ewes were found positive after lambing by both blood smear evaluation and PCR. In the first flock, 87% of the animals were found seropositive before lambing, and the mean antibody titre ($\log_{10} \pm$ SD) to *E. equi* was 2.45 ± 0.401 . In the second flock, 40% were found seropositive before lambing, and the mean antibody titre was 1.93 ± 0.260 . Seroprevalence and mean antibody titre in these 2 flocks were significantly different ($p < 0.001$). The present study indicates that sheep may be a reservoir host for granulocytic *Ehrlichia* infection from one grazing season to the next under natural conditions in Norway.

antibodies; PCR; blood smear; reservoir host; Ehrlichia phagocytophila; tick-borne fever.

Introduction

Granulocytic *Ehrlichia* infection in sheep (tick-borne fever) is endemic in coastal areas of Norway, where the tick *Ixodes ricinus* is abundant (Øverås 1972, Stuen 1997). Most cases of tick-borne fever (TBF) in sheep are diagnosed in May-June (65%) and September-October (23%) (Stuen 1997). Earlier investigations have shown that *Ehrlichia phagocytophila* is transmitted transtadially in *I. ricinus*, and that the rickettsiae may survive for several months in these ticks (MacLeod & Gordon 1933, MacLeod 1936). In addition, earlier observa-

tions indicate that nearly 100% of the sheep grazing on *Ixodes* infested pasture may be infected with granulocytic *Ehrlichia* (Ogden *et al.* 1998), and that the infection could persist for several months after experimental infection (Foggie 1951, Stuen *et al.* 1998).

The purpose of this study was to investigate if sheep grazing on *I. ricinus* infested pasture were still infected with *E. phagocytophila* after having been housed indoors during wintertime in Norway.

Materials and methods

Animals

Sheep from 2 flocks, A and B, from western Norway (Etne, Sunnhordland) were examined for a granulocytic *Ehrlichia* infection by analysis of peripheral blood. Both flocks had been grazing on *I. ricinus* infested pasture the previous years, verified by the local veterinarians. Sheep in flock A had grazed on tick pasture from April to November, while animals in flock B had been on tick pasture from May to July, and from September to November. When the blood samples were collected in April, the animals had been housed indoors for nearly 6 months.

Flock A consisted of 63 winterfed sheep of the old Norwegian short-tailed breed Spel, while flock B consisted of 75 sheep of the mixed breed Norwegian White Sheep. Three years earlier, all sheep in flock B had been slaughtered because of an official scrapie eradication program, and the farmer had purchased replacement lambs from *Ixodes* free parts of Norway. Accordingly, no animal in this flock was older than 3 years and the flock had been on *Ixodes* pastures for only 2 grazing seasons.

Tick-borne fever (TBF) or other tick-associated diseases had not earlier been diagnosed in flock A, and only one lamb had been treated with synthetic pyrethroids (Coopersect vet[®], Schering-Plough) the previous year. In comparison, all sheep in flock B had been treated with synthetic pyrethroids the previous year; twice in the spring and once in the autumn. Four lambs had also been treated against TBF with oxytetracycline (Terramycin vet[®], Pfizer) the last spring. Post mortem examination of one of these lambs showed that it died of a bacterial septicaemia together with a granulocytic *Ehrlichia* infection.

Blood samples

Five lambs (≤ 1 year) and 25 adults (> 1 year)

were sampled in flock A and 30 adults (≥ 2 years) were sampled in flock B. The animals were sampled twice within a 3-week period before they were put onto pasture, the first time before and the second time after lambing. Blood samples were collected in EDTA on both occasions, while the heparinised blood and serum samples were collected before lambing only.

Experimental inoculation

The heparinised blood samples were stabilised with 10% dimethyl sulphoxide (DMSO) and frozen at -70°C (Foggie *et al.* 1966) in aliquots of 10 ml containing 1 ml from each of 10 animals from the same flock. These 6 stabilates were later inoculated intravenously into 2-month-old lambs, such that each aliquot was inoculated into each of 6 susceptible and not previously grazing lambs.

