Veterinary Microbiology 136 (2009) 372–377

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Molecular characterization of two different strains of haemotropic mycoplasmas from a sheep flock with fatal haemolytic anaemia and concomitant *Anaplasma ovis* infection

Sándor Hornok [a,*], Marina L. Meli [b], András Erdős [c], István Hajtós [c], Hans Lutz [b], Regina Hofmann-Lehmann [b]

[a] Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István University, Budapest, Hungary
[b] Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland
[c] County Veterinary Station, Borsod-Abaúj-Zemplén, Miskolc, Hungary

ARTICLE INFO

Article history:
Received 3 August 2008
Received in revised form 27 October 2008
Accepted 29 October 2008

Keywords:
Sheep
Anaemia
Haemotropic mycoplasmas
Mycoplasma ovis
Anaplasma ovis

ABSTRACT

After the first outbreak of fatal *Mycoplasma ovis* infection (eperythrozoonosis) in a sheep flock in Hungary (1997), a second wave of the disease was noted in 2006, with different seasonal pattern and affected age group, as well as increased mortality (5.5%). The aim of the present study was to molecularly characterize the causative agent and to reveal underlying factors of the second wave of the disease. Remarkably, among the 33 sheep examined, 17 were infected with two strains of haemotropic mycoplasmas. Cloning and sequencing isolates of the latter showed that one of the strains was 99.4–99.8% identical to *M. ovis* (AF338268), while the second was only 96.8–97.9% identical and contained a 17-bp deletion. Different isolates of both strains were demonstrated in the same animal. When analyzing possible risk factors for fatal disease outcome, we found that among sheep born prior to the 1997 outbreak significantly more animals survived the second outbreak than succumbed to disease. In addition, locally born sheep were less frequently diseased than sheep introduced into the flock from other places. This suggests an immunoprotective effect in some animals. Concurrent infection with *Anaplasma ovis* was detected in 24 of the 33 evaluated sheep. In conclusion, this is the first study to demonstrate the existence of and characterize two genetically distinct ovine haemotropic mycoplasma strains in a sheep flock with fatal haemolytic anaemia.

1. Introduction

Haemotropic mycoplasmas are unculturable bacteria that may cause haematological disorders in various hosts (Ristic and Kreier, 1984). They had been classified as members of the Anaplasmataceae family (order Rickettsiales) and were further divided into two genera: *Haemobartonella*, characterized by mostly epierythrocytic, coccoid forms, and *Eperythrozoon*, existing mostly in ring forms on red blood cells and free in the plasma. However, analysis of 16S rRNA gene sequences revealed the close relationship of these bacteria with the genus *Mycoplasma* (Neimark et al., 2001). Until now, the only species recognized in sheep was *Mycoplasma ovis* (formerly *Eperythrozoon ovis*), which may induce poor weight gain, severe anaemia, and even mortality in lambs and, occasionally, young adult sheep (Campbell et al., 1971; Daddow, 1979). Mild clinical signs and persistent bacteraemia associated with chronic infection have also been described in older animals (Gulland et al., 1987; Mason and Statham, 1991).

In Hungary, the first and thus far only reported case of ovine eperythrozoonosis resulted in 8.5% (35 out of 412)

* Corresponding author at: Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István University, István u. 2, 1078 Budapest, Hungary. Tel.: +36 1 478 4187; fax: +36 1 478 4193.
E-mail address: Hornok.Sandor@aotk.szie.hu (S. Hornok).

0378-1135/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2008.10.031

morbidity and 2.2% (9 out of 412) mortality (Hajtós et al., 1998). The disease manifested between August and November of 1997, and mainly affected yearlings. *M. ovis* infection apparently persisted in the same flock of sheep, with only sporadic and mild pathogenic effects for several years. However, in July and August of 2006, a new disease outbreak occurred. The aim of the present study was to characterize the causative agent of the latter using molecular methods and to reveal underlying factors that may have contributed to the more severe outcomes, as well as to the altered seasonal pattern and affected age group of the disease.

