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FIRST MOLECULAR IDENTIFICATION OF *MYCOPLASMA OVIS* AND '*CANDIDATUS* M. HAEMOOVIS' FROM GOAT, WITH LACK OF HAEMOPLASMA PCR-POSITIVITY IN LICE

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In order to investigate haemotropic *Mycoplasma* (formerly *Eperythrozoon*) infection of goats, blood samples and blood-sucking lice (*Linognathus stenopsis*) were collected in two goat herds. DNA was extracted from 20 blood samples and from 49 lice allocated to six pools according to host individuals. Haemoplasma infection was detected in four goats by real-time PCR. From the sample with the highest bacterial load the simultaneous presence of *M. ovis* and '*Candidatus* M. haemoovis' was demonstrated by cloning and sequencing. Louse pools were haemoplasma negative, including those from bacteraemic animals. However, not only were *Anaplasma* inclusion bodies seen in blood smears from goats, but relevant PCR-positivity was also detected among lice. This is the first report of a molecular investigation on caprine haemoplasmas, including analysis of their blood-sucking lice. In summary, goats are susceptible to both molecularly characterised ovine haemoplasmas. On the other hand, goat sucking lice (*L. stenopsis*) do not appear to be potential vectors of these agents.

Key words: *Mycoplasma,* haemoplasma, eperythrozoon, goat

Haemotropic mycoplasmas or haemoplasmas (Mycoplasmatales: Mycoplasmataceae) are Gram-negative, wall-less epierythrocytic bacteria that were formerly called *Haemobartonella* and *Eperythrozoon* species (Neimark and Kocan, 1997; Neimark et al., 2001). They may cause haemolytic anaemia and other clinical signs in various mammals (Messick, 2004). In general, haemoplasmas are regarded host-specific, i.e. each member of the group can establish only in one or a few closely related host species. In sheep, previously only *Mycoplasma* (*E.*) *ovis* was described, to which goats also appear to be susceptible (Daddow, 1979). Although morphological differences between ovine and caprine

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eperythrozoa were noted (Daddow, 1979), they were regarded as the same species. Recently, however, a new haemoplasma genotype, '*Candidatus* M. haemoovis' has also been demonstrated from sheep (Hornok et al., 2009). With relevance to the clinical significance of the latter, not only do strains of *M. ovis* vary in their ability to cause disease (Messick, 2004), but pathogenic effects of a simultaneous infection with both ovine haemoplasmas may be exacerbated by concurrent anaplasmosis (Hornok et al., 2009).

Epidemiological data on ovine haemoplasmosis are scarce in the literature. There is indication that only certain blood-sucking insects may be involved in the transmission of *M. ovis*. For instance, *Culex* mosquitoes were shown to be competent vectors (Daddow, 1980), unlike sheep keds (*Melophagus ovinus*) (Foggie and Nisbet, 1964). However, highly sensitive molecular tools to analyse the vector potential of further species have only recently been developed.

While *M. ovis* has a long-known worldwide occurrence, '*Candidatus* M. haemoovis' has only been recently described from Northeast Hungary. Therefore, the present study was undertaken to evaluate (a) if goats are naturally infected with haemoplasmas in the relevant region, (b) if – based on sequence similarity – these bacteria belong to the same species as those isolated from sheep, (c) if goats are susceptible to both haemoplasma genotypes/species of sheep, and (d) if blood-sucking lice collected from bacteraemic goats are potential vectors based on PCR-positivity. It adds to the significance of these investigations that both ovine haemoplasmas have been recently isolated from a human clinical case (Sykes et al., 2010).

Materials and methods

Sample collection

In November 2010, EDTA-anticoagulated blood samples were collected in two herds of goats in Northeast Hungary. In herd A consisting of 150 she-goats/does and 6 males/bucks (that were in good physical condition) 24 animals were sampled (Rudabánya), whereas in herd B 26 goats out of 160 (Szőlősardó). In the latter group sporadic mortality was noted by the local veterinarian, and animals were in weaker physical condition. Giemsa-stained smears were prepared from all blood samples.

With respect to the occurrence of louse infestation the animal owners were consulted. The hair coats of ten goats in both herds were also carefully examined for the presence of blood-sucking lice (Phthiraptera: Anoplura). The species of these was determined according to standard keys, and all specimens were stored in 70% ethanol until evaluation.

DNA extraction and molecular methods

Twenty blood samples were chosen for molecular analysis (nine from herd A, and 11 from herd B). DNA was extracted from 200 µl EDTA-blood with the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. In addition, lice were pooled separately for host individuals and their DNA extracted (Hornok et al., 2010). The presence of amplifiable DNA was tested by using an 18S rRNA gene TaqMan real-time PCR (Applied Biosystems, Rotkreuz, Switzerland) as described previously (Boretti et al., 2009). Real-time PCR evaluation of samples for ovine haemoplasmas was done in an assay designed for the amplification of the 16S rRNA gene of *M. wenyonii* (Meli et al., 2010), but which also detects *M. ovis*. Tenfold serial dilutions of cloned plasmid DNA with known copy number were used for quantitation. Cloning and sequencing were performed from the sample with the lowest threshold cycle (Ct) value, modified from the methods already reported (Meli et al., 2010). In brief, primers MycWen.15f: 5'-ACA CAT GCA AGT CGA ACG AG-3' (20 bp) and Mycwen.1374r: 5'-ATT GAA TGT GGT TTT GAC TAG TAC TTT-3' (27 bp) were used to amplify the almost complete 16S rDNA sequence. The 1360 bp long amplicons were cloned into the pCRII-Topo Vector, followed by isolation of DNA from six clones and checking them by restriction digestion. The screening of louse DNA samples for Anaplasmataceae was carried out as described previously (Hornok et al., 2009).

