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25 factors of the second wave of the disease. Remarkably, among the 33 sheep examined, 17
26 were infected with two strains of haemotropic mycoplasmas. Cloning and sequencing isolates
27 of the latter showed that one of the strains was 99.4-99.8% identical to *M. ovis* (AF338268),
28 while the second was only 96.8-97.9% identical and contained a 17-bp deletion. Different
29 isolates of both strains were demonstrated in the same animal. When analyzing possible risk
30 factors for fatal disease outcome, we found that among sheep born prior to the 1997 outbreak
31 significantly more animals survived the second outbreak than succumbed to disease. In
32 addition, locally born sheep were less frequently diseased than sheep introduced into the flock
33 from other places. This suggests an immunoprotective effect in some animals. Concurrent
34 infection with *Anaplasma ovis* was detected in 24 of the 33 evaluated sheep. In conclusion,
35 this is the first study to demonstrate the existence of and characterize two genetically distinct
36 ovine haemotropic mycoplasma strains in a sheep flock with fatal haemolytic anaemia.

37

38 *Keywords:* sheep; anaemia; haemotropic mycoplasmas; *Mycoplasma ovis*; *Anaplasma ovis*

39

40 **1. Introduction**

41

42 Haemotropic mycoplasmas are unculturable bacteria that may cause haematological
43 disorders in various hosts (Ristic and Kreier, 1984). They had been classified as members of
44 the Anaplasmataceae family (order Rickettsiales) and were further divided into two genera:
45 *Haemobartonella*, characterized by mostly epierythrocytic, coccoid forms, and
46 *Eperythrozoon*, existing mostly in ring forms on red blood cells and free in the plasma.
47 However, analysis of 16S rRNA gene sequences revealed the close relationship of these
48 bacteria with the genus *Mycoplasma* (Neimark et al., 2001). Until now, the only species
49 recognized in sheep was *Mycoplasma ovis* (formerly *Eperythrozoon ovis*), which may induce

50 poor weight gain, severe anaemia, and even mortality in lambs and, occasionally, young adult
51 sheep (Campbell et al., 1971; Daddow, 1979). Mild clinical signs and persistent bacteraemia
52 associated with chronic infection have also been described in older animals (Gulland et al.,
53 1987; Mason and Statham, 1991).

54 In Hungary, the first and thus far only reported case of ovine eperythrozoonosis
55 resulted in 8.5% (35 out of 412) morbidity and 2.2% (9 out of 412) mortality (Hajtós et al.,
56 1998). The disease manifested between August and November of 1997, and mainly affected
57 yearlings. *Mycoplasma ovis* infection apparently persisted in the same flock of sheep, with
58 only sporadic and mild pathogenic effects for several years. However, in July and August of
59 2006, a new disease outbreak occurred. The aim of the present study was to characterize the
60 causative agent of the latter using molecular methods and to reveal underlying factors that
61 may have contributed to the more severe outcomes, as well as to the altered seasonal pattern
62 and affected age group of the disease.

63

64 **2. Materials and methods**

65

66 **2.1. Case history and sample collection**

67

68 Clinical signs of fatal *M. ovis* infection (anaemia, submandibular oedema, weight loss) were
69 noted between July and August of 2006 in 39 animals from a flock of 546 sheep (designated
70 flock A) in Northeast Hungary. Data obtained for the animals, including the 30 sheep that
71 died during the outbreak (group A1), were recorded. Ten months after the first clinical
72 manifestation, EDTA anticoagulated blood samples were collected by jugular venipuncture
73 from a total of 33 sheep: 9 sheep with typical clinical signs that survived the outbreak (group
74 A2); 16 animals with no characteristic clinical signs (group A3); and 8 animals from a

75 neighboring flock (designated flock B) in which clinical signs attributable to *M. ovis* had
76 never been observed. Blood smears were prepared from fresh EDTA anticoagulated blood and
77 stained with Giemsa. Haematological values were determined using an Abacus haematology
78 analyzer (Diatron GmbH, Vienna, Austria). Sheep in both flocks (A and B) were supervised
79 by the same veterinarian and received the same nutrition and prophylactic medication, but
80 were housed in separate buildings on the same farm and grazed in different pastures.