The susceptible lambs were followed for 21 days after inoculation. Rectal temperatures were measured daily, and EDTA-blood was sampled on days 0, 6, 8, 9, 10, and 14 post inoculation. In addition, blood samples were collected from individual lambs on days when rectal temperature $\geq 39.5^{\circ}\text{C}$ was recorded. Serum samples were collected on days 0, 14 and 28.

Hematology and PCR analysis

Hematological values including hematocrit, hemoglobin, erythrocyte counts, and total and differential leucocyte counts were determined electronically from the EDTA-blood samples (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 cells containing *Ehrlichia* inclusions was recorded. The blood samples were also tested for granulocytic *Ehrlichia* infection by a polymerase chain reaction (PCR) technique according to Stuen & Olsson Engvall

(1999). In addition, blood smears were stained with acridine orange (Gulland *et al.* 1987) and tested for *Eperythrozoon ovis* (*Ep. ovis*) infection.

Serological methods

Serum was analysed by an indirect immunofluorescence antibody assay (IFA) to determine the antibody titre to *E. equi* (Artursson *et al.* 1999). Briefly, 2-fold dilutions of sera were added to slides precoated with *E. equi* antigen (Protatek International and Organon Teknika). Bound antibodies were visualised by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-sheep immunoglobulin (Cappel, Organon Teknika). 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. Serum was also analysed for *Ep. ovis* infection by an enzyme-linked immunosorbent assay (ELISA) test (Lang *et al.* 1987).

Data analysis

Statistical calculations were done by using Statistix[®], version 4.0 (Analytical software). Statistical analyses on seroprevalence were performed using a chi-square contingency test and the antibody titres were compared using a Students *t*-test for independent samples. Significance was set at $p < 0.05$.

Results

The animals showed no signs of disease when sampled. Except for a low number of erythrocytes, hematocrit and hemoglobin concentration in one animal, the hematological values were within normal limits (Jain 1984).

When the 6 aliquots of DMSO-stabilised blood were inoculated into each of 6 susceptible lambs, only one reacted with fever ($\geq 40^\circ\text{C}$), rickettsemia (infected neutrophils), neutropenia and seroconversion. The other 5 susceptible lambs showed no clinical or hematological

Table 1. Number of granulocytic *Ehrlichia* infected animals in 2 sheep flocks. Thirty animals in each flock were examined by both blood smear evaluation and polymerase chain reaction (PCR) analysis.

Flock	Before lambing (n = 30)		After lambing*	
	Blood smear	PCR	Blood smear	PCR
A	2**	2**	2	3
B	0	0	2	2

* only 24 and 28 sheep in flock A and B were examined, respectively

** both animals were in the same pool of blood that gave positive result by inoculation

signs of *E. phagocytophila* infection, nor any seroconversion within one month after inoculation, and *Ehrlichia* could not be found by either blood smear evaluation or PCR technique.

The results of blood smear investigation and PCR analysis in the 2 flocks are shown in Table 1. By blood smear examination a low degree of rickettsemia was found (<1% infected neutrophils). In flock A, 2 animals were found positive by blood smear evaluation both before and after lambing, but only one of these was found positive on both samplings. All animals found positive by blood smear investigation were also positive by PCR. Altogether 4 animals in flock A were found positive (13%, 1 lamb and 3 adults). In comparison, none of the sheep in flock B were found positive before lambing, and only 2 were positive after lambing (7%).

The mean antibody titres to *E. equi* in seropositive animals are shown in Table 2. No difference in these titres was observed between seropositive lambs and adults. In flock A, 87% of the animals were found seropositive, and the mean antibody titre was 2.45 ± 0.401 , while only 40% were found seropositive in flock B with a mean antibody titre of 1.93 ± 0.260 . The seroprevalence in these 2 flocks was significantly different ($p < 0.001$), and also the mean

Table 2. The indirect immunofluorescence antibody titre to *Ehrlichia equi* in various age groups in 2 sheep flocks, after the sheep had been housed indoors for nearly 6 months. A titre below 1:40 ($\log_{10} = 1.6$) was considered negative.

