

Molecular Evidence of *Anaplasma phagocytophilum* in *Ixodes ricinus* Ticks and Wild Animals in Austria

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Real-time PCR analysis of a *groESL* heat shock operon segment showed the presence of two genetic lineages of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks as well as one variant in wild red and roe deer, the latter supposedly representing the natural reservoir of one variant of *A. phagocytophilum*.

In 2001, the human granulocytic ehrlichiosis agent, *Ehrlichia equi*, and *Ehrlichia phagocytophila* were classified into a single species named *Anaplasma phagocytophilum* and are now treated as variants of one species (2). The life cycle of *A. phagocytophilum* in Europe involves *Ixodes ricinus* ticks as vectors and wild animal hosts as natural reservoirs. The natural reservoir of the genetic variants is still unknown.

In Austria, isolation of *A. phagocytophilum* DNA from *I. ricinus* ticks (W. Sixl, M. Petrovec, R. Schweiger, D. Stünzner, G. Wüst, E. Marth, and T. Avsic Zupanc, poster presentation P-2, International Conference on Rickettsiae and Rickettsial Diseases, Ljubljana, Slovenia, 2002) and roe deer (M. Petrovec, R. Schweiger, S. Mikulasek, G. Wüst, D. Stünzner, K. Strasek, T. Avsic Zupanc, E. Marth, and W. Sixl, poster presentation P-5, International Conference on Rickettsiae and Rickettsial Diseases, Ljubljana, Slovenia, 2002) has been reported before. As the *groESL* heat shock operon sequence of *A. phagocytophilum* shows more variance than the widely used 16S rRNA gene, it offers a tool for subtyping of genetic lineages. The heat shock sequences deposited in GenBank attracted attention to a homologue region containing one single nucleotide polymorphism, thus allowing differentiation between two genetic lineages of *A. phagocytophilum*, one associated with anaplasmosis in humans and horses and the other associated with a febrile disease in ruminants.

The aim of this study was to isolate and genetically characterize European strains of *A. phagocytophilum* obtained from *I. ricinus* ticks and wild mammals.

I. ricinus ticks were collected by flagging vegetation. The wild animals were killed by hunters, and all horses were derived from one geographic area and were killed in one slaughterhouse (Table 1). The samples were collected from the individual species at different representative sites within each region, except that samples from red and roe deer were collected at one site in Salzburg. DNA was extracted from ticks and animal tissue with the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Vienna, Austria). Amplification was performed in a LightCycler instrument. The primers

groEF1 (5'-AGACGAAATTGCACAAGTT-3') and groER1 (5'-AGCCTTTGCTTTCTTCAAC-3') amplified a *groESL* gene segment. Two hybridization probes, groEFL (5'-CTTTA ACACACTGTGCAATCTTACTTCC-3'-fluoresceine) and groELC640 (LCRed640-5'-TGTTCTTGTCCTCCATTCGCA G-3'-phosphorylated), allowed differentiation of two genetic lineages according to a difference in melting temperatures T_m of 8°C. A 20- μ l reaction mixture contained 2 μ l of LightCycler Fast Start DNA master hybridization probes mix; 2 μ l of DNA template; hybridization probes groEFL and groELC640, each at a concentration of 400 nM; primer groEF1 at a concentration of 500 nM; primer groER1 at a concentration of 250 nM; and 4 mM MgCl₂. The following PCR program was used: 10 min at 95°C and 55 cycles of 5 s at 95°C, 10 s at 64°C, and 5 s at 72°C. The program for analytical melting was 5 s at 95°C, 2 min at 40°C, and an increase to 80°C at an 0.2°C/s ramp rate. Sequencing was done with an AbiPrism 310 capillary sequencer (Applied Biosystems, Vienna, Austria). The G-test of independence was performed with SPSS 7.0.

PCR showed positive results for 8.7% of the examined *I. ricinus* ticks ($n = 880$). Significant differences in prevalences of infection in vectors did not arise between the sexes (10.9%, females; 6.7%, males), nor were there differences in infection rates among ticks at different developmental stages (8.7%, adults; 5.8%, nymphs). Comparison of the seasonal rates of infestation of *I. ricinus* ticks with *A. phagocytophilum* showed a decrease from spring (7.6%) to summer (3.6%) followed by an increase in fall (5.7%). Real-time PCR and partial sequence analysis of 29 tick samples showed one variant of *A. phagocytophilum*, associated with anaplasmosis in humans and horses, in 4 ticks and the second genetic lineage, associated with disease in ruminants, in 25 ticks. Only one genetic variant could be found in 52 *Capreolus capreolus* individuals ($n = 121$) and two *Cervus elaphus* specimens ($n = 7$). No *Anaplasma* DNA was isolated from tissues of hares, horses, and wild boar. The infection rates in roe deer among different age classes as well as at different sampling sites showed no significant differences. No remarkable differences in prevalences of infection could be seen between sexes of roe deer (42.4%, females; 39.1%, males). Roe deer showed highest prevalences of infection in August, September, and December (Table 2).