2. Materials and methods

2.1. Case history and sample collection

Clinical signs of fatal *M. ovis* infection (anaemia, submandibular oedema, weight loss) were noted between July and August of 2006 in 39 animals from a flock of 546 sheep (designated flock A) in Northeast Hungary. Data obtained for the animals, including the 30 sheep that died during the outbreak (group A1), were recorded. Ten months after the first clinical manifestation, EDTA anticoagulated blood samples were collected by jugular venipuncture from a total of 33 sheep: 9 sheep with typical clinical signs that survived the outbreak (group A2); 16 animals with no characteristic clinical signs (group A3); and 8 animals from a neighboring flock (designated flock B) in which clinical signs attributable to *M. ovis* had never been observed. Blood smears were prepared from fresh EDTA anticoagulated blood and stained with Giemsa. Haematological values were determined using an Abacus haematology analyzer (Diatron GmbH, Vienna, Austria). Sheep in both flocks (A and B) were supervised by the same veterinarian and received the same nutrition and prophylactic medication, but were housed in separate buildings on the same farm and grazed in different pastures.

2.2. Nucleic acid extraction

DNA was purified from 33 blood samples using the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). First, 100 μl of EDTA anticoagulated blood was diluted in the same volume of PBS without $MgCl_2$ and $CaCl_2$ (Invitrogen, Basel, Switzerland). Then, 300 μl of lysis buffer containing guanidinium thiocyanate and Triton X-100 (Roche Diagnostics) was added. The lysed samples were loaded into wells on the automated MagNA Pure LC Instrument. Two PBS controls were included in each run to monitor potential cross-contamination. The elution volume was 100 μl. Samples were stored at −20 °C until further analysis. The presence of amplifiable DNA was confirmed for each sample using an 18S rRNA gene real-time TaqMan PCR assay (Applied Biosystems, Rotkreuz, Switzerland) (data not shown).

2.3. Real-time TaqMan PCR to detect M. ovis infection

A quantitative real-time TaqMan PCR assay developed for *M. wenyonii* (AF016546) was used to screen the DNA samples for *M. ovis* (Meli et al., submitted for publication). The PCR amplified a 119-bp long fragment of the 16S rRNA gene of *M. wenyonii*. Primer and probe sequences (forward primer, 5′-CCA CGT GAA CGA TGA AGG TCT T-3′; reverse primer, 5′-GGC ACA TAG TTA GCT GTC ACT TAT TCA A-3′; and probe, 5′-6FAM-AGT ACC ATC AAC GCG CGC TGA TTT CCT AG-MGB-3′) demonstrated 100% identity with the sequence of the 16S rRNA gene of *M. ovis* (AF338268). The 25 μl PCR reaction mixture contained 900 nM of each primer, 0.25 μM probe, 12.5 μl of 2× qPCR MasterMix (Eurogentec, Seraing, Belgium), and of 5 μl template DNA. The amplification mixture contained dUTP for use with uracil-N-glycosylase to prevent carryover of the PCR amplicons. The cycling conditions were: incubation for 2 min at 50 °C and then a 10 min initial denaturation at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C using the ABI Prism 7700 sequence detection system (Applied Biosystems).

2.4. Amplification and sequencing of the 16S rRNA gene of ovine haemotropic mycoplasmas

Initially, a conventional PCR was used to amplify an approximately 200-bp long region of the 16S rRNA gene as described elsewhere (Jensen et al., 2001). PCR products were visualized with ethidium bromide in a 2.5% agarose gel.

