Detection of antibodies to tick salivary antigens among patients from a region of Spain.

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Abstract

With a view to determine which tick species bite humans most frequently in the province of Soria (Spain) and to know whether these species act as vectors of *Rickettsia conorii* and/or *Borrelia burgdorferi*, we analysed the presence of antibodies against salivary proteins of several tick species and of antibodies against *R. conorii* and *B. burgdorferi sensu lato* in 102 samples of serum. The sera were collected from 63 patients who were treated for tick bites at the health services of the Soria Health Area.

Thirty-nine percent of the patients displayed antibodies against *Dermacentor marginatus*; 21% against *Ixodes ricinus*, and 6% against *Rhipicephalus sanguineus*. IgM antibodies against *R. conorii* were found in 15 patients, but none of the patients included in the study displayed IgG/IgM Abs against *B. burgdorferi sensu lato*. In 30% of the patients bitten by *I. ricinus* and in 24% bitten by *D. marginatus*, antibodies against *R. conorii* were found. None of the patients who had been bitten by *R. sanguineus* expressed antibodies against this pathogen.

The results obtained here suggest that *D. marginatus* and *I. ricinus* are the main vectors of the spotted fever group rickettsiae in our environment and that the role of *R. sanguineus* in the transmission of these rickettsiae is debatable.

Key words: ticks, spotted fever group rickettsiae, epidemiology.

Introduction

Ticks are blood-sucking parasites that act as reservoirs and vectors of an important number of pathogenic bacteria for humans. To date, 869 species of ticks are known although only species belonging to the genera *Ixodes*, *Rhipicephalus*, *Amblyomma*, *Dermacentor*, *Hyalomma*, *Haemaphysalis* and *Ornithodoros*¹ have been declared vectors of bacterial diseases transmitted to humans.

Each species of tick requires optimum environmental conditions and biotopes for its development and these parameters govern the distribution of ticks and the geographic regions in which they act as vectors. This means that such zoonoses are restricted to localised zones of the world: *Rickettsia rickettsii* in America; *Rickettsia sibirica* in the northwest of central Asia and China; *Rickettsia conorii* in the south of Europe, southeast Asia, India and Africa, and *Borrelia burgdorferi sensu stricto* in Europe and North America. This distribution may vary owing to dissemination of the vector and/or reservoir due to macroclimatic changes, deforestation and the migration of humans and animals².

The detection of antibodies against tick salivary proteins in humans is a good biological marker of exposure to tick bites. It is also a very useful tool for epidemiological and clinical studies of tick-borne diseases^{3,4}.

The province of Soria (Spain) shows a high prevalence of tick-transmitted infections such as infection by *R. conorii*^{5,6} or by *Coxiella burnetii*⁷ or Lyme disease^{8,9} in humans and in dogs. The aim of the present work is to gain knowledge of the tick species that most frequently bite humans in this area and to determine whether these species act as vectors of *R. conorii* and/or *B. burgdorferi*.

Materials and Methods.

The study was carried out on 102 samples of serum from 63 patients who had attended the Soria Health Area services for one or more tick bites between March 1, 1996, and December 31, 1997. A serum sample was withdrawn from all patients at the time of consultation and the patients were asked to return to provide another one at 21-25 days later.

Each serum sample was divided into two aliquots, which were conserved at –20°C until analysis. In one of the aliquots the presence of IgM antibodies (Abs) against

Rickettsia conorii and of IgG/IgM against *Borrelia burgdorferi sensu lato* was analysed. The other aliquot was sent to the School of Pharmacy of the University of Salamanca, where the presence of anti-tick Abs was analysed.

The detection of IgM Abs against *R. conorii* was accomplished by indirect immunofluorescence, using a suspension of *R. conorii* fixed on a slide (*R. conorii*-Spot IF; bioMerieux, Marcy-l'Etoile, France) as antigen. Serum samples were diluted 1/10 in phosphate buffered saline pH 7.2 with 0.05% Tween-80 (Sigma Chemical Company, St. Louis, Missouri) (TPBS). IgG Abs were removed by absorption (RF-Absorbens; Dade Behring, Marburg, Germany). The sera that showed intra- and extracellular fluorescence upon staining with anti-IgM conjugate (Fluoline-M, bioMerieux, Marcy-l'Etoile, France) but no fluorescence after staining with anti-IgG (Fluoline-G, bioMerieux, Marcy-l'Etoile, France) were considered positive. In all the assays two positive and two negative controls were included. The positive controls were the sera from two patients with clinical signs and symptoms of Mediterranean spotted fever whose anti-*R. conorii* IgM Abs titers were $\geq 1/64$. The titers of these sera were confirmed at the National Reference Centre (Instituto Carlos III, Majadahonda, Madrid). As negative control TPBS was used. All the slides were examined separately by two researchers.