81

82 **2.2. Nucleic acid extraction**

83

84 DNA was purified from 33 blood samples using the MagNA Pure LC total nucleic acid
85 isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). First, 100 µl of EDTA
86 anticoagulated blood was diluted in the same volume of PBS without MgCl₂ and CaCl₂
87 (Invitrogen, Basel, Switzerland). Then, 300 µl of lysis buffer containing guanidinium
88 thiocyanate and Triton X-100 (Roche Diagnostics) was added. The lysed samples were loaded
89 into wells on the automated MagNA Pure LC Instrument. Two PBS controls were included in
90 each run to monitor potential cross-contamination. The elution volume was 100 µl. Samples
91 were stored at -20°C until further analysis. The presence of amplifiable DNA was confirmed
92 for each sample using an 18S rRNA gene real-time TaqMan PCR assay (Applied Biosystems,
93 Rotkreuz, Switzerland) (data not shown).

94

95 **2.3. Real-time TaqMan PCR to detect *M. ovis* infection**

96

97 A quantitative real-time TaqMan PCR assay developed for *M. wenyonii* (AF016546) was
98 used to screen the DNA samples for *M. ovis* (Meli et al., submitted for publication). The PCR
99 amplified a 119-bp long fragment of the 16S rRNA gene of *M. wenyonii*. Primer and probe

100 sequences (forward primer, 5'-CCA CGT GAA CGA TGA AGG TCT T-3'; reverse primer,
101 5'-GGC ACA TAG TTA GCT GTC ACT TAT TCA A-3'; and probe, 5'-6FAM-AGT ACC
102 ATC AAC GCG CGC TGA TTT CCT AG-MGB-3') demonstrated 100% identity with the
103 sequence of the 16s rRNA gene of *M. ovis* (AF338268). The 25 µl PCR reaction mixture
104 contained 900 nM of each primer, 0.25 µM probe, 12.5 µl of 2× qPCR MasterMix
105 (Eurogentec, Seraing, Belgium), and of 5 µl template DNA. The amplification mixture
106 contained dUTP for use with uracil-N-glycosylase to prevent carryover of the PCR
107 amplicons. The cycling conditions were: incubation for 2 min at 50°C and then a 10 min
108 initial denaturation at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C using
109 the ABI Prism 7700 sequence detection system (Applied Biosystems).

110

111 **2.4. Amplification and sequencing of the 16S rRNA gene of ovine haemotropic** 112 **mycoplasmas**

113

114 Initially, a conventional PCR was used to amplify an approximately 200-bp long region of the
115 16S rRNA gene as described elsewhere (Jensen et al., 2001). PCR products were visualized
116 with ethidium bromide in a 2.5% agarose gel.

117 In a second step, two samples from each group (A2, A3, and B) were chosen for
118 further analysis by cloning and sequencing. Samples were selected according to Ct values
119 (low Ct value = high load), and assessed by conventional PCR using species-specific primers
120 for *M. ovis* (forward: 5'-AGAGTT TGA TC(A/C) TGG CTC AG-3', reverse: 5'-CGG TTA
121 CCT TGT TAC GAC TT-3'), which amplify a 1,485-bp region of the 16S rRNA gene, as
122 described elsewhere (20). The 25 µl reaction mixture contained 5 µl of 5× High Fidelity PCR
123 buffer (Finnzymes: BioConcept, Allschwil, Switzerland), 0.5 µM of each primer, 0.2 mM
124 dNTP mixture (Sigma-Aldrich, Buchs, Switzerland), 1 U of Phusion High Fidelity DNA

