Title

Detection and Identification of *Rickettsia helvetica* and *Rickettsia* sp. IRS3/IRS4 in *Ixodes ricinus* Ticks found on humans in Spain.

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New rickettsia species are continuously being isolated from ticks around the world, but in most cases their pathogenicity remains to be determined. Some rickettsiae first thought to be nonpathogenic have later been associated with human disease, such as *R. slovaca* [1], *R. helvetica* [2], *R. aeschlimannii* [3] and, more recently, the Spanish strain Bar29 (*R. massiliae* genogroup), which seems to play a role in the pathogenesis of Mediterranean Spotted Fever [4].

There are many other rickettsiae that, at least to date, have only been found in ticks; namely, the genotypes IRS3/IRS4, firstly isolated in *I. ricinus* ticks from Slovakia [5]; the genotypes RpA4 and DnS14, DnS28, DnS79, DnS94 (belonging to the *R. massiliae* genogroup), which were first isolated, respectively, in *Rhipicephalus pumilio* and *Dermacentor nutalli* ticks from the former Soviet Union [6], and -more recently- the SFG rickettsiae detected in *D. marginatus* ticks collected on vegetation in Jaén and La Rioja (Spain), which are closely related to the genotypes DnS14/DnS28 [7]. The pathogenicity of all these rickettsiae remains uncertain, but since other tick-isolated rickettsiae of previously unknown pathogenicity have been shown to cause human disease, the pathogenic potential of these new rickettsiae certainly deserves specific attention.

From 1997 to 2002, throughout the region of Castilla y León (Northwestern Spain) we collected and identified 3,059 ticks that were attached to people living in this territory (unpub. data). These ticks belonged to 15 species, although 44.15% of them (1320 specimens) were in fact *I. ricinus*, meaning that this species is the most anthropophilic and a serious hazard to human

health in this region of Spain. To determine whether the people bitten by *I. ricinus* were at risk of contracting tick-borne disease, we analyzed all the ticks by PCR to detect those infected with *Rickettsia* spp., *Borrelia burgdorferi* and *Anaplasma phagocytophila*. Here we report the results of our quest for SFG rickettsiae in *I. ricinus* ticks, because these have allowed us to identify, for the first time in Spain, the pathogenic species *R. helvetica* as well as the genotypes IRS3 and IRS4.

Along this 6-year study, each tick found on patients who sought medical advice in the hospitals and healthcare centers of Castilla y León was removed and referred to our laboratory. Each tick was first disinfected in 70% alcohol, rinsed in sterile water and dried on sterile filter paper, after which its DNA was extracted in 5% Chelex-100. In our search for rickettsiae, we proceeded as described previously [3]: briefly, DNA samples were tested for a fragment of the rickettsial *glt*A gene and then, in *glt*A-positive samples, a fragment of the rickettsial *omp*A gene was amplified, sequenced, and compared for identification. When *omp*A was not successfully amplified, the *glt*A amplicon was sequenced and compared. DNA contamination and carry-over of amplified products were prevented by using sterile tools at all times and carrying out each step of the analysis in separate work areas. Two negative controls (Milli-Q water and DNA from laboratory-reared uninfected ticks) were included in each amplification trial. These controls never amplified.

We amplified and sequenced 49 rickettsial amplicons (42 *glt*A, 7 *omp*A) from 48 *I. ricinus* ticks; in one tick, both amplicons were obtained. Eight *glt*A amplicons had 100% sequence identity with the *glt*A of *R. helvetica*

(GenBank, U59423). The remaining 34 *glt*A amplicons had the following identities: 24 were identical to the *glt*A of IRS3 (GenBank, AF140706); 4 were identical to the *glt*A of IRS4 (GenBank, AF141906); 1 was identical and 3 were >99% identical to the *glt*A of *R. massiliae*/Bar29 (GenBank, U59719/U59720); 1 was >99% identical to the *glt*A of *R. aeschlimannii* (GenBank, U59722, see ref. 3), and one was >99% identical to the *glt*A of *Rickettsia* RpA4/DnS14 (GenBank, AF120029/AF120028). Of the 7 *omp*A amplicons sequenced, 2 shared >99% identity to the *omp*A of *R. aeschlimannii* (GenBank, U43800, see ref. 3); 1 was identical to the *omp*A of IRS3 (GenBank, AF141909); 1 was identical and 3 were >99% identical to the *omp*A of IRS4 (GenBank, AF141911). The two amplicons (*glt*A and *omp*A) sequenced from the same tick were both identified as genotype IRS4.

Thus, we found 48 rickettsiae-positive *I. ricinus* specimens among the 1320 analyzed (infection rate: 3.6%). Of the infected *I. ricinus*, fifteen (31,2%) carried pathogenic rickettsiae: 8 *R. helvetica* (16.7%), 4 *R. massiliae*/Bar29 (8.3%) and 3 *R. aeschlimannii* (6.3%). The remaining 33 specimens (66.8%) carried rickettsiae of uncertain pathogenicity: 32 (66.7%) were genotypes IRS3 and IRS4 and only one (2,1%) was genotype RpA4 or DnS14. We did not find ticks infected with more than one rickettsia, but we did find *Borrelia burgdorferi* in one *I. ricinus* tick infected with *R. helvetica*, in 5 ticks infected with IRS3 and in one tick infected with *R. massiliae*/Bar29. We also found *A. phagocytophila* in another IRS4-infected tick.

The 48 rickettsiae-positive ticks were removed in the first 12 post-attachment hours, before they could have ingested any blood, thus

indicating that they were previously infected with the bacteria. The people bitten by these specimens were asymptomatic at the moment of tick removal and they did not develop any symptoms after their tick bites.

R. helvetica is widely distributed in Europe, and possibly also in Japan [8], but it has never been detected in Spain; hence, our finding constitutes their first citation in this country. The genotypes IRS3 and IRS4 have been found in *I. ricinus* from Slovakia [5], Italy [9], and some countries of southeastern Europe [10], and hence this is the first time that these genotypes have been reported in Spain. These observations expand the geographic distribution of these three bacteria and seem to support their specificity for *I. ricinus*, since they were not found in any other tick species. By contrast, our observations apparently enlarge the range of potential tick vectors of *R. massiliae*/Bar29 (to date only associated with the genus *Rhipicephalus*) and that of the genotypes RpA4 and DnS14 (to date associated with the genera *Rhipicephalus* and *Dermacentor*).

In conclusion, our results indicate that people living in Castilla y Leon (Spain) are frequently bitten by *I. ricinus* and that the people bitten have a 1.12% risk of becoming infected with pathogenic rickettsiae (i.e. *R. helvetica*, *R. aeschlimannii* and Bar29) as well as a 2.48% risk of becoming infected with rickettsiae of unknown pathogenicity. Furthermore, since several of the *I. ricinus* ticks were co-infected with more than one tick-borne pathogen, their simultaneous transmission to people during a single *I. ricinus* bite cannot be discarded.

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