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Presence of granulocytic ehrlichia in ticks and serological evidence of human infection in La Rioja, Spain

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SUMMARY

In order to estimate the risks of human granulocytic ehrlichiosis (HGE) in an endemic area for Lyme disease in the North of Spain (La Rioja), we collected and investigated by PCR specific to the *E. phagocytophila* group DNA, a total of 6870 *Ixodes ricinus* ticks. We also used an indirect immunofluorescence (IFI) test to study the presence of antibodies to the HGE agent in 147 human serum samples including patients with Lyme disease (LD), forestry workers, and persons with history of previous tick bite. Fifty serum samples from healthy people resident in urban areas and with no history of tick-bite disorder and without tick exposure were used as controls. Four of 76 adults and 49 of 203 nymphs pools carried *E. phagocytophila* DNA. This result, and the finding of 1·4% of sera reacting in the IFI test confirms that this tick-borne agent is present in La Rioja, and that humans show evidence of contact with it. HGE should be considered in the differential diagnosis of flu-like syndromes in the study area in the north of Spain.

INTRODUCTION

Ehrlichiosis is a group of emerging tick-borne infectious diseases caused by obligate intracellular Gram-negative rickettsia which infect leucocytes. Cases of human granulocytic ehrlichiosis (HGE) were first described in 1994 in Minnesota and Wisconsin [1, 2]. Three years later their presence in Europe was also reported [3]. HGE is a non-specific febrile illness characterized by headache, myalgias, malaise, thrombocytopenia, leucopenia and elevated levels of hepatic transaminases [4, 5]. The illness is clinically indistinguishable from human monocytic ehrlichiosis (HME), which is caused by *E. chaffeensis* [6]. The HGE agent is closely related to *E. equi*, the agent of equine ehrlichiosis, and *E. phagocytophila* the agent of tick-borne fever in ruminants [2, 7]. The latter has been reported in regions in the North of Spain causing abortion in sheep and non-specific illness in cattle [8, 9]. To date there have been no convincing reports of HME infection in humans in Europe.

The natural history of HGE is still being defined, but it is possible that the agent is maintained in nature in a tick-ruminant-rodent cycle, with humans being involved only as incidental 'dead-end' hosts. The main vectors are ticks of the *Ixodes ricinus* complex which are found in regions where HGE and Lyme disease (LD) occur [10–13]. In this study we evaluated the risk of disease in humans in La Rioja (an endemic area for LD in the North of Spain) [14, 15] by investigating the presence of *E. phagocytophila* in *I. ricinus* ticks and by seeking the presence of anti-HGE agent antibodies in human groups potentially exposed to this infection.

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MATERIALS AND METHODS

Tick collection

Ixodes ricinus were collected in La Rioja, a small region in the North of Spain where LD is endemic, from March 1995 to May 1998 by flannel cloth dragging over vegetation. A total of 26 recreation areas were selected for sampling on the grounds that they were the most likely places of contact between humans and ticks. All ticks on the blanket were collected and identified in the laboratory.

Serum samples

Serum samples from patients with previous LD (43 samples), tick-bite history (54 samples) and foresters (50 samples) were frozen and stored at −40 °C until testing. People living in rural and urban areas were sampled. As a control group, we also analysed 50 serum samples from healthy people living in urban environments and with no history of tick-bite disorder or tick exposure.

Processing of ticks and PCR

Once classified, ticks were frozen and stored at −80 °C awaiting DNA extraction. Adult ticks were processed individually and nymphs and larvae were pooled, with each pool containing 10 or 100 individuals, respectively. Individual ticks and pools were digested with proteinase K (200 μg/ml) and sodium dodecyl sulphate (final concentration 1 %). DNA was purified by extraction with phenol-chloroform and then precipitated with sodium acetate and ethanol. Multiple water samples were included as negative controls. DNA concentration and purity was determined in a spectrophotometer at 260 and 280 nm.

A set of primers using the 16S rRNA sequence of the granulocytic ehrlichlia as the target (*E. phagocytophila*, *E. equi* and the HGE agent (E1: 5′- GGC ATG TAG GCG GTT CGG TAA GTT -3′, and E2: 5′- CCC CAC ATT CAG CAC TCA TCG TTT A -3′) amplifying a 262 bp product specific to the *E. phagocytophila* genogroup was used in all the PCR tests [16]. No PCR product was amplified from blood from non-infected 1-month-old lambs, from local strains of ovine *Babesia* (*B. ovis* and *B. motasi*) infected blood, from cultures of tick-borne local agents (*Borrelia burgdorferi*, ovine encephalitis virus similar to Louping ill), from laboratory ticks free of infection (larvae, nymphs and adults of *Ixodes ricinus* and *Ornithodorus erraticus*), or from tissue culture extracts (from BHK, PK15, SCP, myeloma cells). Sensitivity of the PCR protocol was evaluated by serial dilution of purified DNA from a blood sample with a known number of infected neutrophils. The last positive dilution was chosen and the limit for detection was 72 infected neutrophils/mm^3, equivalent to percentages of infection between 0·01 % and 0·001 %.