	Age				
	Flock A			Flock B	
	1 year	2 year	>2 year	2 year	3 year
Number of animals	5	10	15	10	20
Number of seropositive animals	4 (80%)	9 (90%)	13 (87%)	2 (20%)	10 (50%)
Mean antibody titre of seropositive animals (\log_{10}) \pm SD	2.58 \pm 0.250	2.41 \pm 0.401	2.44 \pm 0.428	2.20 \pm 0.301	1.87 \pm 0.211
Mean antibody titre in persistent infected animals (\log_{10}) \pm SD*		2.58 \pm 0.250		2.35 \pm 0.151	

*four animals in flock A, 2 animals in flock B.

antibody titre between seropositive animals in the two herds was significantly different ($p < 0.001$).

In addition, an *Ep. ovis* infection was found in all animals by blood smear investigation, and all animals were also found seropositive for *Ep. ovis* infection.

Discussion

In the present study, the number of sheep that is actually infected with TBF at the time of sampling is unknown. Both flocks had been grazing in areas where *I. ricinus* is abundant. In flock A, 4 sheep (13%) out of 30 were found infected in blood smears and by PCR, i.e. 15% of the seropositive sheep. In comparison, only 2 animals (7%) in flock B were found infected, i.e. 16.7% of the seropositive animals. The sensitivity of the tests used may have been increased either by examination of more neutrophils or by use of a nested PCR technique (Barlough et al. 1996).

An earlier experimental study showed that at least 4 out of 5 *E. phagocytophila* infected lambs were still infectious by experimental inoculation 6 months after the initial infection (Stuen et al. 1998). However, in the present in-

vestigation, the sheep could have been infected with granulocytic *Ehrlichia* up to 12 months before the blood was collected.

In the present study, more animals were found infected after lambing than before. It is difficult to judge whether the immunosuppression occurring in ewes around lambing may have caused a relapse of the infection. These results indicate, however, that granulocytic *Ehrlichia* infection in peripheral blood varies in infected sheep, as earlier observed in experimental TBF infection (Foggie 1951, Stuen et al. 1998). More sheep might therefore have been found positive by increasing the number of samples per animal.

The hematological values of the animals were within normal limits, except for a low number of erythrocytes in one lamb. Since a subclinical *Ep. ovis* infection was found in all sheep, the low number of red blood cells in that animal might have been caused by this infection (Øverås 1969).

E. equi was used as antigen in the serological analysis. 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). It is therefore possible to use any of these closely related antigens to obtain acceptable results in serosurvey, but the IgG titres may differ noticeably depending on the source of the antigen (Bjoersdorff *et al.* 1999, Walls *et al.* 1999).

In flock A, 87% of the sheep were found seropositive 6 months after last exposure to ticks. In contrast, after a stable period of 6 months, the seroprevalence in cattle was reduced by more than 40% and no animals were found infected (Pusterla *et al.* 1998). One explanation of this difference could be that sheep are a more competent and natural host for *E. phagocytophila* than cattle.

While most of the animals in flock A were found to be seropositive against *E. equi*, less than half of the sheep in flock B were seropositive. In addition, the antibody titres were highest in flock A. This result is in accordance with earlier observations on TBF infection in cattle grazing on tick pasture, where the increase in titre was parallel to an increase in seroprevalence (Pusterla *et al.* 1998).

The present study indicates that most problems associated with TBF based on herd history seem to occur in the flock with the lowest seroprevalence and titres. No obvious reasons for this observation were found, other than flock A may have been more thoroughly infected and therefore better immunized than flock B. Sheep in flock A were annually grazing for 3 months longer on *Ixodes* pastures than those in flock B. In addition, animals in flock B had only been on tick pasture for 2 seasons, and lack of sufficient immunity against TBF after restocking may be one explanation for disease problems the previous spring and low seroprevalence.