In a second step, two samples from each group (A2, A3, and B) were chosen for further analysis by cloning and sequencing. Samples were selected according to Ct values (low Ct value = high load), and assessed by conventional PCR using species-specific primers for *M. ovis* (forward: 5′-AGAGTT TGA TC(A/C) TGG CTC AG-3′, reverse: 5′-CGG TTA CCT TGT TAC GAC TT-3′), which amplify a 1485-bp region of the 16S rRNA gene, as described elsewhere (20). The 25 μl reaction mixture contained 5 μl of 5× High Fidelity PCR buffer (Finnzymes: BioConcept, Allschwil, Switzerland), 0.5 μM of each primer, 0.2 mM dNTP mixture (Sigma–Aldrich, Buchs, Switzerland), 1 U of Phusion High Fidelity DNA polymerase (Finnzymes), 15.25 μl of nuclease-free water, and 2.5 μl of template DNA. Amplification was performed using a T-personal 48 Thermocycler (Biometra GmbH, Goettingen, Germany). The thermal profile consisted of an initial denaturation step at 98 °C for 3 min, followed by 35 cycles of 10 s at 98 °C, 30 s at 60 °C, and 1 min at 72 °C, with a final elongation at 72 °C for 10 min. A portion of the PCR product was visualized in a 1% agarose gel stained with ethidium bromide after electrophoresis. The remaining portion was purified with the GenElute™ PCR Clean-Up Kit (Sigma–Aldrich) and cloned into the pCR®4-TOPO® (Invitrogen) vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). Clones were screened by colony PCR, i.e. the *M. wenyonii* TaqMan assay as described above. The fifteen positive clones that produced the lowest Ct values were selected, and plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen, Hombrechtikon, Switzerland). Cycle sequencing was performed with 4 μl of DNA, 3.3 pmol of plasmid-specific primers, an additional internal primer (5′-GGG AGG CTG ATC CAT TGT TA-3′), and the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1

(Applied Biosystems). The cycling conditions were as follows: 1 min at 96 °C, followed by 25 cycles of 96 °C for 10 s and 50 °C for 5 s, and a final incubation at 60 °C for 4 min. Products were purified using SigmaSpin™ Post-Reaction Purification Columns (Sigma–Aldrich) and analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Obtained sequences were edited and aligned with a consensus sequence using SeqScape (Version 1.1, Applied Biosystems), and then compared to the 16S rRNA gene of *M. ovis* (AF338268). For phylogenetic analysis, the sequences were aligned with known mycoplasma sequences from GenBank using ClustalW (Thompson et al., 1994) and, if necessary, manually adjusted. Only the nucleotides available for all included sequences were used in the phylogenetic analysis. A bootstrap phylogenetic tree demonstrating the relationship of sheep isolates to other haemoplasma species was created by the Neighbor-Joining method (Saitou and Nei, 1987) using a distance matrix corrected for nucleotide substitutions based on the Kimura 2-parameter model. The dataset was resampled 1000 times to generate bootstrap values. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Kumar et al., 2004).

New sequences were submitted to GenBank (accession numbers: EU165509–EU165513, EU828579–EU828582).

2.5. Evaluation of Anaplasma ovis infection

The blood samples were screened for the presence of members of the Anaplasmataceae family by conventional PCR with the following primers: Ehr1 (5′-TTT ATC GCT ATT AGA TGA GCC TAT G-3′) and Ehr2 (5′-CTC TAC ACT AGG AAT TCC GCT AT-3′), as described elsewhere (Goodman et al., 1996). In addition, the presence of *A. phagocytophilum* was excluded based on a specific real-time TaqMan assay (Wicki et al., 2000). Five samples were further analyzed by conventional PCR with primers specific for the *msp4* gene of *A. ovis* and *A. marginale* (de la Fuente et al., 2001) using the same conditions as indicated above for the 16S rRNA gene of *M. ovis*. The PCR products were then sequenced.

2.6. Statistical analyses

Calculations were performed with the software Analyse-it (Analyse-it Software Ltd., Leeds, United Kingdom) for Microsoft Excel. Risk factors for haemoplasma infections were evaluated using the Mann–Whitney *U*-test for continuous variables and the Fisher's exact test (cell frequencies ≤ 5) or Chi^2 test (cell frequencies >5) for categorical variables. Differences were regarded as significant at $P < 0.05$.

3. Results

3.1. Clinical outbreak and characteristics of affected animals

In July and August of 2006, 39 adult animals from a Hungarian sheep flock (flock A) showed typical signs of eperythrozoonosis. All affected animals were 5–12 years of age. In their blood smears two morphologically distinguishable moieties were visible: multiple small forms characteristic of *M. ovis* and individual smaller or larger coccoid bodies (Fig. 1). A total of 30 sheep died within 4–30 days after the onset of clinical signs despite initiation of oxytetracyclin treatment at 2- to 4-day intervals. Both mortality (30 out of 546; 5.5%) and lethality (30 out of 39; 77%) were significantly higher ($P = 0.0124$ and $P < 0.0001$, respectively) as compared to the previous outbreak in the same flock in 1997 (mortality 2.2%: 9 out of 412, lethality 25.7%: 9 out of 35).