The detection of IgG/IgM Abs against *B. burgdorferi* was carried out by means of a commercial EIA (Wampole-Innogenetics Diagnostica) according to the manufacturer's instructions. All the assays included the positive and negative controls supplied by the manufacturer as well as a commercial positive control (Accurum 132 *Borrelia burgdorferi* IgM positive control, Boston Biomedica, West Bridgewater, MA).

The detection of anti-tick Abs (IgG, IgM, IgE) was performed with indirect ELISA, using a previously reported procedure⁸. As antigens, we used protein extracts from the salivary glands of six species of ixodids common in the province of Soria (*Dermacentor marginatus*, *Rhipicephalus bursa*, *R. turanicus*, *R. sanguineus*, *Ixodes ricinus* and *Hyalomma marginatum*) and of two species of argasids (*Ornithodoros erraticus* and *O. moubata*), which, because they were not present in the province, served as negative controls. As the protein composition of the saliva of the ixodid ticks vary along the feeding period we obtained the salivary antigens from pools of ticks containing both unfed specimens and specimens in several degrees of engorgement. The

salivary antigens of the argasid ticks were obtained only from unfed specimens. The ELISA plates were coated with 1 µg of antigen per well. The sera were diluted at 1/50 for IgG and IgM or at 1/20 and 1/10 for IgE in PBS containing 0.05% Tween-20 (TPBS). Then, they were incubated one hour at 37 °C. Peroxidase-labeled anti-human immunoglobulin antibodies were also diluted in TPBS (at 1/4000 for anti-IgG and anti-IgM and at 1/500 for anti-IgE) and incubated one hour at 37 °C. As chromogen substrate for peroxidase we used ortho-phenilen-diamine (OPD) and the reactions were stopped with 3N sulphuric acid. In all the ELISA plates we included six negative control sera obtained from people that have never been bitten by any tick. When available we also include one or two positive control sera, as was the case for *D. marginatum*, *I. ricinus*, *R. bursa*, *O. erraticus* and *O. moubata*. The sample sera were considered positive when their optical density surpassed a threshold calculated as the mean optical density of the negative control sera plus three times the standard deviation (mean OD + 3 x standard deviation).

The following data were collected from each patient: age, sex, the date on which the bite had occurred, and the time elapsed between the tick bite and the blood sample extraction(s). These variables, together with the results obtained in the study were processed using the Microsoft Access database. Statistical treatment of the data was accomplished using the SPSS 8.0 program.

Results.

Out of the 63 patients attended at the Soria Health Services for tick bites, 27 were female and 35 were male. The mean age ($\pm$ standard deviation) was 45.9 ± 21.2 years (range, 5-96). All patients were asymptomatic at the time of the study. In 51 patients (81%) the time elapsed between the tick bite and the blood collection was longer than 21 days; 12 of these patients were bled once and the other 39 were bled in two occasions. In a further 12 patients one serum sample was taken into the following 21 days after the tick bite (10.5 ± 6.4 days).

Of the patients studied, 25 (39%) had Abs (IgG, IgM or both, but never IgE) against *D. marginatus*, 13 (21%) against *I. ricinus* and 4 (6%) against *R. sanguineus*. In 21 patients (33%), no Abs of any isotype were detected against any of the 8 tick species included in the study (table 1). Out of the 42 patients carrying anti-tick antibodies, 14 were bled once. The other 28 were bled two times and they all showed anti-tick antibodies in both serum samples.

There were no significant differences between the patients with or without anti-tick Abs in the post-bite time at which these patients were bled, nor were significant differences in the bleeding time between the patients carrying Abs against the three different ixodids.