125 polymerase (Finnzymes), 15.25 μ l of nuclease-free water, and 2.5 μ l of template DNA.
126 Amplification was performed using a T-personal 48 Thermocycler (Biometra GmbH,
127 Goettingen, Germany). The thermal profile consisted of an initial denaturation step at 98°C
128 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
129 elongation at 72°C for 10 min. A portion of the PCR product was visualized in a 1% agarose
130 gel stained with ethidium bromide after electrophoresis. The remaining portion was purified
131 with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and cloned into the pCR®4-TOPO®
132 (Invitrogen) vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). Clones
133 were screened by colony PCR, i.e. the *M. wenyonii* TaqMan assay as described above. The
134 fifteen positive clones that produced the lowest Ct values were selected, and plasmid DNA
135 was purified using the QIAprep Spin Miniprep kit (Qiagen, Hombrechtikon, Switzerland).
136 Cycle sequencing was performed with 4 μ l of DNA, 3.3 pmol of plasmid-specific primers, an
137 additional internal primer (5'-GGG AGG CTG ATC CAT TGT TA-3'), and the BigDye
138 Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems). The cycling
139 conditions were as follows: 1 min at 96°C, followed by 25 cycles of 96°C for 10 s and 50°C
140 for 5 sec, and a final incubation at 60°C for 4 min. Products were purified using SigmaSpin™
141 Post-Reaction Purification Columns (Sigma-Aldrich) and analyzed on the ABI Prism 310
142 Genetic Analyzer (Applied Biosystems).

143 Obtained sequences were edited and aligned with a consensus sequence using
144 SeqScape (Version 1.1, Applied Biosystems), and then compared to the 16S rRNA gene of *M.*
145 *ovis* (AF338268). For phylogenetic analysis, the sequences were aligned with known
146 mycoplasma sequences from GenBank using ClustalW (Thompson et al., 1994) and, if
147 necessary, manually adjusted. Only the nucleotides available for all included sequences were
148 used in the phylogenetic analysis. A bootstrap phylogenetic tree demonstrating the
149 relationship of sheep isolates to other haemoplasma species was created by the Neighbor-

150 Joining method (Saitou and Nei, 1987) using a distance matrix corrected for nucleotide
151 substitutions based on the Kimura 2-parameter model. The dataset was resampled 1,000 times
152 to generate bootstrap values. Phylogenetic and molecular evolutionary analyses were
153 conducted using MEGA version 4 (Kumar et al., 2004).

154 New sequences were submitted to GenBank (accession numbers: EU165509-165513,
155 EU828579-EU828582).

156

157 **2.5. Evaluation of *Anaplasma ovis* infection**

158

159 The blood samples were screened for the presence of members of the Anaplasmataceae family
160 by conventional PCR with the following primers: Ehr1 (5'- TTT ATC GCT ATT AGA TGA
161 GCC TAT G -3') and Ehr2 (5'- CTC TAC ACT AGG AAT TCC GCT AT -3'), as described
162 elsewhere (Goodman et al., 1996). In addition, the presence of *A. phagocytophilum* was
163 excluded based on a specific real-time TaqMan assay (Wicki et al., 2000). Five samples were
164 further analyzed by conventional PCR with primers specific for the *msh4* gene of *A. ovis* and
165 *A. marginale* (de la Fuente et al., 2001) using the same conditions as indicated above for the
166 16S rRNA gene of *M. ovis*. The PCR products were then sequenced.

167

168 **2.6. Statistical analyses**

169

170 Calculations were performed with the software Analyse-it (Analyse-it Software Ltd., Leeds,
171 United Kingdom) for Microsoft Excel. Risk factors for haemoplasma infections were
172 evaluated using the Mann-Whitney U Test for continuous variables and the Fisher's exact test
173 (cell frequencies ≤ 5) or Chi² Test (cell frequencies > 5) for categorical variables. Differences
174 were regarded as significant at $P < 0.05$.

175

176 **3. Results**

177

178 **3.1. Clinical outbreak and characteristics of affected animals**

179

180 In July and August of 2006, 39 adult animals from a Hungarian sheep flock (flock A) showed
181 typical signs of eperythrozoonosis. All affected animals were 5 to 12 years of age. In their
182 blood smears two morphologically distinguishable moieties were visible: multiple small
183 forms characteristic of *M. ovis* and individual smaller or larger coccoid bodies (Fig. 1). A total
184 of 30 sheep died within 4 to 30 days after the onset of clinical signs despite initiation of
185 oxytetracyclin treatment at two- to four-day intervals. Both mortality (30 out of 546; 5.5%)
186 and lethality (30 out of 39; 77%) were significantly higher ($P = 0.0124$ and $P < 0.0001$,
187 respectively) as compared to the previous outbreak in the same flock in 1997 (mortality 2.2%:
188 9 out of 412, lethality 25.7%: 9 out of 35).