Merino sheep represented only 22% (120 out of 546) of flock A, whereas the neighboring flock (flock B: never diseased) consisted exclusively of this breed (Table 1). The remaining sheep in flock A were predominantly Ille de France and Bábolna Tetra crossbreeds. Significantly fewer sheep that had been affected by the disease (groups A1 and A2), were born locally (38%: 15 out of 39) as compared to those in group A3 that demonstrated no clinical signs (65%: 330 out of 507; $P = 0.0016$; Table 1). Among the group of sheep that had died during the 2006 outbreak (A1), there were significantly fewer sheep born prior to the 1997 outbreak (33%: 10 out of 30) than in group A2, which consisted of the survivors of the 2006 disease outbreak (78%: 7 out of 9; $P = 0.0262$; Table 1).

Haematological values were within the normal range, except for haematocrit and haemoglobin concentrations in the recently diseased sheep (group A2). These sheep had significantly lower haematocrit (0.25 ± 0.08) and haemoglobin (76.5 ± 24.5 g/l) levels than those in healthy control group A3, which exhibited values of (0.38 ± 0.10; $P = 0.0168$) and (105.1 ± 13.5 g/l; $P = 0.018$), respectively. In group B the haematocrit was 0.325 ± 0.05 and the haemoglobin concentration 100.25 ± 16.41 g/l.

3.2. Sample prevalence of ovine haemotropic mycoplasmas

Using a real-time PCR assay, 52% of the examined animals (17 out of 33) tested positive, including sheep

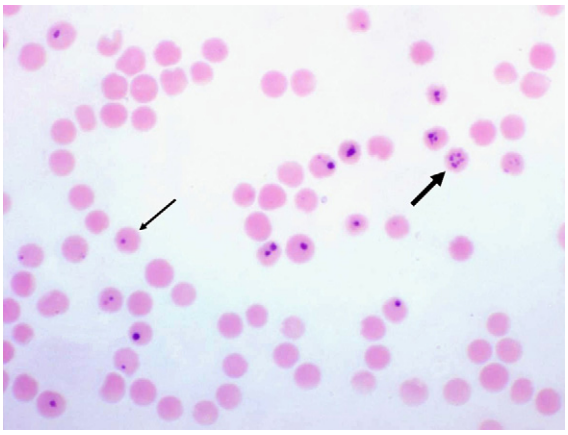

Fig. 1. Blood smear of a sheep concomitantly infected with *Anaplasma ovis* (thin arrow) and haemotropic mycoplasmas (thick arrow). The former are usually individually situated in red blood cells (dark basophilic), whereas the latter tend to occur in multiplicity (clustered or separated) on erythrocytes (therefore brightly basophilic).

Table 1
Data obtained for sheep in the two flocks (A and B) and results of molecular evaluation of their samples. Flock A consisted of 546 sheep which were divided into three groups (A1–A3) according to the outcome in the 2006 disease outbreak.

Group	Outcome in 2006 outbreak	Total number of sheep	Number (%) of merino	Number (%) born locally	Number (%) born before the 1997 outbreak	Number of samples for PCR	Number (%) of haemoplasma-positive samples	Number (%) of anaplasma-positive samples
A1	Sick, died	30	9 (30%)	13 (43%)	10 (30%)	n.a.	n.a.	n.a.
A2	Sick, survived	9	3 (30%)	2 (22%)	7 (78%)	9	6 (67%)	7 (78%)
A3	Not diseased	507	108 (27%)	330 (65%)	176 (35%)	16	7 (44%)	14 (88%)
B	Never diseased	545	545 (100%)	n.a.	n.a.	8	4 (50%)	3 (38%)

Abbreviation: n.a.—not available.

from all three sample groups: A2, A3, and B (Table 1). No significant differences were found in sample prevalence among the three groups. All sheep that tested positive by real-time PCR were also positive in conventional PCR and showed PCR products of different lengths.

