PREVALENCE OF *BORRELIA BURGDORFERI* SENSU LATO GENOMOSPECIES AND OF THE HUMAN GRANULOCYTIC EHRLICHIOSIS (HGE) AGENT IN *IXODES RICINUS* TICKS COLLECTED IN THE AREA OF MONTI LEPINI, ITALY

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Ticks are obligate hematophagous arthropods that are parasites in every class of vertebrates in most regions of the world. They are also considered to be important vectors for the transmission of human infectious diseases. In the present study we used polymer chain reaction (PCR) amplification analysis to determine the prevalence of *Borrelia burgdorferi* and *Ehrlichia phagocytophila*, the agents of, respectively, Lyme borreliosis and human granulocytic ehrlichiosis, among ticks inhabiting the area of Monti Lepini, a wild area located in the Latium Region of Italy. A total of 141 *I. ricinus* ticks (125 nymphs and 16 adults) were collected in the studied area. Total DNAs were extracted from *I. ricinus* nymphs (pooled in groups of five) and from individual adults. The DNA samples were examined for the presence of *B. burgdorferi* sensu lato and *E. phagocytophila* by PCR using two specific pairs of oligonucleotides that specifically amplify distinct DNA regions of the 16S rRNA genes of the two species. The prevalence of vectors infected with *B. burgdorferi* s. l. was 16% in pooled nymphs samples, and 12.5% in adult ticks, while *E. phagocytophila* was found only in pooled nymphs samples (8%). Three genomospecies were identified, namely *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia valaisiana*, in samples found positive for *B. burgdorferi* s. l. No sample was found positive for *Borrelia burgdorferi* sensu stricto.

Lyme borreliosis (LB) is a human tick-borne infection caused by *Borrelia burgdorferi* sensu lato, a group of genetically diverse spirochetes, while human granulocytic ehrlichiosis (HGE) is a recently discovered disease caused by microrganisms belonging to the *Ehrlichia phagocytophila* group (1). In Europe the principal vectors of these infections are ticks belonging to the *Ixodes ricinus* complex (2). Transmission of *B. burgdorferi* s.l. and *E. phagocytophila* group to humans occurs primarily by bites of infected ticks and manifests LB and HE disease state, which show a rather high incidence in the developed countries of the Northern Hemisphere (3). In Italy, cases of LB have been frequently reported, while cases of clinically documented HGE have not been observed. However, a study conducted by our group in the Latium Region, specifically in the area of Tenuta Presidenziale of Castel Porziano, showed *I. ricinus* ticks infected with both *B. burgdorferi* and *E. phagocytophila* (4). These findings led to further investigation of the prevalence of these microrganisms in ticks inhabiting the area of Monti Lepini. Monti Lepini is a wild area also located in the Latium Region not far from the Tenuta Presidenziale of Castel Porziano. This area provides suitable habitats for ticks that were not included in our previous study (4). The main

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objectives of this study were to evaluate of the prevalence of *B. burgdorferi*. genomospecies and *E. phagocytophila* group microrganisms in *I. ricinus* ticks and to compare their prevalence in ticks inhabiting this area with that reported for ticks in other areas of the same region.

MATERIALS AND METHODS

Study area

The area of Monti Lepini is situated in the Latium Region, at the 42th parallel, 50 km south of Rome. The whole district extends over an area of about 80.000 ha (of these, 60.000 ha are mountainous). The mountains are characterized by a typically wild environment with harsh landscapes almost devoid of surface water. The area includes a large plateau spotted with basins covered by residual red soils. The regional climate is temperate; however, substantial differences in temperature and humidity are found in this region relative to the coast (lower temperatures, more abundant rainfall, snow in winter, etc.). Vegetation varies according to sun exposure and altitude. Mediterranean scrub (myrtle, strawberry, heather and many other kinds of bush vegetation), holly oaks, and dwarf palms growing on rocks cover the "warm zone" in the south side of the park the land is covered with. In contrast, the "cold zone" on the north side is comprised of woods of holly, white oaks and underwood (broom, etc.). Ovine and cattle herds graze in the region's pastures. The wild fauna includes boar, beech-marten, weasel, wolf, as well as many species of reptiles and amphibians. Of the 160 species of birds, 64 are considered resident populations.

Tick collection. A total of 141 *I. ricinus* ticks (125 nymphs and 16 adults) were collected in the studied area. Investigators collected ticks by dragging a blanket over vegetation. Ticks attached to the blanket were removed, placed into tubes, and stored individually at –20°C (*I. ricinus* nymphs were pooled in groups of five, while adults were stored individually) until DNA extraction was performed.