Fifteen patients, with a mean age ($\pm$ standard deviation) of 35.1 ± 17.9 years had Abs against *R. conorii* (Table 1). Ten were male and five were female. Of the patients bitten by *D. marginatus*, 24% had Abs against *R. conorii* while among those bitten by *I. ricinus*, the percentage of patients with Abs against *R. conorii* was 30%. No significant differences between the likelihood of a patient with *R. conorii* infection having been bitten by *D. marginatus* or by *I. ricinus* were found ($p < 0.05$). In 5 (33%) of the seropositive patients to *R. conorii*, the species of tick that acted as the vector of the infection was unknown. None of the patients bitten by *R. sanguineus* showed Abs against *R. conorii*.

None of the patients included in the study displayed IgG/IgM Abs against *B. burgdorferi sensu lato*.

Forty-eight percent of the tick bites occurred in spring; 24% in autumn; 17% in winter, and 11% in summer. Among the patients in which the presence of Abs against

R. conorii was detected, 7 (46%) had been bitten in spring; 6 (40%) in autumn; 1 (7%) in winter, and 1 (7%) in summer (Figure 1).

Discussion

The prevalence of human infections transmitted by ticks in a given area depends on the abundance of reservoirs for that infection, the vector density, the degree of anthropophilia of the vectors and the likelihood of humans entering their biotope. The frequency with which different tick species bite humans varies significantly from one zone to another and is, in fact, a function of the above indicated factors. In our study, the most common species was *D. marginatus*, followed by *I. ricinus* and *R. sanguineus*. By contrast, in Liguria (Italy), Manfredi et al.⁹ have reported that the most frequent species is *I. ricinus*, followed by *R. sanguineus* and *D. marginatus*, whereas in Maine (USA) Smith et al.¹⁰ found that the most frequent species is *Dermacentor variabilis* (45%), followed by *Ixodes cookei* (34%) and *Ixodes dammini* (17%).

The province of Soria has optimum ecological characteristics for the development of several species of ticks. It is located in the inner Iberian Peninsula (central-northern region) at a mean altitude of 1,000 m a.s.l. Its climate is continental, with cold dry winters and mild summers. It has extensive forested areas (*Pinus* sp., *Quercus* sp., *Juniperus* sp.) and shrubs occupied by several species of wild (foxes, deer, hares, rats, moles...) and domestic (sheep, goats, dogs, cattle) animals. Humans enter into contact with the tick habitat for both professional (the population of the province is mainly rural) and recreational (hunting, fishing, trekking, picnicking...) activities.

The hosts of the three species of ticks against which we detected the presence of Abs in the population studied are well represented in Soria. Large mammals are the main source of food for the adult stages of *D. marginatus* and *I. ricinus*, while small mammals and birds host the larval and nymph stages of these two species. Dogs, the main host of both adult and immature *R. sanguineus*², are also common in the province of Soria⁷.

One of the main factors responsible for variations in the frequency of the ticks found in the present study is the density of the different species of tick in each biotope. The province of Soria represents an optimum ecological niche for *D. marginatus* (steppe area, temperate forest, meadows and grazing land) and *I. ricinus* (humid microhabitats and high-ground forests) but not for *R. sanguineus*, which prefers forests located in Mediterranean climates².

Another key factor is tick affinity for humans, which is very high in *D. marginatus* and *I. ricinus*² but low in the case of *R. sanguineus*^{2,11}, especially in areas where dogs are frequent. The forms of *R. sanguineus* with the greatest affinity for humans are the immature stages¹², whose bites usually pass unnoticed² and hence would be a population not included in the present study.

The spring and autumn are the seasons in which ticks bite humans most frequently in the studied area, these seasons match with those of greatest infection by *R. conorii*. These months also overlap with the time when people are most in contact with the natural environment and able to enter the tick biological cycle, which is regulated by environmental parameters such as temperature, relative humidity and photoperiodicity². These factors explain why the months with the greatest transmission of tick-borne zoonosis vary from one region to another in the world. In this sense, Carpenter et al.¹³ reported that human parasitism due to *R. sanguineus* in San Antonio (Texas, USA) is higher in the summer and autumn months while Roberts¹⁴ states that in Kenya the parasitism due to this organism is more prevalent in the coldest months.