3.3. Molecular characterization of ovine haemotropic mycoplasmas

Cloning and sequencing of the near-complete 16S rRNA gene of haemotropic mycoplasmas was successful for four

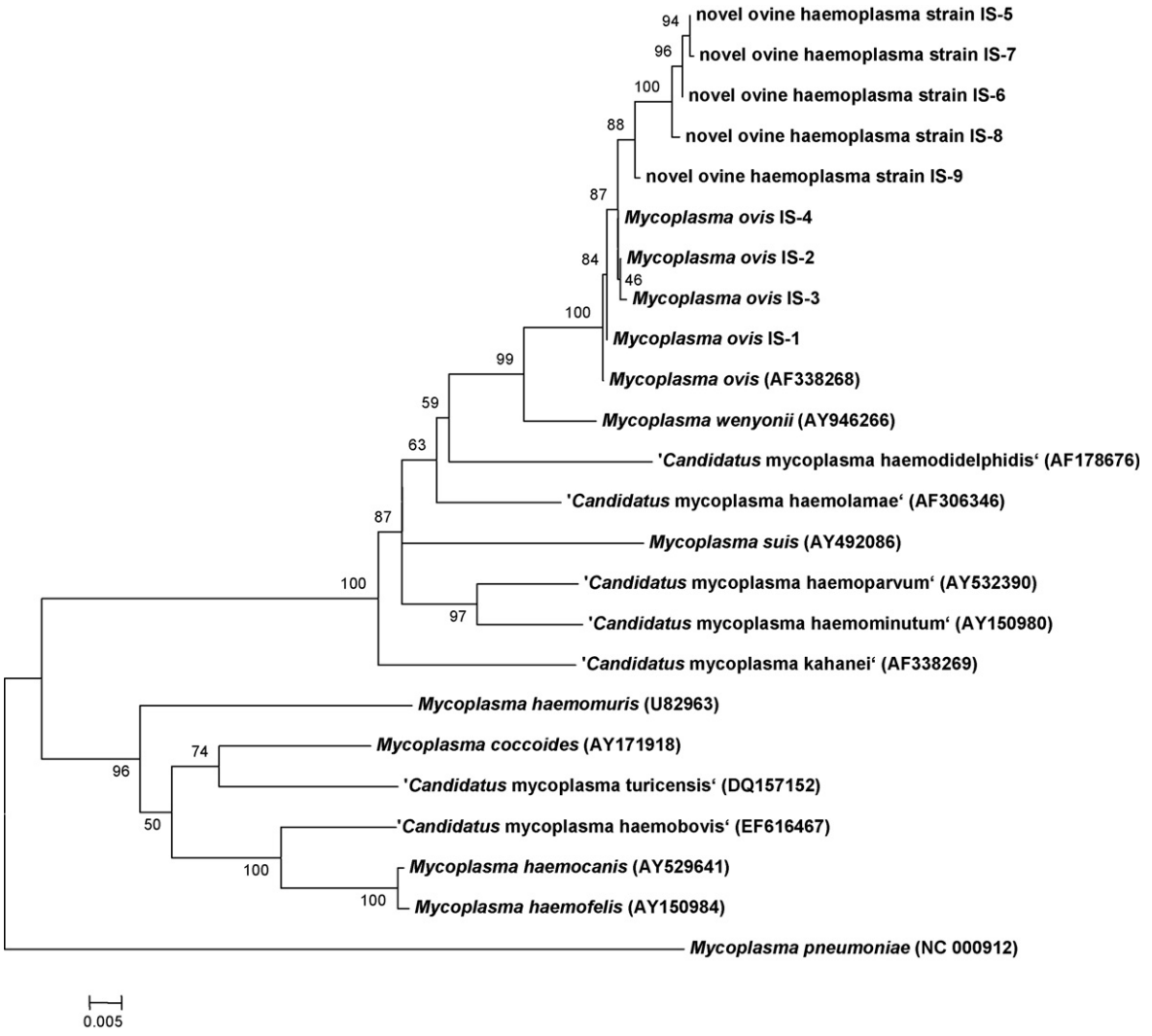

Fig. 2. Phylogenetic tree of the near-complete 16S rRNA gene sequences of haemotropic mycoplasmas available in GenBank in comparison with those of the isolates from the Hungarian sheep. The numbers at the nodes were generated from 1000 bootstrap resamplings. The bar represents the mean number of differences per 50 sites.

