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Anaplasma phagocytophilum groEL Gene Heterogeneity in *Ixodes ricinus* Larvae Feeding on Roe Deer in Northeastern Italy

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Abstract

Anaplasma phagocytophilum is an emerging tick-borne pathogen with both veterinary and human health implications. The role of wildlife hosts for this pathogen are not well defined, even thought roe deer (*Capreolus capreolus*) has been suggested to contribute to the occurrence of this tick-borne diseases in Europe. Therefore the aim of the present study was to investigate the potential role of this ungulate species as a reservoir of human pathogenic strains of *A. phagocytophilum* in a tick-borne diseases endemic area in Northeastern Italy. *Ixodes ricinus* feeding on roe deer were collected and analyzed for the presence for *A. phagocytophilum* by a molecular approach targeting 16S rRNA and *groEL* genes. The mean prevalence of *A. phagocytophilum* recorded was 5.11%, highlighting the ability of roe deer to infect the *I. ricinus* larval stage. The results of further genetic characterization of the strains of *A. phagocytophilum* herein isolated, based on phylogenetic information contained in *groEL* gene sequences, showed substantial heterogeneity among sequences analyzed. Nevertheless, these findings suggest that the roe deer population of the Trentino region of Italy harbors strains of *A. phagocytophilum* of unknown pathogenicity for humans.

Key Words: Anaplasma; Epidemiology; Genetics; Ixodes.

Introduction

Anaplasma phagocytophilum is of growing concern in human and veterinary medicine as an emerging tick-borne pathogen in Europe and in North America, causing an acute disease in several species. Recent taxonomic changes have reclassified the families *Rickettsiacecae* and *Anaplasmatacecae* in the order *Rickettsiales* and the agents formerly known as *Ehrlichia phagocytophila*, *E. equi*, and human granulocytic agent under the unique species *A. phagocytophilum* on the basis of the high degree of similarity in the 16S rRNA (Dumler et al. 2001). Consistently, the disease caused by *A. phagocytophilum* is now reported as granulocytic anaplasmosis, formerly designated as granulocytic ehrlichiosis.

Human granulocytic anaplasmosis (HGA) was first described in Europe in 1996 (Petrovec et al. 1997), and more than 60 cases of HGA have been recorded since (Blanco and Oteo 2002, Strle 2004, Lotric-Furlan et al. 2006). In humans, the clinical symptoms are nonspecific and are usually characterized by fever, headache, malaise, and myalgias accompanied by altered ematochimic laboratory findings (Lotric-Furlan et al. 2006).

A. phagocytophilum is maintained in nature in an enzootic cycle including *Ixodes* spp. ticks as the main competent vector and, because transovarial transmission in ticks does not occur (Massung et al. 2005), a wide range of mammalian species act as reservoirs (Petrovec et al. 2003, de la Fuente et al. 2005, Woldehiwet 2006, Stuen 2007).

Several epidemiological studies carried out in Europe have shown molecular evidence of *A. phagocytophilum* in questing ticks and mammals, suggesting in particular the important role of wild ungulates in the maintenance of the infection in nature (Alberdi et al. 2000, Liz et al. 2002, Petrovec et al. 2002). Roe deer (*Capreolus capreolus*) are among the most important feeding hosts for both mature and immature stages of the sheep tick *I. ricinus* (Matuschka et al. 1993, Carpi et al. 2008), and therefore this species seems to be a major contributor to the occurrence of *A. phagocytophilum* in Europe (Alberdi et al. 2000, Stuen et al. 2001, Liz et al. 2002, Petrovec et al. 2002, 2003, Polin et al. 2004). Nevertheless, the role of this species as natural reservoir of human pathogenic strains of *A. phagocytophilum* has not yet been clarified.

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In Italy, despite sporadic cases of HGA reported since 2004 (Ruscio and Cinco 2003, Beltrame 2006, Mastrandrea et al. 2006), *A. phagocytophilum* has been widely detected in questing *I. ricinus*, in domestic animals, and in pets (Lillini et al. 2006). This incongruity was also recorded in the Trentino region, in the Eastern Italian Alps, an endemic area for several tick-borne diseases that is characterized by high wild ungulate density and that is a suitable habitat for sheep ticks (Chemini et al. 1997, Rizzoli et al. 2002). In the Trentino area, a high prevalence and a wide geographic distribution of *A. phagocytophilum* was reported, both in questing *I. ricinus* and in roe deer (Beninati et al. 2006, Mantelli et al. 2006), despite the absence of human cases of HGA.