DNA extraction

After thawing, individual ticks and pooled nymphs were mechanically crushed in sterile Eppendorf vials by the aid of sterile micro-pestles in 20 ml TES buffer (50 mM Tris-HCl, 1mM EDTA, 15% sucrose, pH 8). Proteinase K was added at a final concentration of 1 mg/ml and samples were incubated overnight at 37°C. After centrifugation, DNA was extracted twice with equal volumes of a phenol/chloroform solution, and ethanol precipitated. Precipitated DNA samples were suspended in 50 ml of double distilled water and used as templates in PCR experiments.

PCR-based analysis

All DNA samples were examined for the presence of *B. burgdorferi* s. l. and *E. phagocytophila* microrganisms by PCR using two specific pairs of oligonucleotides which specifically amplify distinct regions of the 16S rRNA genes of the two species, as described by Marconi and Garon (5) and Massung et al. (6). A typical PCR mixture (25 ml) contained 0.5 units of Taq Gold DNA polymerase (Perkin-Elmer), 0.2 mM each deoxynucleotide, 2.5 mM $MgCl_2$, 1 ml of diluted DNA template in 1x Taq-polymerase buffer, and 50 pmol of each primer. PCR-mixtures were treated for 12 min at 94°C followed by 35 cycles of 30s at 94°C, 45s at 60°C, 45s at 72°C and then 7 minutes at the same temperature of 72°C. PCR-products were examined on a 1.4% (wt/vol) agarose gel and visualized, after electrophoresis, by ethidium bromide staining. Total DNA preparations extracted from reference strains of *B. burgdorferi* s. l. and *E. phagocytophila* (4) were used as positive control templates. Positive and negative (no DNA template added to PCR mixtures) controls were included in each set of PCR experiments. The expected sizes of the amplified fragments was 357 bp for *B. burgdorferi* s. l., and 919 bp for *E. phagocytophila*, respectively. Samples that scored positive for *B. burgdorferi* s. l. were further characterized by PCR using species-specific primers for *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, as described by Marconi and Garon (5) and for *B. valaisiana*, as described by Liebisch et al. (7). PCR products amplified from DNA templates obtained from reference strains of *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, and *B. valaisiana* were included in each set of PCR experiments as positive controls.

RESULTS

LB and HGE are currently the most important vector-borne diseases in the developed countries of the Northern Hemisphere (3). In Europe these diseases are most common in central and eastern countries, with a rather high incidence. In Italy, cases of LB have been frequently reported, while cases clinically documented HGE were not, in spite of the findings indicating the presence of *E. phagocytophila* in ticks (4, 8).

In this study nymphs and ticks inhabiting the area of the Monti Lepini were collected and analyzed for the presence of *B. burgdorferi* and *E. phagocytophila*. The results obtained indicated that four (16%) out of the 25 pools of nymphs processed were positive for *B. burgdorferi* s. l., and two (8%) were positive for the HGE agent. Of the 16 adults ticks, two (12.5%) were positive for *B. burgdorferi* s. l., and none for the HGE agent (Tab. I). The positivity found for *B. burgdorferi* s.l. and for the HGE agent in vectors inhabiting the area of Monti Lepini, confirmed previous reports regarding the circulation and the prevalence of the two pathogenic agents among ticks in Italy (4, 8). Furthermore, PCR-analysis conducted with oligonucleotides species-specific for *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii* and *B. valaisiana* (5, 7), using total DNAs extracted from the six samples found positive for *B. burgdorferi* s. l. (the 4 nymphs pools and the 2 adult ticks) as DNA templates allowed detection of three *B. burgdorferi* genomospecies, namely, *B. afzelii*, *B. garinii* and *B. valaisiana*. The different genomospecies were concurrently present in three out of the six infected samples (mixed infections): two pools amplified DNA fragments specific for *B. valaisiana*, *B. afzelii*, and *B. garinii* while one female adult tick amplified fragments specific for *B. afzelii* and *B. garinii* (Tab. I).

DISCUSSION

The presence of *B. valaisiana* in ticks has been reported in different european countries but the pathogenicity of this genovariant for humans has not been clearly determined (3). These results lead us to conclude that at least three different genomospecies of *B. burgdorferi* s.l. are circulating among ticks inhabiting the area of Monti Lepini. This finding is not surprising since a study conducted in a different area of the same region, the Tenuta Presidenziale of Castel Porziano, identified even the four genomospecies of *B. burgdorferi* in individually infected ticks (4).