PCR reactions were performed on a Robocycler 40 (Stratagene, La Jolla, CA, USA) according to the following thermal profile: 96 °C for 2 min, then 30 cycles comprising denaturation at 96 °C for 30 s, annealing and extension at 70 °C for 90 s, and a final extension at 70 °C for 10 min. The specific size product was visualized following electrophoresis on ethidium bromide stained agarose gels. Multiple water samples were included between test samples as controls for PCR amplicon contamination, and also a positive blood sample from an experimentally infected lamb.

For pooled samples, prevalence was expressed as minimum-maximum percent of infected ticks assuming that either only one or all the ticks in each pool yielded a positive reaction.

Serological assay

Serum samples were tested by an IFI (HGE IFA IgG MRL Diagnostics, CA, USA) as described previously [17]. The antigen in this test is prepared from a human promyelocytic leukemia cell line (HL-60) infected with the HGE agent. The assay was performed according to manufacturer's instructions. To screen the samples only two dilutions (1/64 and 1/128) of each were made. Antibody titres were confirmed by a second microscopist reading coded slides. Titres were reported as the reciprocal of the highest dilution showing specific fluorescence. A titre of 64 or greater to the HGE agent was considered to be positive evidence of specific antibody [18]. Serological cross-reactions to *Rickettsia conorii*, *Coxiella burnetti* and *E. chaffeensis* were investigated in those serum samples with positive results on IFI.

RESULTS

Prevalence of *E. phagocytophila* infection in ticks

A total of 6870 *Ixodes ricinus* ticks were collected and tested for *E. phagocytophila* group: 76 adults, 2005 nymphs (203 pools) and 4789 larvae (42 pools). Four

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Table 1. *Prevalence of granulocytic* Ehrlichia *infection in active* I. ricinus, *in the areas found positive*

Area		Positive adults/total	Prevalence in adults (%)	Positive nymph pools/total pools (total nymphs)	Prevalence in nymphs (%)
1	El Rajao	1/12	8·3	8/25 (269)	3–32
2	Moncalv. Monte	1/4	25	1/11 (113)	0·9–9·1
3	Nestares	1/9	11·1	11/31 (303)	3·6–35·5
4	Montemediano	1/12	8·3	3/25 (238)	1·3–12
5	Valdezcaray	–	–	1/2 (16)	6·3–50
6	Moncalvillo Granja	–	–	3/12 (115)	2·6–25
7	Ortigosa	0/3	0	1/11 (102)	1–9·1
8	Peña los Cintos	0/16	0	6/21 (204)	2·9–28·6
9	La Pilatoba	0/14	0	1/29 (278)	0·4–3·5
10	Venta Piqueras	0/3	0	2/11 (112)	1·8–18·2
11	Carbonera	0/1	0	12/18 (187)	6·4–66·7

Table 2. *Serological examination of human sera for HGE using indirect immunofluorescence* (*IFI*)

Group	No. sera	IFI positive
Lyme disease	43	1 (2·3%)
Tick-bite	54	1 (1·9%)
Forestry	50	0
Control group	50	0

adults (2 males and 2 females) and 49 nymph pools were positive. No larva pool was positive. *Ehrlichia phagocytophila* DNA was identified in 11 sampling areas. Among them, the percentage of adult infection was 0–25%. Infection in nymphs was detected in 2·4–24·1% of individual ticks. Prevalence of nymph infection in different areas ranged between 0·4–3·5% and 6·4–66·7% (Table 1).

HGE serum sample antibodies

Serum samples of 147 patients and 50 controls were analysed. Forty-three had LD, 54 had a history of previous tick-bite (without evidence of tick-borne disease), and 50 were forestry workers. Two individuals (1·4%) reacted with the HGE agent antigen at a titre of 64: one patient with previous neuroborreliosis (2·3%) of LD group, and one hunter with previous tick-bite and absence of *B. burgdorferi* antibodies (1·9%) (Table 2). Neither sample was positive to *R. conorii*, *C. burnetti* or *E. chaffeensis*. We also analysed a previous serum sample of the hunter and found no evidence of HGE infection. He had therefore had an apparently asymptomatic seroconversion to HGE agent. Neither individual had a titre greater than 64. None of the serum samples from the control group was positive.

DISCUSSION

In this study we have demonstrated the presence of ehrlichias of the *Ehrlichia phagocytophila* group in ticks and the immune response of humans to the HGE agent in La Rioja, Spain. To date, no study had demonstrated this infection in humans in our country. In Spain many different tick-borne diseases are present (LD, Mediterranean spotted fever, babesiosis, tick paralysis and tularemia [15, 19–22]. Here we show evidence of possible risk of HGE as we have suggested in earlier communications [23]. Recently, we also reported the first confirmed case of HGE in the south of Europe [24].

