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Fatal bovine anaplasmosis in a herd with new genotypes of *Anaplasma marginale*, *Anaplasma ovis* and concurrent haemoplasmosis

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ABSTRACT

Haematological and molecular analysis of blood samples was carried out during an outbreak of bovine anaplasmosis in Hungary. Acute disease was observed in five animals, two of which died. Anaplasma-carrier state was diagnosed in 69 (92%) of cattle. Further evaluation of 24 blood samples revealed concurrent infections with *Mycoplasma wenyonii* and '*Candidatus M. haemobos*' in 22 and 21 animals, respectively. In addition, two cows were identified with rickettsaemia. Regarding molecular investigation of potential hard tick vectors, *Haemaphysalis inermis* and *Dermacentor marginatus* males collected from the animals were PCR-negative. However, in one pool (out of 18) of *Ixodes ricinus* males, and in six pools (out of 18) of *D. reticulatus* males the *msp4* gene of *Anaplasma marginale* was detected. In the same *I. ricinus* pool *Anaplasma ovis* was also identified. All ticks were negative for haemoplasmas. Anaplasma sequences yielded 97–99% homology to sequences deposited in the Genbank. This is the first report of fatal bovine anaplasmosis associated with divergent *A. marginale* genotypes and concurrent '*Candidatus M. haemobos*' infection, as well as of an *A. ovis* strain in ticks collected from cattle.

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1. Introduction

Representatives of the family Anaplasmataceae (order Rickettsiales) are obligate intracellular bacteria. The type of host cell they infect depend on their genus or species (Dumler et al., 2001). In ruminants, *Anaplasma marginale* and *Anaplasma ovis* invade erythrocytes, which are later removed from the circulation by reticuloendothelial cells, resulting in varying degree of extravascular haemolysis and anaemia (Kocan et al., 2003). Bovine anaplasmosis is caused by *A. marginale*, whereas *A. ovis* can be found naturally in sheep and goat. On the other hand, these species cannot be regarded as strictly host-specific, since both of them infect a broad range of wild ruminants (Kuttler, 1984). In carrier animals *A. marginale* and *A. ovis* can persist subclinically, providing the source for the infection of their arthropod vectors. Biological transmission of both species occurs by hard ticks (Acari: Ixodidae), particularly by males feeding repeatedly and not necessarily on the same animal (intrastadial route). Their interstadial transmission (ingestion by nymphs, inoculation by adults) is regarded as less efficient (Eriks et al., 1993). Mechanical carry-over is also possible by haematophagous flies (Wilson and Meyer, 1966).

Bovine anaplasmosis is endemic to tropical–subtropical countries (Kocan et al., 2003), and in Europe it is reported up to the northern latitude of Switzerland (Hofmann-Lehmann et al., 2004), Austria (Baumgartner et al., 1992) and Hungary (Hornok et al., 2007). In Hungary only subclinical autochthonous bovine anaplasmosis was hitherto known (Hornok et al., 2007). However, in the autumn of 2009, morbidity (anaemia) and mortality attributable to *A. marginale* was noted in a different part of the country. Since geographic strains of *A. marginale* have been shown to differ in their genome and pathogenicity (Kocan et al., 2003; de la Fuente et al., 2005a), the present study was undertaken to investigate the causes that led to this situation, also considering a possible emerging character of bovine anaplasmosis in the region. Since formerly potential mechanical vectors, but not biological vectors were identified (Hornok et al., 2008), it was within the scope of this study to

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evaluate the transmission potential of large numbers of ixodid ticks collected from cattle.

2. Materials and methods

2.1. Sample collection

The relevant herd of 75 limousine beef cattle is kept extensively in northern Hungary, but in previous years several animals were purchased and introduced from other parts of the country. Mortality and morbidity attributable to anaplasmosis were observed in the autumn (at the end of August, September, beginning of October) of 2009. Within two weeks of notification hard ticks have been removed from all cattle. Ticks were also collected with the cloth-dragging method on three pastures grazed by the animals that year. All ticks were identified according to Babos (1965), then stored separately for each cattle in 70% ethanol until evaluation. Questing ticks from the vegetation were pooled according to sexes and species, with five ticks in each pool, the last containing the remainder of specimens. For host-derived ticks only males and nymphs were molecularly analysed. Except for *I. ricinus* (of which the pools contained ten males from one to five animals) ticks collected from different animals were grouped separately, and up to four individuals were processed together.