sheep (one from group A2, one from A3, and two from flock B), with two to five clones obtained per sheep. Four sequences (designated IS-1 to IS-4) showed 99.4–99.8% identity with *M. ovis* (AF338268). In contrast, five isolates (designated IS-5 to IS-9) were found to be only 96.8–97.9% identical to *M. ovis* (Table 2). On a phylogenetical tree four of the latter made up a separate cluster, while one occupied an intermediate position between the *M. ovis* cluster and the new cluster (Fig. 2). The 16S rRNA gene sequence of the new isolates differed from that of *M. ovis* (AF338268) at several nucleotide positions along the full length of the gene (Table 2). In addition, a gap of 17 bp, including nucleotides 440–456, was demonstrated in IS-5 to IS-9. Identical isolates were found in more than one animal, but dissimilar isolates of both strains were also detected in the same animal (Table 2).

3.4. Co-infection with an Anaplasma sp.

Out of the 33 blood samples tested by molecular methods, 24 were found to be positive for an *Anaplasma* sp., as determined by conventional PCR, but negative for *A. phagocytophilum* based on specific real-time PCR. Sequencing results for part of the *msp4* gene from five sheep confirmed the presence of *A. ovis* (data not shown). Concomitant *A. ovis* infection was significantly more prevalent in flock A (21 out of 25) than flock B (3 out of 8; $P = 0.0201$; Table 1). Characteristic inclusion bodies could be observed by microscopy (Fig. 1).

4. Discussion

This is the first demonstration of the existence of two genetically distinct ovine haemotropic mycoplasma strains.

M. ovis infection is usually accompanied by mild clinical signs and no fatalities among adult sheep (Neimark et al., 2004). This is in contrast to the outbreak reported in the present study. However, the prevalence of haemotropic mycoplasma infection in the diseased flock (A) was not significantly different from that in flock B, where clinical signs attributable to *M. ovis* have never been observed. Besides differences in breed composition of the two flocks, the most important factor that could have influenced the clinical manifestation is the co-infection with *A. ovis*. This turned out to be significantly less prevalent in the healthy flock (B) than in the diseased flock (A). Although simultaneous eperythrozoonosis and anaplasmosis have been documented (Splitter et al., 1955), no reports are currently available on the potential synergism between these two haemotropic bacteria. Decreased haemoglobin concentrations and haematocrit values reported for eperythrozoonosis (Gulland et al., 1987) are usually not detected in *A. ovis*-infected adult sheep (Splitter et al., 1956; Hornok et al., 2007). Therefore, these changes detected in the present study were most likely caused by haemotropic mycoplasmas.

In the unaffected group (A3) there was a higher proportion of locally born sheep than in the two groups affected by *M. ovis* (A1, A2). This raises the possibility of a certain degree of innate immunity (natural resistance) associated with endemic foci, which is known to exist

Table 2
Result of the alignment of the nearly complete 16S rRNA gene of nine ovine haemotropic mycoplasma isolates (IS-1 to IS-9) and their comparison with the *Mycoplasma ovis* sequence. Upper index small letters next to the name of an isolate indicate animals from which it was obtained.

Position[1]	101	118	119	125	139	188	218	224	272	288	319	340	395	440–456	507	510	514	635	648	673	690	701	753	763	953	1183	1274	1302	1337
M. ovis	G	T	C	G	G	G	G	G	G	G	C	G	C	AG..GA	C	C	G	C	G	C	A	C	G	C	G	C	C	C	T
IS-1[a]	A	C	*	T	*	*	*	*	*	*	*	*	*	**..**	*	*	*	*	*	*	*	*	*	*	*	*	T	T	A
IS-2[a]	A	C	*	T	*	*	*	*	*	*	*	*	*	**..**	*	*	*	*	*	*	*	*	*	*	A	T	T	T	A
IS-3[a,b,d]	A	C	*	T	*	*	*	*	*	*	*	*	*	**..**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
IS-4[c]	A	C	*	T	*	*	*	*	*	*	*	*	*	**..**	*	*	*	*	*	*	*	*	*	*	*	*	*	T	A
IS-5[a]	*	C	T	T	*	T	A	A	A	T	T	A	T	---..---	T	T	*	T	A	T	G	A	A	T	A	T	T	T	A
IS-6[a]	*	C	T	T	*	T	A	A	A	T	T	A	T	---..---	T	T	*	T	A	T	G	A	A	T	A	T	T	T	A
IS-7[b]	*	C	T	T	A	T	A	A	A	T	T	A	T	---..---	T	T	*	T	A	T	G	A	A	T	A	T	T	T	A
IS-8[c,d]	*	*	*	T	*	T	*	A	A	T	T	A	T	---..---	T	T	T	T	A	*	G	A	*	T	*	T	T	T	A
IS-9[c]	A	C	*	T	*	*	*	*	*	*	*	*	*	---..---	T	T	T	T	A	*	G	A	*	T	*	*	*	*	*

