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1 **First serological and molecular evidence on the endemicity**
2 **of *Anaplasma ovis* and *A. marginale* in Hungary**

3
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25 **Abstract**

26

27 Recurring and spontaneously curing spring haemoglobinuria was recently reported in a
28 small sheep flock in a selenium deficient area of northern Hungary. In blood smears of two
29 animals showing clinical signs, *Anaplasma*-like inclusion bodies were seen in erythrocytes.
30 To extend the scope of the study, 156 sheep from 5 flocks and 26 cattle from 9 farms in the
31 region were examined serologically with a competitive ELISA to detect antibodies to
32 *Anaplasma marginale*, *A. centrale* and *A. ovis*. The seropositivity in sheep was 99.4%, and in
33 cattle 80.8%. *A. ovis* and *A. marginale* were identified by PCR and sequence analysis of the
34 major surface protein (*msp*) 4 gene in sheep and cattle, respectively.

35 Haemoglobinuria, an unusual clinical sign for anaplasmosis might have been a
36 consequence of transient intravascular haemolysis facilitated by selenium deficiency in
37 recently infected sheep, as indicated by the reduction of mean corpuscular haemoglobin
38 concentration (MCHC). Membrane damage was also demonstrated for parenchymal cells,
39 since their enzymes showed pronounced elevation in the plasma. Ticks collected from
40 animals in the affected as well as in neighbouring flocks revealed the presence of
41 *Dermacentor marginatus*, *Ixodes ricinus* and *D. reticulatus*, with the dominance of the first.

42 The present data extend the northern latitude in the geographical occurrence of ovine
43 anaplasmosis in Europe and reveal the endemicity of *A. ovis* and *A. marginale* in Hungary.

44

45 **Keywords:** Anaplasmosis; *Dermacentor*; *Ixodes*; tick; haemoglobinuria; selenium deficiency

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49

50 1. Introduction

51

52 Representatives of the genus *Anaplasma* belong to the order Rickettsiales and are
53 obligate intracellular etiological agents of tick-borne diseases of mammals. In red blood cells
54 of ruminants three closely related species occur: the most pathogenic *A. marginale* and the
55 less pathogenic *A. centrale* in cattle, and the moderately pathogenic *A. ovis* in small ruminants
56 (Kuttler, 1984; Lew et al., 2003). Although anaplasmosis is more frequently associated with
57 haemolytic anaemia in goats, *A. ovis* can also cause disease in sheep, particularly in animals
58 exposed to stress or other predisposing factors (Splitter et al., 1956; Friedhoff, 1997).

59 In Europe the geographical distribution of ovine anaplasmosis is restricted to the
60 southern countries, including France (Cuille and Chelle, 1936), Italy (de la Fuente et al.,
61 2005), Turkey (Sayin et al., 1997), Greece (Papadopoulos, 1999), Bulgaria (Christova et al.,
62 2003) and Southeast Romania (Ardeleanu et al., 2003). Similarly, *A. marginale* is endemic
63 mainly to the Mediterranean-Balkanian countries: France (Poncet et al., 1987), Spain and
64 Portugal (de la Fuente et al., 2004; Caeiro, 1999), Italy (de la Fuente et al., 2005), but it has
65 also been reported in the northern latitude of the Alpean region (Baumgartner et al., 1992;
66 Dreher et al., 2005a). In Hungary only sporadic occurrence of bovine anaplasmosis was
67 recognized in an imported herd of cattle (Dankó et al., 1982).

68 During the past few years recurring, transient spring haemoglobinuria was noted in a
69 small flock of sheep in a selenium deficient area of northern Hungary. The aim of the present
70 study was to find the causative agent, and to collect relevant data on local sheep flocks and
71 cattle.

72

73

74

75 **2. Materials and methods**

76

77 **2.1. Clinical history and sample collection**

78

79 The small flock of Merino sheep in the present study consists of 37 animals that have
80 been kept in Domaháza in northern Hungary for the past 5 years, and prior to that in a
81 neighbouring village. No animals were introduced from outside this area.

82 In the spring of 2006 samples were collected as soon as the notification on clinical
83 signs was received from the local veterinarian. Fresh anticoagulated (EDTA-containing and
84 heparinized) blood was taken from two sheep (A and B) noted with haemoglobinuria this year
85 (from sheep A 2 and 24 days, from sheep B 2 days after the appearance of clinical signs) and
86 from three randomly selected others (C-E) at the same time as from sheep B. Serum samples
87 were collected from all animals in this and from 119 sheep in four neighbouring flocks (kept
88 within a distance of 1 km). Serum and blood samples were also obtained from 26 local cattle
89 (Hungarian Pied, from 9 farms) grazing the same pastures. The age of the animals was
90 ascertained whenever possible.