Fresh EDTA-anticoagulated blood samples were collected on two occasions: first from all 75 cattle in order to prepare blood smears (fixed with ethanol and stained with Giemsa), then secondly from those 24 animals which gave negative or inconclusive results during evaluation of their blood smears, or were inaccessible at the first sampling. DNA was extracted and PCR was performed from the latter samples.

2.2. DNA extraction

DNA was obtained using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. From 24 individual blood samples 200 μl was used. Prior to DNA extraction all hard ticks were washed three times: in detergent containing water, then in tapwater and finally in distilled water. Air-dried specimens were minced with pointed scissors at the bottom of Eppendorf-tubes, in 100 μl of phosphate-buffered saline (PBS). For DNA extraction from ticks an overnight digestion step (incubation at 56 °C for at least 8 h) with tissue lysis buffer and Proteinase-K was also included.

2.3. Conventional PCR for the Anaplasmatic 16S rRNA gene

For preliminary evaluation (DNA from 58 tick pools, 24 blood samples) primers EHR16SD and EHR16SR were used, which amplify an approximately 345 bp fragment from the 5′ region of the 16S rRNA gene from various members of the family Anaplasmataceae (Brown et al., 2001). Five microliters of extracted DNA was included in a 25 μl reaction mixture containing 25 pmol of each primer, 2 mM $MgCl_2$, 200 μM of each dNTP, 1 × Green GoTaq reaction buffer and 0.5 unit of GoTaq DNA polymerase (GoTaq Core System I., Promega, Madison, USA). Amplification was performed using a T-personal 48 thermal cycler (Biometra GmbH, Göttingen, Germany). An initial denaturation step at 94 °C for 15 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s. Final extension was done at 72 °C for 5 min. PCR products were subjected to electrophoresis in a 1.5% agarose gel (100 V, 40 min), pre-stained with ethidium bromide and visualized with ultra-violet light.

2.4. Conventional PCR for the major surface protein 4 (msp4) gene, cloning, sequencing

Amplification of the *msp4* gene of *A. marginale*, *A. centrale* and *A. ovis* was attempted from all samples positive in the 16S gene PCR. One microliter DNA and 10 pmol of each primer were used in a 50 μl PCR volume containing 1.5 mM $MgSO_4$, 0.2 mM dNTP, 1 × AMV/*Tfl* reaction buffer, 5u *Tfl* DNA polymerase. PCR amplification, cloning, sequencing were done as reported previously (de la Fuente et al., 2004, 2005b). For each sample, at least two independent clones were sequenced.

2.5. Real-time PCRs for haemotropic mycoplasmas

The presence of amplifiable DNA was confirmed for each sample using an 18S rRNA gene TaqMan real-time PCR (Applied Biosystems, Rotkreuz, Switzerland). Screening for haemotropic mycoplasmas (involving DNA from 58 tick pools and 24 cattle blood) was done with a universal SYBR Green assay as described (Willi et al., 2009). Positive samples were further evaluated with species specific real-time TaqMan PCR assays for bovine haemoplasmas (Meli et al., 2010).

2.6. Real-time PCRs for Rickettsia spp.

Two-step evaluation of cattle-blood samples was based on the amplification of the 23S rRNA gene and the detection of the citrate synthase (*gltA*) gene as described earlier (Boretti et al., 2009).

2.7. Phylogenetical analysis

The bootstrap values were calculated from 1000 resamplings and show the percentage of replicate trees in which the associated taxa clustered together. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are given in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 855 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

2.8. Statistical analysis

Prevalence data were compared by using Fisher's exact test. Exact confidence intervals were calculated according to Sterne's method. For correlation analyses, the Spearman rank correlation test was used. Statistical probes were performed using the Excel add-in Analyse-it (Analyse-it Software, Leeds, UK). Differences were regarded significant when $P < 0.05$.