For each tick-borne disease there may be one or several vectors and a single vector may transmit one or several diseases¹⁵. In relation with this it has been observed recently that some tick species are not specific vectors of only certain diseases and that their role as vectors of other infectious agents has been underestimated¹⁶. Accordingly, *D. marginatus* is not only a vector of *Francisella tularensis* and *Rickettsia slovaca*² but it is also a vector of *B. burgdorferi*¹⁷ and *R. conorii*¹⁸.

The usual method for the diagnosis of rickettsiosis in most clinical microbiology laboratories is the serologic testing, and the indirect immunofluorescence assay (IFA) is considered the “gold standard” of this kind of assays. However, IFA has low specificity because of the presence of the group-shared lipopolysaccharide antigen, which originates extensive intra-group cross-reactivity. Although serological cross reactions are common among the spotted fever group rickettsiae, the background knowledge, the data on the geographic distribution and the clinical signs and symptoms have allowed to place even closely related organisms in separate species.

Prior studies in the province of Soria found seroprevalences to *R. conorii* around 6% in humans⁵ and 8% in dogs⁶. Traditionally *R. conorii* has been considered the main

agent of human rickettsiosis in Spain, but recently other pathogenic rickettsiae, as *R. slovaca*, have also been identified in this country.

The results obtained here suggest that *D. marginatus* and *I. ricinus* might be implicated in the transmission of spotted fever group rickettsiae to humans in our environment and that the role of *R. sanguineus* is debatable, as has been already posited by other authors^{16,18}.

Although high seroprevalences of Lyme disease have been observed in Soria in humans and dogs (respectively, 13 %⁸ and 11 %⁷), we have not detected any Abs against *B. burgdorferi* in the patients analysed, even in those bitten by the main vector of the Lyme spirochete, *I. ricinus*. Several factors could have been the responsible of this result: first, the ticks could have been removed from people in the first 48 hours post-attachment thus preventing the transmission of the spirochete (Ohnishi et al., 2001, ver cita más abajo); second, even in the case of an effective transmission, the patients could have been bled before the synthesis of the anti-*B. burgdorferi* antibodies have occurred. In any case, as the distribution of some tick-borne diseases depends on factors that remain to be elucidated², consequently the presence of the vector and the reservoir of a given rickettsia in a particular area does not necessarily mean that human infections by that agent will be produced.

In our study, 21 (29%) patients were bitten by an unknown tick species, and in 17 of them the serum sample was withdrawn more than 21 days after the tick bite. It would be of great interest to identify the tick species involved since in 5 of those patients Abs against *R. conorii* were detected.

To determine which tick species are really implicated in the transmission of the different species of spotted fever group rickettsiae further studies need to be made in which ticks removed from the patients must be directly identified and the rickettsial strains present in the ticks and in their human hosts must be genotyped.

OHNISHI, J.; PIESMAN, J. & DE SILVA, A.M. (2001). Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. **Proceedings of the National Academy of Sciences**, 98: 670-675