91 Ticks were removed from at least 30% of sheep in this as well as in 12 other flocks of
92 the region (within 50 km) for species identification.

93

94 **2.2. Clinical laboratory procedures**

95

96 Thin blood films were prepared from samples of sheep A and B, fixed with methanol
97 and stained with Giemsa. Haematological values were determined using an Abacus
98 haematology analyser (Diatron GmbH, Vienna, Austria), and biochemical parameters with an

99 automatic spectrophotometer (RX Daytona, Randox Laboratories Ltd., Crumlin, UK). Stained
100 blood smears were also made from samples of cattle included in this survey.

101

102 **2.3. Serology for *Anaplasma* spp.**

103

104 A competitive enzyme-linked immunosorbent assay (cELISA) was performed with
105 samples of 156 sheep and 26 cattle using the *Anaplasma* Antibody Test Kit from VMRD Inc.
106 (Pullmann, WA, USA) following the manufacturer's instructions. This assay detects serum
107 antibodies to a major surface protein (MSP5) of *A. marginale*, *A. centrale*, *A. ovis* and *A.*
108 *phagocytophilum*. Although approved only for bovines by the U.S. Department of
109 Agriculture, it could detect seroconversion of experimentally infected sheep, since their
110 antibodies compete successfully for free binding sites with monoclonal antibodies present in
111 the detection system of the test kit (Dreher et al., 2005b). Optical density (OD) values were
112 determined using an automatic Multiscan Plus microplate reader (model RS-232 C,
113 Labsystems, Helsinki, Finland), and the percentage of inhibition was calculated as follows: I
114 (%) = $100 - (\text{sample OD} \times 100) / (\text{mean OD of three negative controls})$. Samples with an
115 inhibition $\geq 30\%$ were regarded positive.

116

117 **2.4. Molecular biology**

118

119 **2.4.1. DNA extraction**

120

121 DNA was extracted from 200 μl amounts of EDTA blood from 5 seropositive sheep
122 (A-E) and 12 (including 2 seronegative) cattle using QIAamp DNA blood mini kit (QIAGEN,
123 Hilden, Germany) according to the manufacturer's instructions.

124

125 **2.4.2. *msp4* polymerase chain reaction (PCR) and sequencing**

126

127 The *Anaplasma* spp. *msp4* gene was amplified by PCR and sequenced as reported
128 previously (de la Fuente et al., 2005; 2007). For sheep samples, 1 µl (1-10 ng) DNA was used
129 with 10 pmol of each primer (*A. marginale/A. ovis*:

130 MSP45: 5'GGGAGCTCCTATGAATTACAGAGAATTGTTTAC3' and

131 MSP43: 5'CCGGATCCTTAGCTGAACAGGAATCTTGC3'; *A. phagocytophilum*:

132 MSP4AP5: 5'-ATGAATTACAGAGAATTGCTTGTAGG-3' and

133 MSP4AP3: 5'-TTAATTGAAAGCAAATCTTGCTCCTATG-3') in a 50 µl volume PCR (1.5
134 mM MgSO₄, 0.2 mM dNTP, 1× AMV/*Tfl* reaction buffer, 5u *Tfl* DNA polymerase)

135 employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were
136 performed in an automated DNA thermal cycler (Techne model TC-512, Cambridge,
137 England, UK) for 35 cycles. After an initial denaturation step of 30 sec at 94°C, each cycle

138 consisted of a denaturing step of 30 sec at 94°C, an annealing for 30 sec at 60°C and an
139 extension step of 1 min at 68°C for *A. marginale/A. ovis* and an annealing-extension step of 1

140 min at 68°C for *A. phagocytophilum*. Negative control reactions were performed with the
141 same procedures, but adding water instead of DNA to monitor contamination of the PCR. The

142 program ended by storing the reactions at 10°C. PCR products were electrophoresed on 1%
143 agarose gels to check the size of amplified fragments by comparison to a DNA molecular
144 weight marker (1 Kb DNA Ladder, Promega). Amplified fragments were resin purified

145 (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both
146 strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain).