189 Merino sheep represented only 22% (120 out of 546) of flock A, whereas the
190 neighboring flock (flock B: never diseased) consisted exclusively of this breed (Table 1). The
191 remaining sheep in flock A were predominantly Ille de France and Bábolna Tetra crossbreeds.

192 Significantly fewer sheep that had been affected by the disease (groups A1 and A2), were
193 born locally (38%: 15 out of 39) as compared to those in group A3 that demonstrated no
194 clinical signs (65%: 330 out of 507; $P = 0.0016$; Table 1). Among the group of sheep that had
195 died during the 2006 outbreak (A1), there were significantly fewer sheep born prior to the
196 1997 outbreak (33%: 10 out of 30) than in group A2, which consisted of the survivors of the
197 2006 disease outbreak (78%: 7 out of 9; $P = 0.0262$; Table 1).

198 Haematological values were within the normal range, except for haematocrit and
199 haemoglobin concentrations in the recently diseased sheep (group A2). These sheep had
200 significantly lower haematocrit (0.25 ± 0.08) and haemoglobin ($76.5 \text{ g/l} \pm 24.5 \text{ g/l}$) levels than
201 those in healthy control group A3, which exhibited values of (0.38 ± 0.10 ; $P = 0.0168$) and
202 ($105.1 \text{ g/l} \pm 13.5 \text{ g/l}$; $P = 0.018$), respectively. In group B the haematocrit was 0.325 ± 0.05 and
203 the haemoglobin concentration $100.25 \pm 16.41 \text{ g/l}$.

204

205 **3.2. Sample prevalence of ovine haemotropic mycoplasmas**

206

207 Using a real-time PCR assay, 52% of the examined animals (17 out of 33) tested positive,
208 including sheep from all three sample groups: A2, A3, and B (Table 1). No significant
209 differences were found in sample prevalence among the three groups. All sheep that tested
210 positive by real-time PCR were also positive in conventional PCR and showed PCR products
211 of different lengths.

212

213 **3.3. Molecular characterization of ovine haemotropic mycoplasmas**

214

215 Cloning and sequencing of the near-complete 16S rRNA gene of haemotropic mycoplasmas
216 was successful for four sheep (one from group A2, one from A3, and two from flock B), with

217 two to five clones obtained per sheep. Four sequences (designated IS-1 to IS-4) showed 99.4-
218 99.8% identity with *M. ovis* (AF338268). In contrast, five isolates (designated IS-5 to IS-9)
219 were found to be only 96.8-97.9% identical to *M. ovis* (Table 2). On a phylogenetical tree four
220 of the latter made up a separate cluster, while one occupied an intermediate position between
221 the *M. ovis* cluster and the new cluster (Fig. 2). The 16S rRNA gene sequence of the new
222 isolates differed from that of *M. ovis* (AF338268) at several nucleotide positions along the full
223 length of the gene (Table 2). In addition, a gap of 17 bp, including nucleotides 440 to 456,
224 was demonstrated in IS-5 to IS-9. Identical isolates were found in more than one animal, but
225 dissimilar isolates of both strains were also detected in the same animal (Table 2).

226

227 **3.4. Co-infection with an *Anaplasma* sp.**

228

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

235

236 **4. Discussion**

237

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

240 *Mycoplasma ovis* infection is usually accompanied by mild clinical signs and no
241 fatalities among adult sheep (Neimark et al., 2004). This is in contrast to the outbreak reported