3. Results

3.1. Cattle: clinical status, prevalence of anaplasmosis and rate of tick infestation

Anaplasmosis was first recognized in blood smears of 51 cattle from the herd of 75 animals. Of these five cattle appeared to be clinically affected (morbidity 6.7%; 95% CI: 2.2–14.9%) with lethargy and abruptly developing ruminal atony. Laboratory findings indicated mild to severe anaemia, lymphocytosis, haemolysis and

pathological changes in parenchymal organs (data not shown). Two of the cattle died within three days after the onset of their illness (mortality 2.7%; 95% CI: 0.32–9.3%). All except one cattle affected by anaplasmosis have been new in the herd (purchased and introduced from two other places of the country in the year of the current disease outbreak).

Bacteraemia detected by either positive results of blood smear evaluation or *msp4* PCR indicated that the prevalence of anaplasmosis in the herd was at least 92% (69 out of 75 animals; 95% CI: 83.4–97.0%). One cattle surviving acute disease became PCR-negative following oxytetracycline treatment. On four out of the seven PCR-negative cattle no ticks were found (three of them were constantly stabled). Taken together, tick infestation was significantly more prevalent among anaplasma-infected (57 out of 68) than among anaplasma-negative animals ($P = 0.026$). On the three PCR negative, but tick-infested animals only *I. ricinus* and *H. inermis* were detected.

3.2. Ixodid ticks: prevalence of species and their rate of anaplasma-infection

Questing ticks were present on the vegetation along the margin of one pasture out of three. This area corresponded to the grazing site prior to the clinical outbreak of anaplasmosis. However, all 42 ticks collected from the vegetation and analysed by PCR were negative in the *msp4* PCR (Table 1).

Altogether 673 adult hard ticks belonging to four species were collected from cattle. These were *I. ricinus* (71.3%), *D. reticulatus* (14.6%), *H. inermis* (11.7%) and *D. marginatus* (2.4%). Males of *H. inermis* were significantly less prevalent that those of other species ($P < 0.001$). One pool of *I. ricinus* and six pools of *D. reticulatus* males were *msp4* PCR-positive (Table 1); pools of *D. marginatus* or *H. inermis* were negative. Considering all analysed tick pools, anaplasma-infection was significantly ($P = 0.042$) more prevalent among ticks preferring open country habitats (*Dermacentor* spp.), than among forest-adapted ones (*Ixodes* and *Haemaphysalis* spp.).

3.3. Sequencing and phylogenetic analysis

Two new *A. marginale msp4* sequences (genotypes) were detected in cattle-blood samples (Table 1), differing in up to 22/849 positions of their *msp4* nucleotide sequences. The *msp4* encoded by genotype HU-2009-2 contained an amino acid deletion, indicating further polymorphism compared to others. However, the cow infected with this variant appeared to be healthy. Only three out of the eight cattle harbouring genotype HU-2009-3 could be haematologically evaluated, and one of them was anaemic. Genotype HU-2009-1 was only detected in *D. reticulatus* ticks. Interestingly, in one pool of *I. ricinus* – removed from four cattle including a diseased cow – an *A. ovis* sequence (genotype HU-2009-4) was present. In summary, eight out of eleven cattle in which (or in the ticks of which) new genotypes were detected were recently introduced into the herd.

GenBank accession numbers for the *msp4* sequences are HM063430–063432 (*A. marginale*) and HM063433 (*A. ovis*). Their phylogenetic relationships are shown in Fig. 1. New sequences of *A. marginale* and *A. ovis* clustered together with geographically diverse strains of the same species. However, the three new genotypes of *A. marginale* identified in the relevant herd belonged to different clades of their cluster.

3.4. Evaluation of other haemotropic agents

Of the 24 blood samples tested for the presence of haemotropic *Mycoplasma* spp. 22 (91.7%; 95% CI: 73–99.0%) contained *M. wenyonii*. Out of these 21 (87.5%; 95% CI: 67.6–97.3%) were co-infected with '*Candidatus M. haemobos*'. In the latter group of samples, based on lower CT values, 14 showed predominance of *M. wenyonii*, whereas in seven samples the copy number was higher for '*Candidatus M. haemobos*'. The CT values of the SYBR Green assays correlated well with the CT values for the dominating bovine haemoplasma species assessed by specific real-time PCR ($P < 0.0001$; $Rs = 0.91$).