147 At least two independent clones were sequenced for each PCR.
148 For cattle samples, the same primers were used as for the sheep samples (de la Fuente
149 et al., 2005; 2007) with some differences in the PCR and sequencing. One µl (1–10 ng) of

150 extracted DNA was added to a 49 µl reaction mixture comprised of 10 pmol of each primer,
151 1.5 mM MgCl₂, 0.2 mM dNTP, 5 µl 10× PCR buffer and 5u of GoTaq Flexi DNA polymerase
152 (all Promega). Amplification was performed using a Tpersonal 48 thermal cycler (Biometra
153 GmbH, Göttingen, Germany) under the same conditions as with ovine samples. Amplified
154 DNA was subjected to electrophoresis in a 1% agarose gel (100 V, 40 min), pre-stained with
155 ethidium bromide and viewed under ultra-violet light. After purification with Wizard[®] SV gel
156 and PCR clean-up system (Promega), ABI Prism[®] Big Dye Terminator v3.1 Cycle
157 Sequencing Kit (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) was
158 used for DNA sequencing reactions. Samples were then examined using an ABI Prism[®] 3100
159 Genetic Analyser at the Agricultural Biotechnology Center Gödöllő, Hungary.

160

161 **2.4.3. Sequence analysis**

162

163 Obtained sequences were checked with Chromas v.1.45 and compared to sequence
164 data available from GenBank[®], using the BLAST 2.2.15. program
165 (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignment was performed using
166 the program AlignX (Vector NTI Suite V 5.5, Invitrogen, North Bethesda, MD, USA) with an
167 engine based on the Clustal W algorithm (Thompson et al., 1994). Nucleotides were coded as
168 unordered, discrete characters with five possible character states: A, C, G, T or N and gaps
169 were coded as missing data.

170

171 **2.4.4. Sequence accession numbers**

172

173 New sequences were submitted to GenBank[®] database. The GenBank accession
174 numbers for *msp4* sequences of *A. ovis* and *A. marginale* strains are EF190509-EF190513 and
175 EF190508, respectively.

176

177 **2.5. Statistical analysis**

178

179 Exact confidence intervals for the prevalence rates were calculated according to
180 Sterne's method. Data were compared by using Fisher's exact test, and means of inhibition
181 values by *t*-test. Differences were regarded significant when $P \leq 0.05$.

182

183 **3. Results**

184

185 **3.1. Clinicopathological and haematological findings**

186

187 In each year, usually in May a few sheep of the examined flock showed
188 haemoglobinuria that lasted for 1-3 days and then ceased spontaneously. In 2006 only two
189 animals were noted with such transient clinical signs: sheep A in April and sheep B in May.

190 In blood smears of sheep A and B very few ($< 1\%$) erythrocytes contained one or more
191 small ($< 1 \mu\text{m}$) round, dark staining bodies on the periphery (submarginally), suggestive of
192 infection with *Anaplasma* sp.. Regarding haematological parameters, only reduction of mean
193 corpuscular haemoglobin concentration (MCHC: normal value 310-340 g/l) could be
194 demonstrated in the two sheep with noted haemoglobinuria (277 and 295 g/l, respectively).
195 Biochemical analysis showed pronounced elevation of plasma levels of aspartate
196 aminotransferase (AST) and alanine aminotransferase (ALT) in all three examined animals
197 (A, B and C), and of alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT) in
198 sheep A and B (Table 1). Total protein was slightly increased. Other values were within their
199 normal range (data not shown).

200 In blood smears of examined cattle the percentage of infected erythrocytes was low (<
201 3%), and they contained 1-2 inclusion bodies in marginal, submarginal or central position
202 (with equal approximated proportion). No relevant clinical signs were observed in these cattle
203 during the past years.

204

205 **3.2. Serological and molecular characterization of *Anaplasma* infections**

206

207 Seroprevalence of anaplasmosis in five local flocks of sheep was 99.4% (CI: 96.5-
208 100%), as 155 of 156 animals had inhibition values $\geq 30\%$, decreasing with the advance of
209 age (Table 2). This implies that 1-2 year old sheep had a significantly ($P < 0.005$) higher
210 mean antibody level when compared to that of 6-10 year old sheep. In the cELISA 80.8% (21
211 of 26, CI: 60.7-93.5%) of local cattle were also found positive.

212 *A. phagocytophilum* was not detected in analysed samples. The *Anaplasma* spp. *msp4*
213 gene was successfully amplified from all 5 ovine samples. Sequence analysis of the PCR
214 products established that all of them correspond to *A. ovis*, differing from each other and
215 from those found in GenBank in some positions (Table 3). The only exception was EF190511
216 which showed 100% sequence identity to *A. ovis* obtained from Sicilian sheep (AY702923).