Statistical analysis

For rates of PCR-positivity exact confidence intervals (CI) at the level of 95% were calculated according to Sterne's method (Reiczigel, 2003). Prevalences (for sexes) were compared by using Fisher's exact test, and mean values (for age) by Student's *t*-test. Differences were regarded significant when $P < 0.05$.

Results and discussion

This is the first report of a molecular investigation on caprine haemoplasmas, including analysis of their blood-sucking lice.

Haemoplasma infection could not be ascertained in any of the Giemsa-stained blood smears. However, four goats out of 20, all belonging to herd A, were haemoplasma-positive in real-time PCR (prevalence 20%, CI: 5.7–43.7%) (Table 1). Interestingly, louse infestation was also observed exclusively in herd A, where goats were in better physical condition.

There was no significant difference between the prevalences of *Mycoplasma* infection among male and female animals ($P = 0.2$). However, the mean age was significantly ($P = 0.019$) higher among PCR-positive (10.3 ± 2.1)

she-goats than among uninfected ones (5.9 ± 2.8), supporting the view that goats – like sheep – may enter a prolonged carrier state for haemoplasmas (Foggie and Nisbet, 1964).

From the sample with the lowest Ct value (highest bacterial load) *M. ovis* and '*Candidatus* M. haemoovis' were identified. Their sequences showed 100% homology with former Hungarian isolates (GenBank accession numbers EU165511 and EU828580, respectively). Medium to low levels of bacteraemia in relevant animals (as reflected by copy numbers: Table 1) may be explained by the following factors: (1) goats were reported to have lower levels of bacteraemia than sheep (Daddow, 1979); (2) even the absence of detectable bacteraemia was noted in some infected goats (Mason and Statham, 1991); (3) bacteraemia may subside in the peripheral blood during long-term infections (Foggie and Nisbet, 1964). This may also be a factor preventing mechanical carry-over by blood-sucking insects, since vector-borne transmission necessitates high levels of bacteraemia (Mason and Statham, 1991).

The better physical condition of animals in herd A – where haemoplasma infection was detected – and the lack of relevant clinical signs (anaemia) attests the low pathogenicity of *M. ovis* and/or '*Candidatus* M. haemoovis' in goats. However, in some blood smears marked anisocytosis was noted which may also be attributed to haemoplasmas in goats, as reported in sheep (Ganter et al., 1993). Since sporadic deaths only occurred in herd B, where (in a representative number of samples) no haemotropic mycoplasmas were detected, the mortality most likely was not related to haemoplasmosis.

Table 1

Data of PCR-positive goats and lice

	Goats				Lice		
Goat no.	Sex	Age (year)	Haemoplasma PCR: copy number in 1 µl of blood	*Anaplasma* inclusion bodies in blood smear	number of lice	Haemoplasma PCR: results in lice	Anaplasmataceae PCR: results in lice (obtained sequence)
1	F	11	1160	+++	5	negative	negative
2	F	12	260	+	20	negative	negative
3	F	8	400	+	NA	NA	NA
4	M	3	62	++	NA	NA	NA
5	F	7	0	+++	6	negative	+
6	F	9	NA	+	8	negative	+
7	F	4	NA	NA	7	negative	+
8	F	4	NA	NA	3	negative	+++ (*Anaplasma* sp.)

NA – not available, M – male, F – female; + = low positivity, +++ = high positivity

Forty-nine goat sucking lice (*Linognathus stenopsis*) could be collected from six goats. All louse pools were haemoplasma PCR-negative, including two pools containing DNA of 25 lice that sucked blood on bacteraemic goats (Table 1). Taking into account the high sensitivity of the real-time PCR, this excludes the presence of even low copy numbers of haemoplasma DNA, i.e. viable microorganisms in relevant lice. This may be explained by the very short digestion interval of anopluran lice (Lehane, 2005), and the extracellular location (exposure to enzymes) of haemoplasmas. The present data suggest the lack of mechanical vector potential of *L. stenopsis* for caprine haemoplasmas.

Blood smears from both herds showed *Anaplasma* inclusion bodies in erythrocytes. This was confirmed by PCR-positivity for Anaplasmataceae in four out of six louse pools (Table 1). Partial sequence of the 16S rRNA gene in one pool allowed identifying these agents on the genus level. Accordingly, results of the present study are in line with previous findings that these haematophagous insects may be carriers (i.e. potential vectors) of *Anaplasma* spp. of ruminants (Hornok et al., 2010).

In summary, as molecularly confirmed, goats are susceptible to both ovine haemoplasmas, *M. ovis* and '*Candidatus* M. haemoovis'. Goat sucking lice (*L. stenopsis*) do not appear to be potential vectors of these agents. The above findings, together with the recent isolation of both ovine haemoplasma variants in a veterinarian (Sykes et al., 2010), draw the attention to the possible zoonotic transmission of these bacteria to goat handlers.

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