Concerning cattle with demonstrated pathogenic effect of anaplasmosis, only three could be evaluated for concurrent haemoplasmosis. The clinically most severely affected, but surviving cow was co-infected with haemoplasmas, whereas other two, diseased or mildly anaemic animals were not. All tick pools were negative for *M. wenyonii* and '*Candidatus M. haemobos*'.

Blood smears and PCR results were negative for *A. phagocytophilum* and piroplasms (data not shown). However, two blood samples were rickettsia-positive. The species they contained was/were most likely not *R. helvetica*, but could not be further identified due to the high CT values (37.5 and 39.8).

4. Discussion

This is the first report of fatal bovine anaplasmosis associated with divergent *A. marginale* genotypes and concurrent '*Candidatus M. haemobos*' infection, as well as of an *A. ovis* strain in ticks collected from cattle. The *msp4* gene chosen in this study for analysis of *Anaplasma* spp. is more conserved than other *msp* genes (de la Fuente et al., 2002), making it suitable to distinguish geographical strains, frequently with varying pathogenicity (de la Fuente et al., 2005a). Correspondingly, the highly heterogenous population of *A. marginale* in animals of the relevant herd and their ticks may reflect extensive cattle movement in the region (de la Fuente et al., 2003).

Risks associated with frequent introduction of newly purchased cattle are twofold. Susceptible cattle imported into endemic areas

Table 1
Results of molecular analyses. Only those samples were tested in the *msp4* PCR which showed positivity in the 16S screening assay. Not all *msp4* positive samples were sequenced. Example accession numbers of GenBank sequences to which maximal identity was found are AY666010 for *A. marginale* and EF190511 for *A. ovis*.

Sample type	Sample source	*msp4* PCR (positives/ all tested)	Result of sequencing	Genotype (number of samples containing it)	Maximal identity with GenBank sequences
Individual blood	Cattle	14/19	*A. marginale*	HU-2009-2 (1)	97%
				HU-2009-3 (8)	99%
Pooled ticks from the vegetation (♂, ♀)	*Dermacentor reticulatus*	0/2	–		
	Haemaphysalis inermis	0/4	–		
Pooled ticks from cattle (only ♂, also nymphs*)	*Ixodes ricinus**	1/18	*A. marginale*	HU-2009-3 (1)	99%
			A. ovis	HU-2009-4 (1)	99%
	D. reticulatus	6/18	*A. marginale*	HU-2009-1 (1)	98%
				HU-2009-3 (5)	99%
	D. marginatus	0/7	–		
	H. inermis	0/2	–		