217 The *Anaplasma* spp. *msp4* gene was also successfully amplified from 4 of the 12
218 bovine samples. One of them was sequenced, revealing 99.4% similarity to an *A. marginale*
219 sequence deposited in GenBank (AY127073).

220

221 **3.3. Ticks collected from sheep and cattle**

222

223 The most dominant tick species found during the spring in the affected sheep flock
224 was *Dermacentor marginatus* (95.9%: 70 of 73), followed by *Ixodes ricinus* (2.7%: 2 of 73)

225 and *D. reticulatus* (1.4%: 1 of 73). This prevalence of *D. marginatus* was not significantly
226 different from that observed in other parts of the region (92.8%: 346 of 373).

227 The predominant tick species found on local cattle in the autumn was *D. reticulatus*
228 (data not shown).

229

230 **4. Discussion**

231

232 This is the first report of ovine anaplasmosis in Hungary. Haemoglobinuria in the
233 relevant sheep flock (according to its seasonality) was suspected to be the effect of a tick-
234 borne pathogen. Although data are not available on the occurrence of ovine babesiosis or
235 theileriosis in Hungary, in the present survey the etiological role of piroplasms was excluded
236 by PCR (data not shown). Anaplasmosis was diagnosed on the basis of erythrocyte inclusion
237 bodies seen in blood smears of local sheep (and cattle), and their high rate of seropositivity to
238 *Anaplasma* spp. in the cELISA, but the causative agent could not be identified with these
239 methods, as *A. marginale* can also infect sheep (Kuttler, 1984; Sharma, 1988). Additionally,
240 in order to exclude from the prevalence rates seropositivity due to cross-reacting *A.*
241 *phagocytophilum* (Dreher et al., 2005b), two *msp4* PCR assays were applied: one specific for
242 *A. marginale/A. ovis* (primers MSP45 and MSP43), and another for *A. phagocytophilum*
243 (primers MSP4AP5 and MSP4AP3) (de la Fuente et al., 2005). Identification of the species
244 was done by sequence analysis of *msp4* amplicons (de la Fuente et al., 2005) which revealed
245 the presence of *A. ovis* in sheep and *A. marginale* in cattle, according to their typical hosts
246 (Kuttler, 1984). The sequence heterogeneity between the five Hungarian *A. ovis* isolates
247 suggest that *msp4* genotypes may vary not only among geographic regions and different hosts
248 (de la Fuente et al., 2007), but also between individual sheep of the same flock.

249 The first report of bovine anaplasmosis in Hungary dates back to 1978, when it was
250 diagnosed in an imported herd (Dankó et al., 1982). Those animals were successfully treated,
251 clinical signs disappeared, and no data on the occurrence of *A. marginale* have since been
252 reported in this country. Cattle of the present survey never left the region and were not
253 brought in from abroad, therefore this is the first recognition of an endemic focus and of the
254 autochthonous infection of cattle with *A. marginale* in Hungary. Since bovine anaplasmosis
255 frequently has a fatal outcome or necessitates culling of affected animals (Dreher et al.,
256 2005a), absence of relevant clinical signs in cattle of the study area suggests that the causative
257 agent is a less virulent strain that has been present for several years, allowing the reduction of
258 pathogenicity.

259 On the other hand, ovine anaplasmosis is usually a benign disease, but predisposing
260 factors may aggravate or influence its manifestation (Friedhoff, 1997). This should be taken
261 into account when considering the present results, as all studied animals were kept in a
262 selenium deficient area (Hajtós, 1982). Selenium deficiency is known to have an
263 immunosuppressive effect and promotes susceptibility to bacterial diseases (Van Vleet, 1980).

264 Haemoglobinuria is an unusual clinical sign of anaplasmosis, because anaemia results
265 from extravascular opsonization and phagocytosis of parasitized erythrocytes by
266 reticuloendothelial cells (Allen et al., 1981). However, in case of sheep in the present study a
267 significant role of this mechanism can not be substantiated, because affected animals did not
268 become anaemic, and their total bilirubin concentration was within the normal range. On the
269 contrary, a transient intravascular haemolysis might have occurred when red blood cells were
270 exposed to freshly inoculated *A. ovis*, which was facilitated by selenium deficiency, making
271 erythrocyte membranes more vulnerable (Rotruck et al., 1972). *Anaplasma marginale* was
272 also shown to induce downregulation of enzymes that have a selenium-dependent nature and
273 are important in the oxidant defence system of erythrocytes (Reddy et al., 1988; More et al.,

274 1989), thus the two factors may have acted synergistically. Similarly, haemoglobinuria in
275 ruminants with intraerythrocytic infectious agents was reported to be associated with
276 oxidative stress to erythrocytes (Sahoo et al., 2001).