The strains of *A. phagocytophilum* identified so far in several animal species showed minor genetic variability at the nucleotide level of the 16S rRNA (von Loewenich et al. 2003). At the same time, using phylogenetic information in the *groEL* gene sequences, Petrovec et al. (2003) showed a clear difference between *A. phagocytophilum* strains circulating in roe deer and those from humans.

Based on these results, the aim of the present study was to estimate the prevalence of *A. phagocytophilum* in immature *I. ricinus* feeding on roe deer in the Trentino region. To define the implications for human health in Northeastern Italy, phylogenetic analysis was carried out to investigate and describe the genetic relationship between circulating strains in the roe deer population and the available human origin sequences of HGA.

Material and Methods

Study area

The study was undertaken in the province of Trento (eastern Italian Alps), an endemic area for several tick-borne diseases (Hudson et al. 2001, Rizzoli et al. 2004), in seven hunting districts selected across the whole geographic and altitudinal range and covering a total area of 692.10 km^2 (centroid coordinates: 11.21°E, 46.10°N). The land is 55% coniferous and deciduous forest. In this area, improvements in wildlife management over recent decades have favored the expansion of wild ungulates, in particular roe deer (about 30,000 head) (Autonomous Province of Trento, Game Management Assessment, 2003).

Sample collection and DNA extraction

The study was carried out on pools of feeding ticks, at the larval stage, that were collected with sterile clasps from the lower part of the forelegs (distal to the carpal joint) of roe deer shot during the regular hunting season in September 2004. Ticks were examined microscopically to determine species and stage, based on the reference key by Manilla (1998), and stored at –20°C until DNA extraction. Mean infection prevalence was calculated with methods for estimation of individual-level prevalence based on pooled samples previously described (Cowling et al. 1999).

Of 132 roe deer samples examined for tick infestation, only animals with a minimum of 30 engorged larvae were considered further for molecular analysis. DNA was extracted from pools of five larvae (previously homogenized with a sterile pestle in sterile tubes) with a Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNA was eluted with 80 μL elution buffer. Negative controls were added to each DNA extraction to verify potential contamination.

Molecular analysis

Polymerase chain reaction (PCR) amplification of the tick mitochondrial cytochrome b gene was performed in each sample as a quality control for tick DNA extraction (Derdáková et al. 2003). Only positive samples were processed further.

A fragment of the 16S rRNA gene (546 bp) of *A. phagocytophilum* was amplified by means of nested PCR, with primers previously reported (Massung et al. 1998). Briefly, the amplification was performed on a PerkinElmer Thermal Cycler in a reaction volume of 20 μL containing 1× HotMaster Taq Buffer (with 25 mM Mg), 1.25U of HotMaster Taq DNA Polymerase (Eppendorf, Milan, Itlay), 10 mM of dNTPs, 10 pmoles of each primer in the first-round PCR, and 4 pmoles of each primer in the second-round PCR. All reactions included 2 μL of template DNA.

With the same protocol reported above, samples that were positive in the 16S rRNA assay were tested by nested PCR targeting the *groEL* gene of *A. phagocytophilum*. The primers used in the nested PCR amplified a 1297-bp region of the *groEL* gene (Liz et al. 2002). Amplified DNA was visualized on a 2% agarose gel (Sigma). All positive PCR products for both 16S rRNA and *groEL* genes were purified by enzymatic Exosap-IT (USB Corporation) and sequenced in both directions with an ABI BigDye terminator kit (Applied Biosystems, Monza, Italy) and analyzed on an ABI prism 3130 automated sequencer.

