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Detection of four *Borrelia burgdorferi* genospecies and first report of human granulocytic ehrlichiosis agent in *Ixodes ricinus* ticks collected in central Italy

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SUMMARY

The presence of *Borrelia burgdorferi* s.l. and of *Ehrlichia phagocytophila* group was sought by PCR in *Ixodes ricinus* collected in a protected area of central Italy. Nymphs ($n = 1475$, gathered in 295 pools of 5 nymphs each) and adult ticks ($n = 28$) were examined. *B. burgdorferi* s.l. was detected in 13·8% of the nymph pools; of these, 63·4% were infected by *B. valaisiana*, 26·8% by *B. afzelii*, 7·3% by *B. garinii*, and 2·5% by *B. burgdorferi* s.s. Only a single adult male tick proved to host *B. afzelii*. The agent of human granulocytic ehrlichiosis (HGE) was detected in 2·7% of the nymph pools. Two HGE agent-positive nymph pools were also found to be positive for *B. garinii* and for *B. afzelii*, respectively. This is the first report from central Italy of the finding of the HGE agent in ticks.

INTRODUCTION

Lyme borreliosis and ehrlichiosis are emerging diseases transmitted by ticks, which are increasingly reported from Europe. In Italy, after a first case detected in Liguria [1], Lyme borreliosis has been diagnosed in at least a further 1400 cases [2]. Most reports come from the north-eastern region, which is ecologically well-suited to the growth of large populations of the carrier, reservoirs of the pathogens, and of their wild hosts. In contrast, there have been only occasional reports of Lyme borreliosis from the central and southern regions of Italy [3, 4].

To date, there have been no unequivocal reports of cases of human ehrlichiosis from mainland Italy, though there have been three serologically confirmed cases of infection in Americans who were temporary residents in Sardinia; in each case the clinical picture was compatible with ehrlichiosis [5]. Exposure to *Ehrlichia* sp. has been serologically demonstrated among people living in north-eastern alpine areas of Italy [6] and in a protected area of the Parco Nazionale d'Abruzzo in central Italy [7]. The geographical distribution of the aetiological agents of Lyme borreliosis and of ehrlichiosis, that may be transmitted by the same tick species, *Ixodes ricinus*, and that use the same animal reservoirs, are generally overlapping.

Up to now, four *Borrelia burgdorferi* (sensu lato) species have been isolated from the tick *I. ricinus* in Italy, namely *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, and *B. valaisiana*. All can cause erythema migrans (EM) [8], but each is characterized by a different pathogenic potential. *B. burgdorferi* s.s. is often associated with arthritis, *B. garinii* with neurological symptoms, *B. afzelii* with acrodermatitis chronica atrophicans (ACA) [9], while, although serological results [10] suggest the involvement of *B. valaisiana* in clinical manifestations, no strain has yet been isolated from a human sample.

The genus *Ehrlichia* can be divided into three genogroups. The first to be characterized was the *Ehrlichia canis* group (*E. canis*, *E. chaffeensis*, *E. muris*, and *E. ewingii*). Other groups so far described are the *Ehrlichia phagocytophila* group (*E. phagocytophila*, *E. equi*, human granulocytic ehrlichiosis [HGE] agent, and *E. platys*), and the *Ehrlichia sennetsu* group (*E. sennetsu* and *E. risticii*) [11].

Two *Ehrlichia* species that are deemed responsible for HGE have been identified in Italy in *I. ricinus*

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samples; these are the HGE-like *Ehrlichia* in the north-eastern regions and *Ehrlichia phagocytophila* in Latium [12]. Our study forms part of an extensive investigation carried out in central Italy and aimed at quantifying the distribution of these two groups of agents in ticks. The first area sampled was Capocotta, an area included in the Presidential Reserve of Castel Porziano, where both the ecological conditions and the natural fauna appeared likely to support a wide distribution of *I. ricinus* and therefore with the potential for widespead presence of pathogens carried by these hard ticks.

MATERIALS AND METHODS

Study area

Capocotta, an area of 1080 ha, is on the coast of the Tyrrhenian sea, 30 km from Rome; from January 1997, it has been included in the Presidential Reserve of Castel Porziano (4766 ha). Its climate is sub-mediterranean. The reserve hosts a mosaic of oaks (*Quercus ilex*, *Q. pedunculata*, *Q. suber*), pines (*Pinus halepensis*, *P. pinea*), a scrub made up by species typical of the Mediterranean flora, grassland, pasture lands, water expanses and some buildings. Ungulates (fallow deer, red deer, roe deer, wild boars), many micromammalians species and a great variety of birds comprise the wild fauna of the reserve. Capocotta is usually closed to public, with admission only upon request and under strict control. The 1 ha area selected for this study was chosen carefully; it has been reafforested with *Pinus pinea*, its underwood comprises mainly typical Mediterranean shrubs and most of the ground is overgrown with blackberry (*Rubus ulmifolius*).