In the north of Spain we have obtained evidence of a febrile illness produced by *E. phagocytophila* in sheep [8], cattle [9] and, more recently, a high prevalence of *E. phagocytophila* infection in *Ixodes* and other species of ticks in a neighbouring region [25]. Some authors suggest that the organisms of the *E. phagocytophila* group are very closely related and actually belong to the same species [2, 7].

Conditions permitting the presence of HGE in the North of Spain exist, since the ambient temperatures and humidity support the survival of the tick vector. In addition, the use of a large part of the country for sheep and cattle husbandry and the presence of other wild mammals including deer and rodents provides excellent food sources for this tick species, and allows the maintenance of zoonotic infections in domestic species.

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Of the ticks analysed, up to 24·1% of nymphs and 5·3% of the adults, but no larvae were positive in PCR. This agent is not spread transovarially, horizontal transmission involving a susceptible vertebrate host is necessary [26, 27].

In Europe, the prevalence of HGE agent in *I. ricinus* ticks in Switzerland was 0·5–1·3% (1·6% of the adults and 0·5% of the nymphs), 26·5% of the ticks from cattle with ehrlichiosis, 4·4% of the ticks from healthy cattle and 0·8% of the free-living ticks (both male and female) [28, 29]. In Italy 24·4% (all nymphs) [13, 30], and in the United Kingdom 0·25–7% (all nymphs) were infected [31, 32]. In the United States of America, the prevalence of HGE agent in *Ixodes* sp. is very wide, with 7–53% of the adults ticks and 21% of nymphs showing evidence of infection [10, 12, 33, 34]. This variability could be due to differences among tick species either as adults or during development or to geographical or environmental differences influencing intermediate host species or the infectivity of ticks [29]. Another possible explanation is that there may be differences between the PCR primers employed. Our findings are compatible with these figures, lying in an intermediate position.

In Europe, human exposure to the HGE agent has been reported and patients with illness due to HGE agent have been documented [3, 35, 36]. Sero-epidemiological studies have shown different rates of infection in humans. In Switzerland the overall seroprevalence was 5%, with a prevalence in tick-exposed persons of 7·4–17·1%, hunters of 9%, LD patients of 12·7%, blood donors of 1·1% and neonates of 0·5% [37, 38]. In Bulgaria, 3% of blood donors showed antibodies to HGE agent [39]. In Hungary 12·5% of tick-exposed patients had antibodies to the HGE agent [40]. In an endemic area of *B. burgdorferi* in Italy, prevalence was 8·6–20·5% in forestry workers, 5·5% in hunters, 4·3% in civil protectors and 1·5–4% of the residents in wooded areas of the Alpine area [41, 42]. In Sweden, 11·4% of the inhabitants had antibodies to *E. equi* and 1·1% to *E. chaffeensis* [43]. In Denmark, 3·8% of patients with Lyme neuroborreliosis also had antibodies to *E. equi* while none of blood donors tested was positive [44]. In France, the prevalence of the HGE agent was 1·6% of LD patients and 1% of the forestry workers [40]. In the United Kingdom 7·5% of LD patients, 5% of tick exposed patients and none of blood donors had antibodies to HGE agent [45]. In Germany, antibody to HGE agent has been reported in 14% of forestry workers, in 11·4% of LD patients and in 1·9% of blood donors [46]. In Norway, 10·2% of LD patients showed serological evidence of infection with HGE [4].

Now, in Spain we have found a seroprevalence of 1·4% (lower than other studies), 2·3% of LD patients and 1·9% with previous tick-bite. As none of our patients had ever travelled abroad, we assume that these patients ware exposed in La Rioja. It has been suggested that active HGE agent may cause false positive LD serological results when ELISA and immunoblotting are used [47], but not all studies support this observation [46] and infection of mice with the HGE agent does not induce diagnostically significant *B. burgdorferi* serological cross-reactions [48]. Therefore the possibility of multiple infections with *B. burgdorferi*, HGE agent and other agents as *Babesia* sp. should be considered when diagnosing tick-borne infections in Europe [49]. The purpose of this study was not to evaluate the specificity and sensitivity of the serological test employed, but the absence of cross reactions with the investigated microorganism suggested to us that the test was as specific as others had found [18, 37].

In summary, evidence of the presence of *E. phagocytophila* group in ticks of La Rioja has been obtained and serological responses to the HGE agent have been demonstrated in risk groups (an LD patient and an individual occupationally or recreationally exposed to the risk of tick-bite) in La Rioja (Spain) an endemic area for LD. Physicians need to be aware of this disease in Europe and should consider this diagnosis in febrile patients with tick bites in areas where LD is endemic.

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