Another explanation of the difference in seroprevalence could be that the flocks were infected with *Ehrlichia* at different periods of the grazing season. The sheep had been housed indoors for nearly half a year, but the antibody

titres in *E. phagocytophila* infected lambs seem to last for at least 6 months in experimentally infected lambs (Paxton & Scott 1989, Stuenkel *et al.* 1998).

Four lambs in flock B were treated against TBF in spring the previous year, and if the sheep in this flock were infected mainly at that time, the actual titres may reflect antibody levels developed about 12 months earlier. Unfortunately, no blood samples were collected from the sheep in the autumn to verify this assumption. In one study in humans, the antibodies remained detectable in about half of the HGE patients one year after onset of symptoms (Aguero-Rosenfeld *et al.* 2000).

No difference in seroprevalence and antibody titres was observed between lambs and adults in flock A. This is in accordance with similar observations in cattle (Pusterla *et al.* 1998). However, in flock B, only 20% of the 2-year-old sheep were seropositive, compared with 50% of the 3-year-old sheep. According to the farmer, the youngest animals had the previous year a shorter period on *Ixodes* infested pasture when compared with the oldest animals.

Different strains of granulocytic *Ehrlichia* could be involved in these 2 flocks. Earlier observations indicate that different strains of *E. phagocytophila* may cause differences in both clinical and immunological responses (Foggie 1951, Tuomi 1967). In addition, sheep breed variation in susceptibility to a TBF infection has also been reported (Scott 1983). However, to the authors knowledge, no difference in clinical reaction to an *E. phagocytophila* infection has been observed between sheep breeds in Norway.

All sheep in flock B had been treated 3 times with synthetic pyrethroids the previous year, while only one lamb in flock A was treated. The difference in treatment régime may also have had some effect on the seroprevalence. However, earlier studies indicate that lambs treated

with synthetic pyrethroids may become infected with TBF within 3 weeks on *Ixodes* infested pasture (Hardeng et al. 1992). In addition, the antibody titres in synthetic pyrethroid treated and untreated animals were not significantly different at the end of the grazing season (Stuen et al. in press).

In conclusion, the present study indicates that sheep under natural conditions in Norway may be a reservoir host for granulocytic *Ehrlichia* infection from one grazing season to the next. However, to analyse the epidemiological importance of this, a more detailed study during tick infestation is needed.

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Sammendrag

Persistens av granulocyttaer Ehrlichia-infeksjon i to saueflokker i Norge.

Granulocyttaer *Ehrlichia*-infeksjon (sjodogg) er vanlig i Norge i områder med *Ixodes ricinus*. I denne undersøkelsen ble det tatt blodprøver i 2 saueflokker som hadde beitet på *I. ricinus*-beite forrige sesong. Prøvene ble uttatt etter at dyrene hadde stått inne i ca. 6 måneder. Tredve dyr i hver flokk ble undersøkt for en sjodogg infeksjon ved hjelp av blodpoding, blodutstryk, PCR-analyse og serologi. Det ble tatt blod av de samme dyrene ved 2 anledninger i løpet av en 3 ukers periode; første gang før lamming og andre gang etter lamming. I den ene flokken ble 2 dyr funnet infisert før lamming, og 3 dyr ble funnet positive etter lamming. I den andre flokken ble ingen dyr funnet positive før lamming, men 2 dyr ble funnet positive etter lamming. I den første flokken var 87% av dyrene seropositive og antistoff-titret mot *E. equi* var 2.45 ± 0.401 (middel \pm SA). I den andre flokken var 40% seropositive og titret var 1.93 ± 0.260 . Både seroprevalensen og antistoff-titret mellom de 2 flokkene var signifikant forskjellige. Denne undersøkelsen indikerer at sau kan være et reservoar for sjodogg-smitte mellom 2 beitesesonger i Norge.

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