I. ricinus ticks acquire *A. phagocytophilum* when taking a blood meal from an infected animal and can transfer the

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TABLE 1. Geographic distribution of the ticks and wild animals examined

Species examined (tissue)	No. of individuals examined from the following Austrian region:						Total no. examined
	Upper Austria	Lower Austria	Salzburg	Vienna	Burgenland	Styria	
<i>I. ricinus</i>	174		486		20	200	880
<i>Capreolus capreolus</i> (liver)	113		8				121
<i>Cervus elaphus</i> (liver)			7				7
<i>Lepus europaeus</i> (liver)	20						20
<i>Sus scrofa</i> (spleen)	2	146	7	24			179
<i>Equus</i> sp. (liver)						43	43

pathogen when the next blood meal occurs. In this study, the infestation rate of 6.6% in ticks is in agreement with that of 5.1% reported in Austria before (Sixl et al., International Conference on Rickettsiae and Rickettsial Diseases). Due to transstadial transmission of *A. phagocytophilum*, adult ticks were infested more often (8.7%) than nymphs (5.8%). These results are in agreement with the infection rate of 8.2% observed in adult *I. ricinus* ticks from northern Spain (B. Oporto, H. Gil, M. Barral, P. Saenz, I. del-Pozo, A. Hurtado, R. A. Juste, and A. L. Garcia-Perez, poster presentation P-4, International Conference on Rickettsiae and Rickettsial Diseases, Ljubljana, Slovenia, 2002) and the prevalence of infection of 4.2% perceived in Italian nymphs (1). The observed variations in infection rates throughout the sampling period are in agreement with the seasonal activity of *I. ricinus* ticks (4).

Two genetic lineages of *A. phagocytophilum* could be isolated from ticks. Two adult ticks and two nymphs from the Upper Austria sampling site harbored the variant associated with anaplasmosis in humans. Dual infection of ticks with both genetic lineages of *A. phagocytophilum* did not occur.

No evidence of infection was found in tissue samples obtained from hares, wild boar, and horses. The infection rate of 43% in *Capreolus capreolus* is in agreement with the prevalence of 32% detected in this species in the Czech Republic (Petrovec et al., International Conference on Rickettsiae and Rickettsial Diseases) but much lower than that of 74% reported previously for Austrian roe deer (Petrovec et al., International Conference on Rickettsiae and Rickettsial Diseases). All age classes as well as both sexes were infected with *A.*

phagocytophilum without significant differences in prevalences. Roe deer were infected throughout the whole sampling period, with highest prevalences in August, September, and December.

The infection rate of 28.6% in *Cervus elaphus* was remarkably lower than that of 86% found in red deer from Slovenia (3).

Only one genetic variant of *A. phagocytophilum* could be isolated from red and roe deer samples. All the *groESL* gene sequences of *A. phagocytophilum* were identical to those obtained from most *I. ricinus* ticks and to all *groESL* gene sequences in GenBank obtained from red deer, roe deer, and other ruminants. The genetic lineage obtained from four ticks and associated with human disease could not be demonstrated in wild animals.

The high prevalence of infection of roe deer with *A. phagocytophilum* occurring throughout the year provides evidence that this species serves as a natural reservoir for one genetic lineage of *A. phagocytophilum* in Austria.

Nucleotide sequence accession numbers. The nucleotide sequences of both genetic variants of *A. phagocytophilum*, obtained from *I. ricinus* ticks, have been submitted to GenBank (accession no. AY343485 and AY343486).

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TABLE 2. Seasonal infestation of roe deer with *A. phagocytophilum*

Month	No. of <i>Capreolus capreolus</i> specimens examined	No. (%) positive
June	17	6 (35.3)
July	25	6 (24)
August	9	6 (66.7)
September	19	12 (63.2)
October	29	6 (20.7)
November	16	6 (37.5)
December	6	4 (66.7)