Upper indexes: a—one sheep from group A2; b—one sheep from group A3; c and d—two sheep from flock B.
[1] Position numbers given with respect to *M. ovis* (AF338268). Nucleotides identical to the latter are given as asterisks; mismatches are listed.

against other agents infecting erythrocytes (Gern et al., 1988). At the same time, sheep that survived the current disease outbreak were more frequently born prior to the previous outbreak in 1997 than sheep that succumbed, suggesting the development of an age-related and/or acquired immunity to haemotropic mycoplasmas.

The disease outbreak between August and November of 1997 may have been a consequence of the introduction of infected animals into the flock (Hajtós et al., 1998). However, no animals were introduced into flock A in 2006. Therefore, exclusive manifestation of the disease from July to August may have been associated with the seasonal activity of potential arthropod vectors. Since mosquitoes may transmit *M. ovis* (Daddow, 1980; Howard, 1975) and they depend on water for their development, it may be relevant that the pastures grazed by sheep flocks A and B are flanked by a river. It is documented that heavy rainfalls with floods in 2006 entailed an early summer mosquito invasion in Hungary and promoted vector-borne transmission of different agents in the region (Vasconcelos, 2006), possibly including an upsurge in ovine haemoplasma infection of naive animals. On the other hand, ticks as competent vectors of certain haemotropic mycoplasmas (Seneviratna et al., 1973) were not found in the pastures or on the sheep in 2006 or 2007 (data not shown).

The disease outbreak in 2006 may have also been influenced by the appearance of the second, genetically distinct ovine haemoplasma strain. Reclassification of haemotropic *Mycoplasma* spp. was based on 16S rRNA gene sequences (Neimark et al., 2001). Accordingly, the new sheep haemoplasma strain is most closely related to *M. ovis* (96.8–97.9% identity to AF338268). At the same time, it is also substantially different from it, most notably due to a 17 bp long deletion. Similar genotypic variants of haemoplasmas (with or without a gap in their 16S rRNA sequence) were shown to exhibit higher or lower pathogenicity in other hosts (Jensen et al., 2001; Neimark et al., 2001). The overall ratio of 16S sequence divergence between *M. ovis* and the novel ovine haemotropic mycoplasma strain is close to the value for the declaration of a new species (Drancourt and Raoult, 2005). However, to confirm if the novel strain deserves the status of a *Candidatus*, preferably additional genes should be compared and the pathogenicity of the agent further evaluated in monoinfections.

In conclusion, although *M. ovis* can be regarded as mildly pathogenic, concurrent infection with divergent strains and/or other haemotropic bacteria may result in a more severe clinical manifestation and even mortality among adult sheep.

Acknowledgements

The authors would like to thank the invaluable help of J. Fodor, E. Gönczi, T. Meili, Prof. M. Rusvai, A. Perreten and K. Museux. Laboratory work was performed with logistical support from the Center for Clinical Studies at the Vetsuisse Faculty of the University of Zurich. Regina Hofmann-Lehmann is the recipient of a Swiss National Science Foundation professorship (grant numbers PP00B-102866/1 and PP00P3-119136/1).

References

Campbell, R.W., Sloan, C.A., Harbutt, P.R., 1971. Observations on mortality in lambs in Victoria associated with *Eperythrozoon ovis*. Aust. Vet. J. 47, 538–541.

Daddow, K.N., 1979. *Eperythrozoon ovis*—a cause of anaemia, reduced production and decreased exercise tolerance in sheep. Aust. Vet. J. 55, 433–434.