Tick collection

Ticks were caught by dragging [13] a 1 m^2 cotton cloth through the vegetation twice a month, from April 1997 to September 1998. As far as possible, collections were made fortnightly, choosing windless and rainless days. Ticks were preserved in 70% ethanol and stored until examination. *I. ricinus* nymphs were examined as pools of five, whereas adult ticks were processed individually.

DNA extraction

Specimens were homogenized in sterile Eppendorf vials using sterile micro-pestles in 100 μl of TES buffer (50 mM Tris–HCl, 1 nM EDTA, 15% sucrose, pH 8). Proteinase K was added at a final concentration of 1 mg/ml and samples were incubated overnight at 42 °C. Phenol/chloroform was used for further DNA purification. DNA was precipitated with ethanol and the pellet suspended in 50 μl of double distilled water.

PCR and DNA sequencing

The PCR assay developed was based on specific primers for *B. burgdorferi* s.l. and *Ehrlichia phagocytophila* genogroup as described originally by Marconi and Garon [14] and Massung and colleagues [15], respectively; both sets of primers amplify a region of the 16S rRNA genes.

A reaction mixture with a volume of 25 μl was prepared by mixing together 0·5 units of Taq DNA polymerase, 0·2 mM each dNTP (dATP, dCTP, dGTP and dTTP), 2·5 mM $MgCl_2$, 1 μl of DNA template in the 1 Taq polymerase buffer, and 50 pM of each primer. The reaction mixture was heated at 94 °C for 12 min and then subjected to 35 thermal cycles according to the following scheme: 94 °C for 30 sec, 60 °C for 45 sec, 72 °C for 45 sec, and then kept at the same temperature for 7 min, as described by Favia and colleagues [16].

PCR amplifications were performed using the Perkin–Elmer Taq Gold kit; 10–15 μl of the amplification product was loaded onto a 1·4% agarose gel; after electrophoresis, bands were visualized by ethidium bromide staining. The expected sizes of the specific fragments were 357 bp for *Borrelia* sp. and 919 bp for *Ehrlichia* sp. respectively. Positive controls were purified DNA for *Borrelia burgdorferi* s.l. (kindly provided by M. Cinco, Trieste, Italy) and *Ehrlichia phagocytophila* [16]. Negative controls were PCR reaction with no DNA template. Positive and negative controls were included in each set of PCR analysis.

Positive samples were further analysed by PCR using species-specific primers for *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* as described by Marconi and Garon [14] and for *B. valaisiana* as described by Liebisch and colleagues [17].

Strains of *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. valaisiana* (kindly provided by M. Cinco, Trieste, Italy) were amplified as positive controls.

The 919 bp fragment obtained from the ehrlichia-positive sample was gel purified with the Concert™ Rapid Gel Extraction System (Gibco–BRL) following the manufacturer's instructions. Automatic sequencing was performed by the MWG-biotech (Ebersberg, Germany).

Table 1. *Prevalence of* B. burgdorferi *sensu lato and of HGE agent in 1475 nymphs of the tick* I. ricinus *gathered in 295 pools of 5 specimens each*

Genospecies	No. of positive pools/ examined pools	% of positive pools	Estimated infection rate (%)
B. burgdorferi sensu lato	41/295	13·8	2·9
B. valaisiana	26/41	63·4	18·2
B. afzelii	11/1	26·8	6·0
B. garinii	3/41	7·3	1·5
B. burgdorferi sensu stricto	1/41	2·5	0·4
HGE agent	8/295	2·7	0·5
Coinfection	2/295	0·7	0·1

Statistical analysis

The estimated infection rate was calculated following the method previously described by Cinco and colleagues [18]. A binomial distribution was used ($p = 1 - \sqrt[k]{n^-}/N$), where p is the estimated probability that a single tick is infected, n^- is the number of negative pools, N is the number of samples examined and K is the number of specimens in each pool.

RESULTS

A total of 3612 ticks was collected, belonging to the following species: *I. ricinus*, 2329 (64·48%), *Haemaphysalis concinna*, 1275 (35·30%), *Dermacentor marginatus*, 3 (0·08%), *Rhipicephalus bursa*, 4 (0·11%), *Hyalomma marginatum*, 1 (0·03%). Only specimens of *I. ricinus* were examined in this study, since this species appears to be the main agent responsible for the transmission of *B. burgdorferi* s.l. in Europe.