In addition to the sequences produced in this study, a set of significant sequences of the *groEL* gene of *A. phagocytophilum* from Europe, available on GenBank, were added for the phylogenetic analysis ($n = 29$; accession numbers are reported in Figure 1). The available *groEL* gene sequence of *Ehrlichia canis* was added as outgroup (accession number: NC_007354). Respecting the coding frame, Clustal X was used to provide alignment of sequences (Thompson et al. 1997).

Phylogenetic analysis

To create phylogenetic trees, a model of molecular evolution was estimated by means of a hierarchical likelihood ratio test approach and the Akaike information criterion (Akaike 1973) implemented in the software ModelTest version 3.7 (Posada and Crandall 1998, 2001). Bayesian methods implemented in the computer program MrBayes version 3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003) were used to create phylogenetic trees and assess statistical support for clades. A Markov chain Monte Carlo search for 1,000,000 generations using two runs with four chains (temperature = 0.05) was performed, and results were represented as a 50% majority rule consensus tree. Tree statistics and performed phylogenetic manipulations were calculated with the computer program PAUP* version 4.0b10 (Swofford 2003). Genetic diversity was expressed as nucleotide diversity (Nei 1987) or the mean proportion of nucleotide differences among sequences.

Nucleotide sequence accession numbers

New sequences have been deposited in GenBank with the accession numbers EU552012– EU552023.