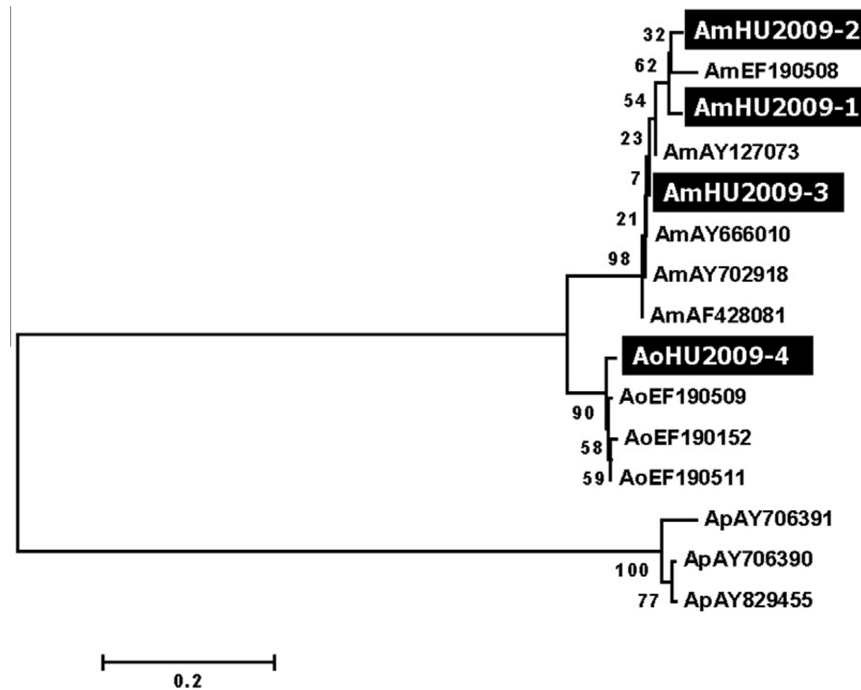

Fig. 1. Phylogenetical relationships of new *A. marginale* and *A. ovis* genotypes with other *Anaplasma* sp. isolates. Bootstrap values are shown next to the branches. Abbreviations: Am – *A. marginale*; Ao – *A. ovis*; Ap – *A. phagocytophilum.* Accession numbers in decreasing order of sequence homology to new genotypes: AY666010, AY127073, AF428081 – USA isolates; AY702918 – Italian isolate. EF . . . formerly published Hungarian isolates.

are known to be at risk for exhibiting severe disease (Figueroa et al., 1998). On the other hand, innate immunity that local cattle have against certain *A. marginale* strains may not cope with heterologous challenge, i.e. may not be protective against genotypes with novel surface antigens, as in case of HU-2009-2. Although newly identified genotypes were found in anaemic animals or in relevant ticks, their role in clinical disease could not be unanimously substantiated, in part due to the inaccessibility to samples of cattle at the time of their acute disease for molecular analysis. Nevertheless, the simultaneous presence of genotypes that are new in a worldwide context may indicate their association with disease in the herd. This was also suggested by anamnesis data: cows that were clinically affected by anaplasmosis and the great majority of those in which divergent *A. marginale* genotypes were detected were both newly introduced into the herd.

Autochthonous cases of clinical bovine anaplasmosis were hitherto unknown in Hungary. According to the first report on the occurrence of subclinical infection with *A. marginale* in the country (Hornok et al., 2007, 2008), lower degree (99%) of genetic variation and high (81%) seroprevalence may have reflected endemic stability (Dreyer et al., 1998; Coleman et al., 2001). That aspect of the situation can be regarded similar to the one in the herd of the present study, as indicated by the high percentage of bacteraemic cattle, as well as by clinical manifestation in older animals (Coleman et al., 2001). Since prior to the disease outbreak no control measures were introduced, which may have interfered with endemic stability (Coleman et al., 2001), the most likely destabilizing effect that entailed acute disease may have been the introduction of naive animals and/or the emergence of new *A. marginale* variants.

Detection of unidentified rickettsiae in blood samples of two cows is irrelevant to the disease outbreak in 2009, since none of these animals were clinically affected. However, to the best of our knowledge, this is the first account of a molecularly confirmed bovine rickettsaemia (*sensu stricto*) in Europe, as observed in wild ruminants (Stefanidesova et al., 2008). These findings may contribute to the emerging veterinary significance of rickettsioses, and underline the importance to evaluate the potential reservoir role of domestic ruminants in synathropic maintenance of zoonotic, tick-borne *Rickettsia* spp. (Jilintai et al., 2008).

This is also the first report of *M. wenyonii* and '*Candidatus M. haemobos*' infection of cattle in Hungary. The good correlation found between the SYBR Green assay and the specific haemoplasma real-time PCR indicates that the SYBR Green signals were most likely due to bovine haemotropic mycoplasmas. These bacteria attach to and damage erythrocytes, but usually cause only mild anaemia and no mortality (Smith et al., 1990; Neimark et al., 2001; Hoelzle et al., 2010). Concerning their role in the present disease outbreak, haemoplasma infection in the relevant herd must have been widespread prior to the clinical cases, since none of their potential vectors (Hofmann-Lehmann et al., 2004; Smith et al., 1990) were observed (lice) or did abound (mosquitoes, flies) at that time of the year. Further on, out of three cattle showing symptoms and/or haematological changes, only the most severely striken was PCR positive: another diseased and one anaemic cow were negative. Therefore *M. wenyonii* and '*Candidatus M. haemobos*' may have contributed to, but were probably not the primary causative agents of morbidity, mortality in this case. On the other hand, haemotropic bacteria are known to enhance the pathogenic effect of each other. For example, concurrent *M. ovis*, '*Candidatus M. haemoovis*' and *A. ovis* infection was reported to entail increased pathogenicity and even mortality among sheep (Hornok et al., 2009). Data obtained in the present study may raise the possibility of a similar, tri-lateral synergism between corresponding agents in cattle. PCR negativity of ticks reflects that these are unlikely vectors, similarly to previous findings (Hofmann-Lehmann et al., 2004; Smith et al., 1990).

