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ScienceDirect
Veterinary Microbiology 122 (2007) 316–322
veterinary microbiology
www.elsevier.com/locate/vetmic

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

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Received 21 December 2006; received in revised form 26 January 2007; accepted 29 January 2007

Abstract

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

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

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

Keywords: Anaplasmosis; *Dermacentor*; *Ixodes*; Tick; Haemoglobinuria; Selenium deficiency

1. Introduction

Representatives of the genus *Anaplasma* belong to the order Rickettsiales and are obligate intracellular

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doi:10.1016/j.vetmic.2007.01.024

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

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

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

2. Materials and methods

2.1. Clinical history and sample collection

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

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

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

2.2. Clinical laboratory procedures

Thin blood films were prepared from samples of sheep A and B, fixed with methanol and stained with Giemsa. Haematological values were determined using an Abacus haematology analyser (Diatron GmbH, Vienna, Austria), and biochemical parameters with an automatic spectrophotometer (RX Daytona, Randox Laboratories Ltd., Crumlin, UK). Stained blood smears were also made from samples of cattle included in this survey.

2.3. Serology for Anaplasma spp.

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

2.4. Molecular biology

2.4.1. DNA extraction

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

2.4.2. msp4 polymerase chain reaction (PCR) and sequencing

The *Anaplasma* spp. *msp4* gene was amplified by PCR and sequenced as reported previously (de la Fuente et al., 2005, 2007). For sheep samples, 1 μl (1–10 ng) DNA was used with 10 pmol of each primer (*A. marginale/A. ovis*: MSP45: 5′GGGAGCTCCTATG - AATTACAGAGAATTGTTTAC3′ and MSP43: 5′CC-CCGGATCCTTAGCTGAACAGGAATCTTGC3′; *A. phagocytophilum*: MSP4AP5: 5′-ATGAATTACAGA-GAATTGCTTGTAGG-3′ and MSP4AP3: 5′-TTAA-TTGAAAGCAAATCTTGCTCCTATG-3′) in a 50 μl volume PCR (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1× AMV/*Tfl* reaction buffer, 5 u *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Techne model TC-512, Cambridge, England, UK) for 35 cycles. After an initial denaturation step of 30 s at 94 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 30 s at 60 °C and an extension step of 1 min at 68 °C for *A. marginale/A. ovis* and an annealing-extension step of 1 min at 68 °C for *A. phagocytophilum*. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The program ended by storing the reactions at 10 °C. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 Kb DNA Ladder, Promega). Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain). At least two independent clones were sequenced for each PCR.

For cattle samples, the same primers were used as for the sheep samples (de la Fuente et al., 2005, 2007) with some differences in the PCR and sequencing. One micro liter (1–10 ng) of extracted DNA was added to a 49 μl reaction mixture comprised of 10 pmol of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 μl 10 × PCR buffer and 5 u of GoTaq Flexi DNA polymerase (all Promega). Amplification was performed using a Tpersonal 48 thermal cycler (Biometra GmbH, Göttingen, Germany) under the same conditions as with ovine samples. Amplified DNA was subjected to electrophoresis in a 1% agarose gel (100 V, 40 min), prestained with ethidium bromide and viewed under ultra-violet light. After purification with Wizard® SV gel and PCR clean-up system (Promega), ABI Prism® Big Dye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) was used for DNA sequencing reactions. Samples were then examined using an ABI Prism® 3100 Genetic Analyser at the Agricultural Biotechnology Center Gödöllő, Hungary.

2.4.3. Sequence analysis

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

2.4.4. Sequence accession numbers

New sequences were submitted to GenBank® database. The GenBank accession numbers for *msp4* sequences of *A. ovis* and *A. marginale* strains are EF190509–EF190513 and EF190508, respectively.

2.5. Statistical analysis

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

3. Results

3.1. Clinicopathological and haematological findings

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

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

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

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 μmol/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. 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
S.D.	±3.44	±5.30	±6.28	±8.50	±14.08

3.2. Serological and molecular characterization of Anaplasma infections

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

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

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

Table 3
Nucleotide differences in nine positions[b] among *msp4* 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.

3.3. Ticks collected from sheep and cattle

The most dominant tick species found during the spring in the affected sheep flock was *Dermacentor marginatus* (95.9%: 70 of 73), followed by *Ixodes ricinus* (2.7%: 2 of 73) and *D. reticulatus* (1.4%: 1 of 73). This prevalence of *D. marginatus* was not significantly different from that observed in other parts of the region (92.8%: 346 of 373).

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

4. Discussion

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

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

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

Haemoglobinuria is an unusual clinical sign of anaplasmosis, because anaemia results from extravascular opsonization and phagocytosis of parasitized erythrocytes by reticuloendothelial cells (Allen et al., 1981). However, in case of sheep in the present study a significant role of this mechanism cannot be substantiated, because affected animals did not become anaemic, and their total bilirubin concentration was within the normal range. On the contrary, a transient intravascular haemolysis might have occurred when red blood cells were exposed to freshly inoculated *A. ovis*, which was facilitated by selenium deficiency, making erythrocyte membranes more vulnerable (Rotruck et al., 1972). *Anaplasma marginale* was also shown to induce downregulation of enzymes that have a selenium-dependent nature and are important in the oxidant defence system of erythrocytes (Reddy et al., 1988; More et al., 1989), thus the two factors may have acted synergistically. Similarly, haemoglobinuria in ruminants with intraerythrocytic infectious agents was reported to be associated with oxidative stress to erythrocytes (Sahoo et al., 2001).

Mild pathogenicity of *A. ovis* was further reflected by the low number of sheep noted with haemoglobinuria, although this may have been influenced by differences between the flocks (e.g. whether they were

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

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

Acknowledgements

This research was partially supported by the grant ‘‘Epidemiología de zoonosis transmitidas por garrapatas en Castilla – La Mancha’’ (06036-00 ICS-JCCM). V. Naranjo is funded by Junta de Comunidades de Castilla – La Mancha (JCCM), Spain. The authors would like to acknowledge the advice of Martin J. Kenny (University of Bristol, UK). Without the contributions of J. Elek and I. Hajtós this work could not be done.

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