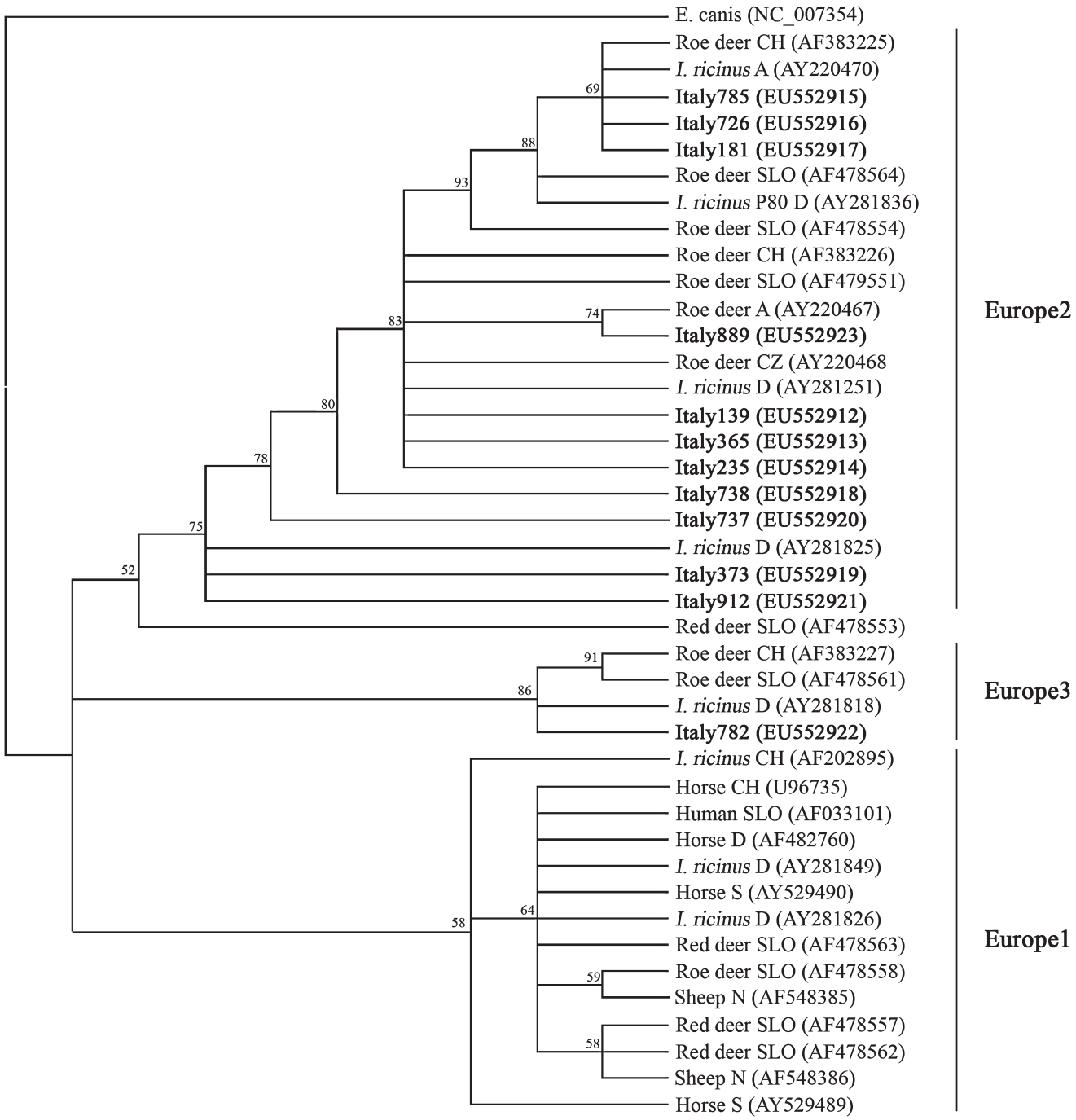

FIG. 1. Phylogenetic tree constructed by Bayesian analysis of 42 *groEL* gene sequences (length 1164 nucleotides). Posterior probabilities of clades are indicated above branches. New Italian sequences are shown in boldface.

Results

Seventy-eight of the 132 roe deer studied were highly infested by *I. ricinus* (tick abundance more than 30 engorged larvae), and 18 of 78 pools of larvae of *I. ricinus* were found positive for the 16S rRNA gene of *A. phagocytophilum*. The mean prevalence of *A. phagocytophilum* in *I. ricinus* larvae feeding on roe deer was about 5.11% (95% confidence interval: 3.04–7.97). Twelve partial sequences of the *groEL* gene were generated. Mean nucleotide diversity among new Italian sequences was 0.66% (range: 0.00%–1.81%). The average difference between Italian samples and the human patient with HGA (accession number AF033101) was 1.79% (range: 0.95%–2.07%). Nucleotide and amino acid substitutions are summarized in Table 1. The phylogenetic tree representing the relationships among these sequences and selected sequences from the literature is shown in Figure 1. A clear separation among European *groEL* sequences is evident, confirming previous studies (Alberti et al. 2005). Eleven of 12 Trentino sequences belonged to the "Europe2" clade, which includes only strains with unknown pathogenicity (Alberti et al. 2005). The "Italy782" sample was included in a new lit-

Table 1. Variable Sites and Differences along *groEL* Gene Sequences of Italian Samples Compared with Human Granulocytic Anaplasmosis (HGA) Strain (GenBank Accession Number AF033101)

	51	*282*	*381*	*447*	*450*	*516*	*660*	*697*	*705*	*720*	*721*	*723*	***724***	*729*	*741*	*756*	*807*	*846*	*849*	*864*	*876*	*888*	*933*	*949*	*951*	*969*	*972*	*996*	*1044*	*1104*	*1113*	*1128*	*1131*
HGA	A	A	T	T	G	T	T	C	C	A	C	G	**T**	A	C	C	C	C	G	T	T	C	C	C	T	A	C	C	T	A	A	T	T
It.139	G	G	.	.	A	.	C	.	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	T	.	.	G	C	.
It.181	G	G	.	.	A	.	C	T	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	.	C	G	G	C	C
It.235	G	G	.	.	A	.	C	T	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	T	.	.	G	C	.
It.365	G	G	.	.	A	C	C	.	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	T	.	.	G	C	.
It.373	G	G	.	.	A	.	C	T	A	G	A	A	**G**	C	.	T	G	T	A	.	.	.	T	.	.	.	.	.	.	.	G	C	.
It.726	G	G	.	.	A	.	C	T	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	.	C	G	G	C	C
It.737	G	G	.	.	A	.	C	T	A	G	A	A	**G**	C	.	T	G	T	A	.	.	.	T	.	.	G	.	T	.	.	G	C	.
It.738	G	G	.	.	A	.	C	T	A	G	A	A	**G**	C	.	T	G	T	A	A	C	.	T	.	.	G	.	T	.	.	G	C	.
It.782	G	.	C	.	A	.	C	.	.	.	.	.	**.**	.	.	.	.	.	A	.	.	T	T	T	G	.	.	.	.	.	G	C	.
It.785	G	G	.	.	A	.	C	T	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	.	C	G	G	C	C
It.889	G	G	.	.	A	.	C	T	A	G	A	A	**G**	T	T	T	G	.	A	A	C	.	T	.	.	G	.	.	.	.	G	C	.
It.912	G	G	.	.	A	.	C	T	A	G	A	A	**G**	C	.	T	G	T	A	.	.	.	T	.	.	.	.	.	.	.	G	C	.