B. burgdorferi s.s. was not detected in this study, while it was detected in ticks inhabiting closely located areas (4, 8). In Europe, *B. burgdorferi* s.s. has not been found uniformly distributed, while this species has been reported to be the most frequently isolated species in North America (9). Since a number of mammals, in particular rodents, have been shown to be reservoir hosts for *B. burgdorferi* s.s. (10-12), the low incidence of this microrganism reported in this study might simply reflect differences in reservoir host populations between the different studied areas.

In conclusion, the results of this study emphasize that ticks infected with *B. burgdorferi* and the HGE agent are currently present in central Italy, and that *E.phagocytophila* infections may well occur in Italy.

Tab. I. *Prevalence of* B. burgdorferi *complex and of HGE agent in nymphs and adult I. ricinus ticks collected in the area of Monti Lepini.*

I. ricinus Stage and sex	No. of *I. ricinus* collected	*I. ricinus* PCR-based identification[a]					
		Positive for *B. burgdorferi* s.l.	*B. valaisiana*	*B. garinii*	*B. valaisiana*, *B. garinii* and *B. afzelii*	*B. garinii* and *B. afzelii*	Positive for HGE agent
Nymph	125 (25 pools)	4 pools	1 pool	1 pool	2 pools	-	2 pools
Adult female	6	1	-	-	-	1	-
Adult male	10	1	1	-	-	-	-

[a] *Total DNAs were extracted from pooled nynphs and adult ticks and subjected to PCR analysis using pairs of oligo-Nucleotides that specifically amplify distinct regions of the 16S rRNA genes of B. burgdorferi s.l. and E. phagocytophila. Identification of B. burgdorferi sensu stricto, B. afzelii, B. garinii, and B. valaisiana was achieved using species-specific oligonucleotides as described in Materials and Methods.*

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REFERENCES

1. **Chen S.M., J.S. Dumler, J.S. Bakken, D.H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. *J. Clin. Microbiol.* 32:589.
2. **Schouls L.M., I. van de Pol, S.G. Rijpkema and C.S. Schot.** 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. *J. Clin. Microbiol.* 37:2215.
3. **Parola P. and D. Raoult.** 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis. 32:897.*
4. **Santino I., M. del Piano M., R. Sessa, G. Favia and A. Iori.** 2002. Detection of four *Borrelia burgdorferi* genospecies and first report of human granulocytic ehrlichiosis agent in *Ixodes ricinus* ticks collected in central Italy. *Epidemiology and Infection.* In press.
5. **Marconi R.T. and C.F. Garon.** 1998. Development of polymerase chain reaction primer sets for diagnosis of Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA signature nucleotide analysis. *J. Clin. Microbiol. 30:2830.*
6. **Massung R.F., K. Slater, J.H. Owens et al.** 1998. Nested PCR assay for detection of granulocytic. *Ehrlichiae. J Clin Microbiol.* 36: 1090.
7. **Liebisch G., B. Sohns and W. Bautsch.** 1998, Detection and typing of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks attached to human skin by PCR. *J. Clin. Microbiol.* 36:3355.
8. **Frusteri L., D. Padovan, R. Murgia, L. Poldini, M. Maroli and M. Cinco.** 1998. Studies on natural infection of *Ixodes ricinus* in Italy. Report of multiple microorganism coinfection (Borrelia spp. and Ehrlichia spp.). *Alpe Adria Microbiol. 7:123.*
9. **Ryffel K., O. Peter, B. Rutti, A. Suard, E. Dayer.** 1999. Scored antibody reactivity determined by immunoblotting shows an association between clinical manifestations and presence of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* in humans. *J. Clin. Microbiol. 37:4086.*
10. **Wang G., A.P. van Dam, I. Schwartz and J. Dankert.** 1999. Molecular typing of Borrelia burgdorferi sensu lato: taxonomic, epidemiological and clinical implication. *Clin. Microbiol. Rev. 12:633.*
11. **Santino I., R. Grillo, M. Nicoletti, D. Santapaola, D. Speziale, R. Sessa, G. Fadda and M. del Piano.** 2002. Prevalence of IgG antibodies against *Borrelia burgdorferi* s.l. and *Ehrlichia phagocytophila* in sera of patients presenting symptoms of Lyme disease in a central region of Italy. *Int. J. Imunopathol. Pharmacol. 15:245.*
12. **S. Frydas, Y. Theodoridis, T. S. Rallis, I. Hatzistilianou, K. K. Adamama-Moraitou, D. Paludi, S. Di Luzio, L. Castellani, A. Grilli and M. Di Gioacchino.** 2001. Leishmania antibodies in children's serum samples in correlation with the disease in dogs. *Int. J. Immunopathol. Pharmacol. 14:173.*