Although 2329 *I. ricinus* (826 larvae, 1475 nymphs and 28 adults) were collected, only nymphs and adults were analysed because they are the most important for transmission of *B. burgdorferi* to humans [19]. For the purposes of testing, the 1475 nymphs of *I. ricinus* were divided into 295 pools of 5 nymphs each; 28 adult ticks (21 male and 7 female) were examined individually. *B. burgdorferi* s.l. was detected in 41 (13·8%) of the 295 pools and in a single male adult of the 28 tested.

A subsequent analysis showed that 63·4% of the *Borrelia* spp. belonged to *B. valaisiana*, 26·8% to *B. afzelii*, 7·3% to *B. garinii*, and 2·4% to *B. burgdorferi* s.s. The adult tick hosted *B. afzelii*. The estimated infection rate is shown in Table 1.

E. phagocytophila genogroup was detected in 8 (2·7%) of the 295 nymph pools examined. The estimated infection rate was 0·5% (Table 1). The subsequent sequencing of PCR-positive products permitted the identification, for the first time in central Italy, of *Ehrlichia* sp. responsible for human granulocytic ehrlichiosis (HGE). Two pools that were positive for the HGE agent were also found to be positive for *B. garinii* and for *B. afzelii*, respectively.

DISCUSSION

The incidence of Lyme borreliosis disease is still low in Italy, except in some regions such as Veneto, Friuli-Venetia-Giulia, Trentino Alto Adige, and Liguria, where the disease is endemic [2]. This could be due to two main reasons – either a lack of familiarity with the disease, or a low incidence of *Borrelia burgdorferi* s.l. infection in ticks. In this study we tested the second hypothesis by collecting and examining ticks in a rural area of Latium, the Presidential Reserve of Castel Porziano. We documented an estimated infection rate with *B. burgdorferi* s.l. in ticks of 2·9%; this finding is consistent with the incidence of Lyme borreliosis in our region [4].

The presence of four *Borrelia* genospecies in ticks taken from a single location in Castel Porziano may reflect the favourable ecology of this particular site, and its ability to support a fauna comprising numerous wild ungulates.

In this study, no tick was infected with two or more species of *Borrelia burgdorferi* s.l., although, in another study in Latium region, we have found different *Borrelia* species in the same pool of ticks.

B. valaisiana was the species found most frequently (63·4%) as also reported by Kirstein and colleagues [20] in Ireland where the predominance of *B. valaisiana* seemed to be associated with a broad range of host species for this genospecies.

There are considerable differences in tick infection rates with *B. burgdorferi* in different geographical areas. Our estimated tick infection rate in Latium (2·9% of ticks infected by *B. burgdorferi* s.l.) is much

lower than that reported from some regions of northern Italy (0–70%) [21], where borreliosis is considered to be endemic, and from other European regions [11, 22–24].

At 2·7%, the rate of tick infection with HGE agent was five times lower than the 13·8% infection rate with *B. burgdorferi*. Our study revealed, for the first time in central Italy, the presence of an *Ehrlichia* sp. responsible for human granulocytic ehrlichiosis.

In contrast with the wide variation between different geographical areas in Italy and in Europe, of the prevalence of tick infection with the agent of Lyme borreliosis, there appears to be much less evidence of variation in the prevalence of tick infection with the HGE agent. Our estimated tick infection rate of 0·5% in Latium is similar to the prevalence of tick infection with *E. phagoytophila* found in other European countries. In Scotland, the prevalence of infection in pools of nymphs and adults ranged from $> 0{\cdot}25\%$ to 2% [25]. In central France the prevalence of infection in adult ticks was 1·3% [24], in western Switzerland [26] and United Kingdom [27] 1·4%. Somewhat higher prevalences have been reported from southern Germany (2·2%) [11], from Slovenia (3·2%), from where the first European HGE case was reported [28] and from the north-eastern regions of Italy, 4·2% [12].

The method of testing pools of nymphs did not allow us to detect, as in other central European countries [11, 29, 30] and in the United States [31, 32], double infections caused by both *Borrelia* spp. and *Ehrlichia* spp. However, the possibility exists that ticks may transmit both infections with a single bite [33, 34–36]. This risk is important because although the clinical symptoms of both infections are more or less similar, the two pathogenic agents do not respond to the same antibiotic therapy [37–39].

Although the reserve that we sampled is closed to the general public, during some seasons (spring and late summer) in which nymphs are particularly abundant, seasonal workers are allowed to enter the area chosen for our investigation [40]. In this study we were able to demonstrate not only the wide distribution of *I. ricinus* in the area under study, but also the prevalence of tick infections with the agents of Lyme borreliosis and of HE in a rural area of Latium in central Italy. Though we did not confirm the presence of double infections (due to the way in which our study was carried out), the possibility of such double infections reinforces the potential risk, not only for the health of the protected animal species living in the reserve but also for man.

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