References

1. Camicas JL, Hervy JP, Adam F, Morel PC. The ticks of the world (Acarida, Ixodida). Orstom, ed. Paris; 1998.
2. Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis 2001; 32: 897-928.
3. Schwartz BS, Ford P, Childs JE, Rothman N, Thomas RJ. Anti-tick saliva antibody: a biologic marker of tick exposure that is a risk factor for Lyme disease seropositivity. Am J Epidemiol 1991; 134: 86-95.
4. Lane RS, Moss RB, Hsu YP, Wei T, Mesirov ML, Kuo MM. Anti-arthropod saliva antibodies among residents of a community at high risk for Lyme disease in California. Am J Trop Med Hyg 1999; 6: 850-859.
5. Saz JV, Bacellar F, Merino FJ, Filipe A. Seroprevalencia de la infección por *Coxiella burnetii* y *Rickettsia conorii* en la provincia de Soria. Enferm Infecc Microbiol Clin 1993; 11: 469-473.
6. **Delgado S and Cármenes P. Canine seroprevalence of *Rickettsia conorii* infection (Mediterranean spotted fever) in Castilla y León (northwest Spain). Eur J Epidemiol 1995; 11: 597-600.**
7. Nebreda T, Contreras E, Merino FJ, Dodero E, Campos A. Brote de fiebre Q y seroprevalencia en una población rural de la provincia de Soria. Enferm Infecc Microbiol Clin 2001; 19: 57-60.
8. **Saz JV, Nuncio S, Merino FJ, Aquisé M, Medina J, Filipe AR. Enfermedad de Lyme en la provincia de Soria: Estudio clinicoepidemiológico. Enf Infecc Microbiol Clin 1994; 12 (2): 52-59**
9. Merino FJ, Serrano JL, Saz JV, Nebreda T, Gegúndez M, Beltrán M. Epidemiological characteristics of dogs with Lyme borreliosis in the province of Soria (Spain). Eur J Epidemiol 2000; 16: 97-100.
10. Pérez-Sánchez R, Oleaga-Pérez A, Encinas-Grandes A. Analysis of the specificity of the salivary antigens of *Ornithodoros erraticus* for the purpose of serological detection of swine farms harbouring the parasite. Parasite Immunology 1992; 14: 201-206.
11. Manfredi MT, Dini V, Piacenza S, Genchi C. Tick species parasitizing people in an area endemic for tick-borne diseases in North-Western Italy.

- Parassitologia 1999; 41: 555-560.
12. Smith RP, Lacombe EH, Rand PW, Dearborn R. Diversity of ticks species biting humans in a emerging area for Lyme disease. Am J Public Health 1992; 82: 66-69.
 13. Goddard J. Focus of human parasitism by the brown dog tick, *Rhipicephalus sanguineus* (Acari: Ixodidae). J Med Entomol 1989; 26: 628-629.
 14. Gilot B, Laforge ML, Pichot J, Roult D. Relationships between the *Rhipicephalus sanguineus* complex ecology and Mediterranean spotted fever epidemiology in France. Eur J Epidemiol. 1990; 6: 357-362.
 15. Carpenter TL. McMeans MV, McHugh. Additional instances of human parasitism by the brown dog tick (Acari: ixodidae). J Med Entomol 1990; 27: 1065-1066.
 16. Roberts JI 1935 The ticks of rodents and their nest and the discovery that *Rhipicephalus sanguineus* Latr. is the vector of tropical typhus in Kenya. J Hyg 1935; 35: 1-22.
 17. Lane SL. Competence of ticks as vectors of microbial agents with an emphasis on *Borrelia burgdorferi*. In: Sonenshine DE, Mather TN, eds. Ecological dynamics of tick-borne zoonoses. New York: Oxford University Press, 1994: 45-67.
 18. Maroli M, Khoury C, Frusteri L, Nanilla G. Diffusione della zeca del cane (*Rhipicephalus sanguineus* Latreille, 1806) in Italia: un problema di salute pubblica. Ann Ist Super Sanità 1996; 32: 387-397.
 19. Anglov L, Dimova P, Berbencova W. Clinical and laboratory evidence of the importance of the tick *D. marginatus* as a vector of *B. burgdorferi* in some areas of sporadic Lyme disease in Bulgaria. Eur J Epidemiol 1996; 12: 499-502.
 20. Genchi C. Arthropoda as zoonoses and their implications. Vet Parasitol 1992; 44: 21-33.

Table 1. Characteristics of the patients attended for tick bites in this study.

	Patients with antibodies against			
	<i>Dermacentor marginatus</i>	<i>Ixodes ricinus</i>	<i>Rhipicephalus sanguineus</i>	no tick
Number (%)	25 (39%)	13 (21%)	4 (6%)	21 (33%)
Age (mean $\pm$ SD)	46.2 $\pm$ 22.2	43.3 $\pm$ 27.8	58.0 $\pm$ 18.1	45.3 $\pm$ 16.1
Sex (% men)	11 (44%)	7 (54%)	3 (75%)	15 (71%)
Bleeding time (mean $\pm$ SD)	32.4 $\pm$ 11.4	23.7 $\pm$ 14.1	36.5 $\pm$ 3.0	33.4 $\pm$ 17.9
Abs to <i>R. conorii</i>	6	4	0	5

Figure 1. Seasonal variation in tick bites and infection by *R. conorii* in the province of Soria.