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

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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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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.