242 in the present study. However, the prevalence of haemotropic mycoplasma infection in the
243 diseased flock (A) was not significantly different from that in flock B, where clinical signs
244 attributable to *M. ovis* have never been observed. Besides differences in breed composition of
245 the two flocks, the most important factor that could have influenced the clinical manifestation
246 is the co-infection with *A. ovis*. This turned out to be significantly less prevalent in the healthy
247 flock (B) than in the diseased flock (A). Although simultaneous eperythrozoonosis and
248 anaplasmosis have been documented (Splitter et al., 1955), no reports are currently available
249 on the potential synergism between these two haemotropic bacteria. Decreased haemoglobin
250 concentrations and haematocrit values reported for eperythrozoonosis (Gulland et al., 1987)
251 are usually not detected in *A. ovis*-infected adult sheep (Splitter et al., 1956; Hornok et al.,
252 2007). Therefore, these changes detected in the present study were most likely caused by
253 haemotropic mycoplasmas.

254 In the unaffected group (A3) there was a higher proportion of locally born sheep than
255 in the two groups affected by *M. ovis* (A1, A2). This raises the possibility of a certain degree
256 of innate immunity (natural resistance) associated with endemic foci, which is known to exist
257 against other agents infecting erythrocytes (Gern et al., 1988). At the same time, sheep that
258 survived the current disease outbreak were more frequently born prior to the previous
259 outbreak in 1997 than sheep that succumbed, suggesting the development of an age-related
260 and/or acquired immunity to haemotropic mycoplasmas.

261 The disease outbreak between August and November of 1997 may have been a
262 consequence of the introduction of infected animals into the flock (Hajtós et al., 1998).
263 However, no animals were introduced into flock A in 2006. Therefore, exclusive
264 manifestation of the disease from July to August may have been associated with the seasonal
265 activity of potential arthropod vectors. Since mosquitoes may transmit *M. ovis* (Daddow,
266 1980; Howard, 1975) and they depend on water for their development, it may be relevant that

267 the pastures grazed by sheep flocks A and B are flanked by a river. It is documented that
268 heavy rainfalls with floods in 2006 entailed an early summer mosquito invasion in Hungary
269 and promoted vector-borne transmission of different agents in the region (Vasconcelos,
270 2006), possibly including an upsurge in ovine haemoplasma infection of naive animals. On
271 the other hand, ticks as competent vectors of certain haemotropic mycoplasmas (Seneviratna
272 et al., 1973) were not found in the pastures or on the sheep in 2006 or 2007 (data not shown).

273 The disease outbreak in 2006 may have also been influenced by the appearance of the
274 second, genetically distinct ovine haemoplasma strain. Reclassification of haemotropic
275 *Mycoplasma* spp. was based on 16S rRNA gene sequences (Neimark et al., 2001).
276 Accordingly, the new sheep haemoplasma strain is most closely related to *M. ovis* (96.8-
277 97.9% identity to AF338268). At the same time, it is also substantially different from it, most
278 notably due to a 17 bp long deletion. Similar genotypic variants of haemoplasmas (with or
279 without a gap in their 16S rRNA sequence) were shown to exhibit higher or lower
280 pathogenicity in other hosts (Jensen et al., 2001; Neimark et al., 2001). The overall ratio of
281 16S sequence divergence between *M. ovis* and the novel ovine haemotropic mycoplasma
282 strain is close to the value for the declaration of a new species (Drancourt and Raoult, 2005).
283 However, to confirm if the novel strain deserves the status of a *Candidatus*, preferably
284 additional genes should be compared and the pathogenicity of the agent further evaluated in
285 monoinfections.