The disease occurred in the autumn. Similar seasonality of anaplasmosis was reported from other parts of the world in the temperate zone (Rodgers et al., 1994). This is relevant to dominant vectors and types of transmission under this climate, as mechanical carry over is more likely during summer months when tabanid horse flies are active (Hornok et al., 2008), whereas biological

transmission by hard ticks tends to occur in the spring or in the autumn when these are most abundant on the vegetation (Hornok, 2009). Thus the seasonality of acute anaplasmosis in the herd of this study, the exclusive presence of ticks along the pasture grazed by the animals prior to the disease outbreak, as well as the statistically substantiated association of tick-infestation with anaplasmosis suggest the vector role of hard ticks in the background.

Male ticks can transmit pathogens intrastadially, which is regarded as the most important means by which *Anaplasma* spp. of ruminants spread from one host to another (Eriks et al., 1993). Consequently, from among individuals that already sucked blood only males were processed. However, taking into account the possibility of transstadial transmission, nymphs collected from animals and both sexes from the vegetation were also evaluated. Based on the present results – from among the species analysed – only *D. reticulatus* appeared to be an important vector of *A. marginale* in the region. The vector competency of this species was also justified by recent transmission experiments (Zivkovic et al., 2007).

Regarding the temporo-spatial distribution of ixodid species in the country, the vector role of *D. reticulatus* is further corroborated by observations on seasonality, as this species is most prevalent on the vegetation during September and October (Hornok, 2009), when the disease outbreak occurred in 2009. For further tick species with significant autumn activity, *H. inermis* males were significantly fewer on cattle than males of other species in the present study, and *I. ricinus* males may mate in the absence of hosts (Kiszewski et al., 2001), in such cases precluding intrastadial transmission of *A. marginale* anyway. Therefore, also taking into account results of their molecular evaluation, these two species appear to be unlikely vectors of *A. marginale*. A plausible explanation for this can be drawn from habitat preferences of the relevant ticks, as characterized by the previously postulated (Uspensky, 2002) division between forest ticks (*Ixodes* and *Haemaphysalis* spp.) and open country ticks (*Dermacentor* spp.). In this respect – and as concluded from this study –, since infection was significantly more prevalent in pools of the latter category, *A. marginale* may have adapted to open country ticks (exemplified by *D. reticulatus*) because of their habitats (including pastures) where the type host, cattle can be found grazing.

To the best of our knowledge, this is the first molecular evidence of *A. ovis* in male *I. ricinus*. Since this is the most important hard tick species that sucks blood on humans in Europe (Gray, 1991), a possible vector role for it in spreading ovine anaplasmas – as raised by the present findings – may have relevance to the recently discovered zoonotic potential of *A. ovis* (Chochlakis et al., 2010). Nevertheless, since the positive pool of *I. ricinus* contained ticks removed from a diseased cow, the resistance of cattle to *A. ovis* (Kuttler, 1984) also needs to be further evaluated and revised.

In summary, emerging character of clinical anaplasmosis in the country warrants regulation to avoid unnecessary cattle movements, and to undertake continuous monitoring of factors in disease epidemiology. At the same time, persons in charge of this must be aware that intervention into the naturally balanced situation (especially treating all animals instead of diseased ones) may entail aggravation of the situation with increased morbidity and mortality (Coleman et al., 2001). Consequently, it is more appropriate to implement strategic control measures (e.g. reducing the chances for tick infestation) and thus to aim at evoking and maintaining endemic stability in the region.

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