Note: Nucleotide position is related to complete gene sequence. Dots indicate equal nucleotide. The only nonsynonymous substitution at position 724 is indicated in boldface.

tle clade with two sequences from roe deer sampled in Slovenia and one from *I. ricinus* sampled in Germany. Because this clade is clearly separated from "Europe1" and "Europe2" clades, we identify it as "Europe3," following the nomenclature previously introduced (Alberti et al. 2005).

Discussion

In the present study we detected and characterized *A. phagocytophilum* in larvae of *I. ricinus* that fed on roe deer in Northeastern Italy. The potential role of roe deer in the ecology and epidemiology of tick-borne diseases has been previously investigated in Europe, and in Italy as well. This species represents a key host by feeding both immature and mature stages of *I. ricinus*, thus modulating local tick abundance and the occurrence of pathogen infection (Hudson et al. 2001, Perkins et al. 2006). In the present study we have confirmed the circulation of *A. phagocytophilum* in roe deer population in the Trentino region as reported by Beninati et al. (2006), as well as its consequent role in infecting *I. ricinus* larvae.

Concerning genetic variability of the strains herein isolated, in view of the relatively small study area and the single host species investigated, sequence analysis of the *groEL* gene showed moderate nucleotide heterogeneity among new Italian sequences. Furthermore, most of them belong to the "Europe2" clade, thus showing a low similarity with HGA.

Separation between the "Europe1" and "Europe2" clades has been confirmed, and our tree suggests the presence of a third clade, "Europe3." The comparison of sequences at the nucleotidic and aminoacidic levels shows that this heterogeneity is mostly caused by synonymous substitutions. Only one amino acid change is present along the analyzed sequences (Ser to Ala in the 242 amino acid position): this change seems to be characteristic of the "Europe2" clade.

In accordance with the findings reported by Petrovec and colleagues (2002), we confirmed a divergence between deer species in the *A. phagocytophilum* lineage in Northeastern Italy. The "Europe2" clade, in particular, was associated primarily with roe deer, whereas the "Europe1" clade (i.e., the HGA strain) was associated with a wider host range, including both domestic and wild animals. Therefore, our phylogenetic analysis evidenced the occurrence of strains of unknown pathogenicity of *A. phagocytophilum* in the roe deer population. This result could explain the absence of HGA cases in the area investigated, even though serological evidence of exposure to *A. phagocytophilum* among forestry rangers in the Trentino region have been recorded with a mean seroprevalence of 4.9% (Versini, personal communication). Thus, according to our results, this might suggest the circulation in the study area of only sylvatic and not pathogenic strains of *A. phagocytophilum* and therefore the necessity to interpret serological investigations of this pathogen with particular care.

Nevertheless, the occurrence of HGA in the Trentino region cannot be excluded, as it is easily underestimated because of the nonspecific symptoms of *A. phagocytophilum* infection in humans. One line of evidence that could support this possibility is the presence of one sequence from roe deer (AF478558) that falls into the "Euorpe1" clade. Moreover, only a few sequences of HGA have been deposited to date, making a general comparison difficult.

In conclusion, additional studies are needed to better define the biological and public health significance of strains with unknown pathogenicity and the one included in the "Europe3" variant in Northeastern Italy. In particular, further research is needed to define the pathogenicity of the different strains at the molecular level, using more informative genes involved in the host–pathogen interaction to better understand the association between host species and strains found in the phylogeny. Moreover, the surveillance of *A. phagocytophilum* should be increased by the development of a surveillance system targeting both people in high-risk infection areas and domestic and wild animals.

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