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

289

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291

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297

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385

386 **Figure captions:**

387

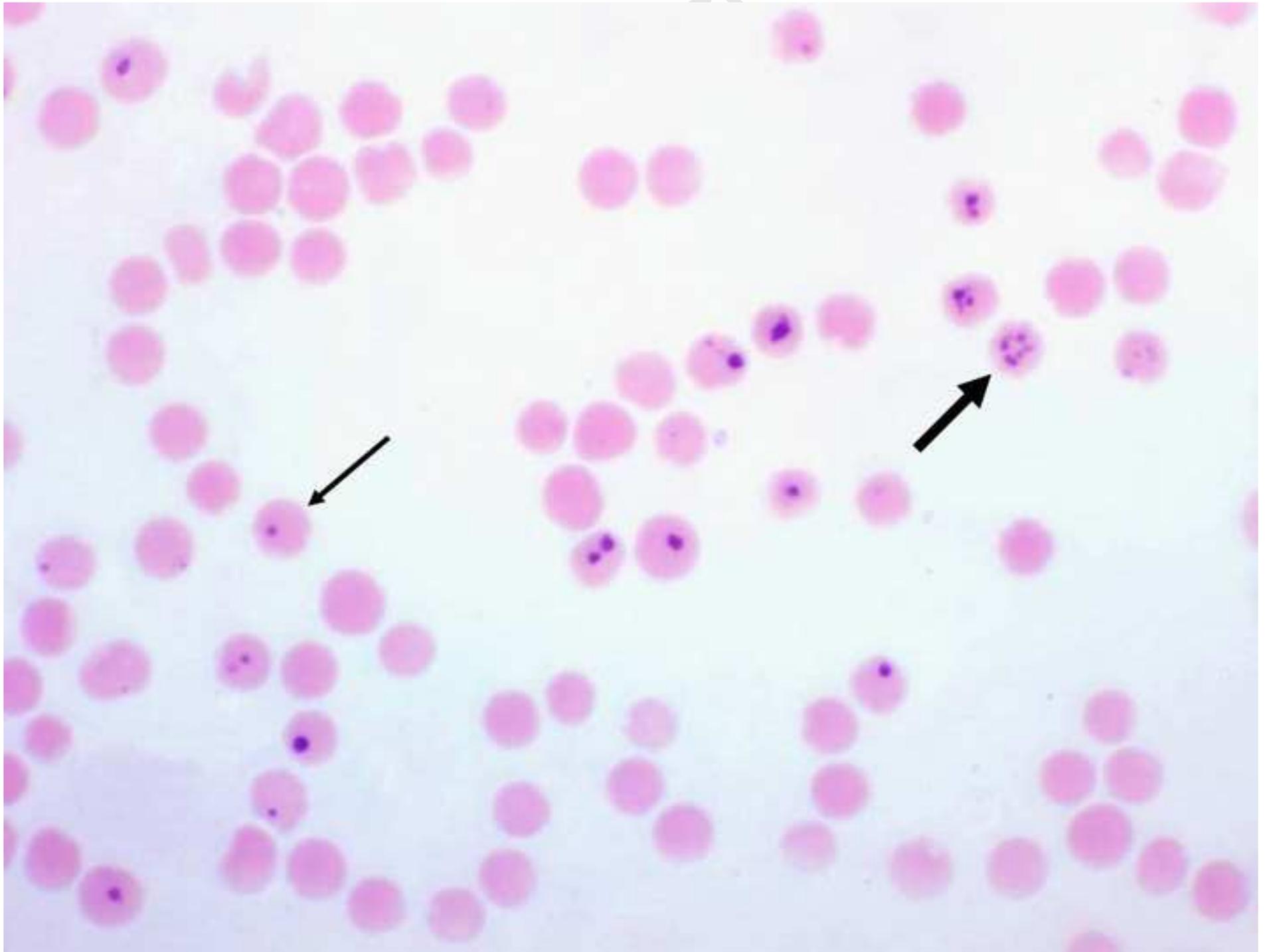
388 **Figure 1.** Blood smear of a sheep concomitantly infected with *Anaplasma ovis* (thin arrow)
389 and haemotropic mycoplasmas (thick arrow). The former are usually individually situated in
390 red blood cells (dark basophilic), whereas the latter tend to occur in multiplicity (clustered or
391 separated)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