277 Mild pathogenicity of *A. ovis* was further reflected by the low number of sheep noted
278 with haemoglobinuria, although this may have been influenced by differences between the
279 flocks (e.g. whether they were continuously monitored or not). Most haematological
280 parameters also remained within the normal range. In accordance with the present results
281 decreased MCHC level during ovine anaplasmosis was reported previously (Ramprabhu et
282 al., 1999). The blood chemistry values obtained in our study suggest pathological changes in
283 the liver and in muscles, but not in the kidney. Both selenium deficiency (Bickhardt et al.,
284 1999) and anaplasmosis (Allen et al., 1981) may induce elevation of AST and ALP, therefore
285 this is most likely attributable to a combined effect of the two. In contrast to
286 hypoproteinaemia frequently observed in case of sheep with selenium deficiency (Bickhardt
287 et al., 1999), the slight elevation in total protein level may have indicated humoral immune
288 response to *A. ovis* (hyperglobulinaemia). This was further justified by finding all except one
289 examined sheep seropositive in the area. The significant decrease of antibody levels with the
290 advance of age, the low percentage of infected erythrocytes compared to other reports
291 (Splitter et al., 1956; Allen et al., 1981), PCR negativity of 6 seropositive cattle and the
292 absence of clinical signs in most sheep as well as cattle may be associated with the carrier
293 state of these animals. Anaplasmosis usually progresses to a lifelong persistent and subclinical
294 infection (Palmer et al., 1998; Kieser et al., 1990), simultaneously providing the source for
295 tick-borne transmission of the pathogen (Kocan et al., 2003). This may also explain the
296 extremely high prevalence of ovine anaplasmosis in our study as observed by others
297 (Shompole et al., 1989), and makes it probable that in the same area both *A. ovis* and *A.*
298 *marginale* might be present in wildlife reservoirs too (Kuttler, 1984; de la Fuente et al., 2004).

299 According to data in the literature (Arthur, 1960) and the prevalence of tick species
300 found on sheep in the affected flock, mainly *Dermaacentor marginatus* could be implicated in
301 the transmission of *A. ovis* in northern Hungary. However, the vector of this species is
302 unknown in many regions of the world (Friedhoff, 1997). The occurrence of tick species in
303 more distant sheep of the region did not differ significantly from that in the affected flock,
304 and lambs are regularly exported to other parts of Hungary, therefore monitoring of ovine
305 anaplasmosis should be extended to a larger area in the country. Whether this stable endemic
306 focus indicates a unique case, or on the contrary, it reflects a northward expansion of the
307 geographical distribution of *A. ovis*, needs further investigation.

308

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310

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316

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Table 1. Blood biochemical parameters of sheep with (A, B) or without (C) haemoglobinuria

Parameter	Normal value	Sheep A	Sheep B	Sheep C
AST	< 60 U/l	140	175	161
ALT	< 10 U/l	16	30	54
bilirubin (total)	< 8 μ mol/l	2.2	1.7	7
ALP	40-200 U/l	382	295	n.a.
GGT	10-30 U/l	44.8	47.0	13.4
protein (total)	60-80 g/l	92.4	83.1	94.7

Blood was collected 24 days and 2 days after haemoglobinuria in sheep A and B, respectively.

Sheep C was sampled at the same time as sheep B. Abbreviations: AST - aspartate

aminotransferase, ALT - alanine aminotransferase, ALP - alkaline phosphatase, GGT -

gamma-glutamyl transpeptidase, n.a. – not available

Table 2. Antibody levels of sheep in the *Anaplasma* spp. cELISA according to age group

	age in years				
	1-2	3	4	5	6-10
sample number	19	38	27	23	34
mean inhibition value (%)	88.6	86.6	84.7	83.8	78.7
standard deviation	± 3.44	± 5.30	± 6.28	± 8.50	± 14.08

Table 3. Nucleotide differences in nine positions^b among *msh4* sequences from *A. ovis* isolates

Sequence ^a	30	139	178	270	287	302	438	470	549
EF190513	T	A	T	G	A	T	G	T	G
EF190509	C	*	*	A	G	*	A	C	C
EF190510	C	G	*	A	*	*	A	C	C
EF190512	C	*	C	*	*	C	A	*	C
EF190511	C	*	*	*	*	*	A	*	C

^a GenBank accession number.

^b The numbers represent the nucleotide position starting at translation initiation codon adenine.

Conserved nucleotide positions with respect to the EF190513 are represented with asterisks.