Daddow, K.N., 1980. *Culex annulirostris* as a vector of *Eperythrozoon ovis* infection in sheep. Vet. Parasitol. 7, 313–317.

de la Fuente, J., Van Den Bussche, R.A., Kocan, K.M., 2001. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). Vet. Parasitol. 97, 65–76.

Drancourt, M., Raoult, D., 2005. Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. J. Clin. Microbiol. 43, 4311–4315.

Gern, L., Kindler, A., Brossard, M., 1988. Annual evolution of cattle immunity against *Babesia divergens* in Northern Switzerland. Prev. Vet. Med. 6, 9–16.

Goodman, J.L., Nelson, C., Vitale, B., Madigan, J.E., Dumler, J.S., Kurtti, T.J., Munderloh, U.G., 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N. Engl. J. Med. 334, 209–215.

Gulland, F.M., Doxey, D.L., Scott, G.R., 1987. The effects of *Eperythrozoon ovis* in sheep. Res. Vet. Sci. 43, 85–87.

Hajtós, I., Glávits, R., Erdős, A., Pálfi, V., 1998. Diagnosis of *Eperythrozoon ovis* infection in sheep in Hungary. Magyar Állatorvosok 120, 697–703 (in Hungarian, with English abstract).

Hornok, S., Elek, V., de la Fuente, J., Naranjo, V., Farkas, R., Majoros, G., Földvári, G., 2007. First serological and molecular evidence on the endemicity of *Anaplasma ovis* and *A. marginale* in Hungary. Vet. Microbiol. 122, 316–322.

Howard, G.W., 1975. The experimental transmission of *Eperythrozoon ovis* by mosquitoes. Parasitology 71, 33.

Jensen, W.A., Lappin, M.R., Kamkar, S., Reagan, W.J., 2001. Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats. Am. J. Vet. Res. 62, 604–608.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform. 5, 150–163.

Mason, R.W., Statham, P., 1991. The determination of the level of *Eperythrozoon ovis* parasitaemia in chronically infected sheep and its significance to the spread of infection. Aust. Vet. J. 68, 115–116.

Neimark, H., Johansson, K.-E., Rikihisa, Y., Tully, J.G., 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus* Mycoplasma haemofelis', '*Candidatus* Mycoplasma haemomuris', '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii'. Int. J. Syst. Evol. Micr. 51, 891–899.

Neimark, H., Hoff, B., Ganter, M., 2004. *Mycoplasma ovis* comb. nov. (formerly *Eperythrozoon ovis*), an epierythrocytic agent of haemolytic anaemia in sheep and goats. Int. J. Syst. Evol. Micr. 54, 365–371.

Ristic, M., Kreier, J.P., 1984. Family III. Anaplasmataceae. In: Krieg, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, Baltimore, pp. 719–729.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Seneviratna, P., Weerasinghe, N., Ariyadasa, S., 1973. Transmission of *Haemobartonella canis* by the dog tick, *Rhipicephalus sanguineus*. Res. Vet. Sci. 14, 112–114.

Splitter, E.J., Twiehaus, M.J., Castro, E.R., 1955. Anaplasmosis in sheep in the United States. J. Am. Vet. Med. Assoc. 125, 244–245.

Splitter, E.J., Anthony, H.D., Twiehaus, M.J., 1956. *Anaplasma ovis* in the United States. Am. J. Vet. Res. 17, 487–491.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Vasconcelos, P., 2006. Flooding in Europe: a brief review of the health risks. Euro. Surveill. 11, E060420.2. http://www.eurosurveillance.org/ew/2006/060420.asp#2.

Wicki, R., Sauter, P., Mettler, C., Natsch, A., Enzler, T., Pusterla, N., Kuhnert, P., Egli, G., Bernasconi, M., Lienhard, R., Lutz, H., Leutenegger, C.M., 2000. Swiss Army Survey in Switzerland to determine the prevalence of *Francisella tularensis*, members of the *Ehrlichia phagocytophila* genogroup, *Borrelia burgdorferi* sensu lato, and tick-borne encephalitis virus in ticks. Eur. J. Clin. Microbiol. Infect. Dis. 19, 427–432.