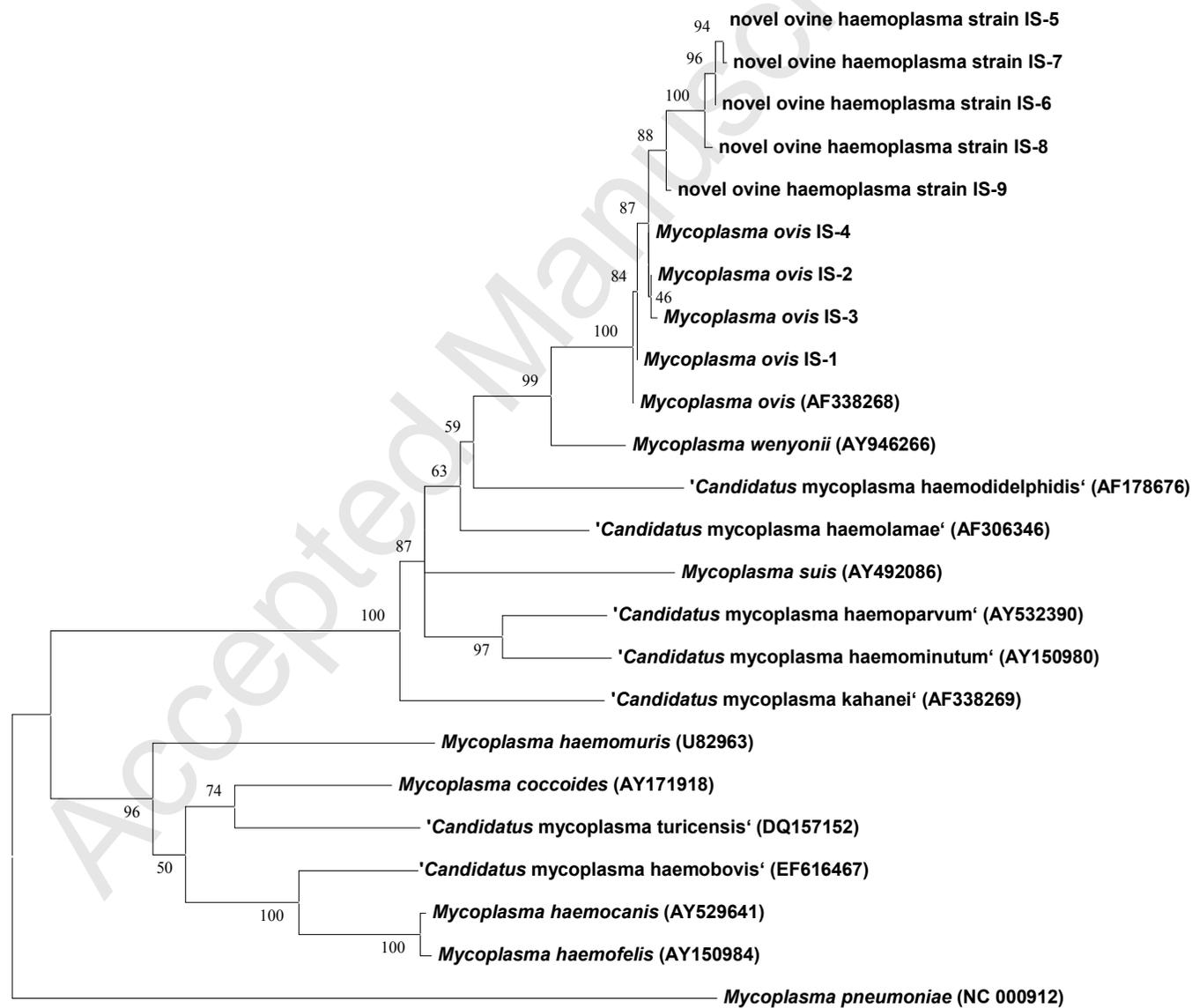
392

393 **Figure 2.** Phylogenetic tree of the near-complete 16S rRNA gene sequences of haemotropic
394 mycoplasmas available in GenBank in comparison with those of the isolates from the
395 Hungarian sheep. The numbers at the nodes were generated from 1,000 Bootstrap
396 resamplings. The bar represents the mean number of differences per 50 sites.

397

Figure 1





0.005

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 (A1-3) 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

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	
<i>M. ovis</i>	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	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	*	*	*	*	*	

¹ Position numbers given with respect to *M. ovis* (AF338268). Nucleotides identical to the latter are given as asterisks; mismatches are listed.

Upper indexes: a – one sheep from group A2; b – one sheep from group A3; c and d – two sheep from flock B