Title: **Seroprevalence of *Rickettsia slovaca* infection in an area of northern Spain**

Running title: *Rickettsia slovaca* infection

Authors: Lourdes Lledó[1], María Isabel Gegúndez[1], Jesús Medina[1], Nelia Fernandes[2], Jesús Vicente González[3], Rufino Álamo[3], Pedro Fernández-Soto[4], Ricardo Pérez-Sánchez[4], Fátima Bacellar[2]

1-Department of Microbiology and Parasitology, Faculty of Medicine, Alcalá University, Spain.

2-Centro de Estudos de Vectores e Doenças Infecciosas do Instituto Nacional de Saúde, Aguas de Moura, Portugal.

3-Servicio Territorial de Sanidad y Bienestar Social de la Junta de Castilla y León, Spain.

4-Department of Parasitology, Faculty of Pharmacy, Salamanca University, Spain.

Corresponding author and address for reprints:

- Lourdes Lledó, MD, PhD, Lecturer in Microbiology,
- Departamento de Microbiología y Parasitología, Universidad de Alcalá,
- Ctra. Madrid-Barcelona, Km 33.6, 28871- Alcalá de Henares, Madrid, Spain.
- Phone: 34 91 885 47 94
- Fax: 34 91 885 46 63
- E-mail: lourdes.lledo@uah.es

SUMMARY

An epidemiological survey was undertaken in two provinces of northern Spain to assess the number of people that have been exposed to *Rickettsia slovaca*, a member of the disease-causing spotted fever group (SFG). Seroprevalence was determined in 200 subjects of the general population by indirect immunofluorescence; six (3.33%) were found to have *R. slovaca* antibodies. In addition, serum samples were taken from 183 further subjects who had bitten by ticks and *R. slovaca* antibodies screened for in the same way. Thirty one of these subjects (16.93%) were seropositive. Immunoblotting was used to confirm the presence of antibodies in subjects with acute infections. The difference in seroprevalence between the general and the tick-bitten population was significant. Sex had no influence on seroprevalence in either population, although it was significantly influenced by age and occupation in the tick-bitten group. The five patients detected with acute infection all showed IgM antibodies to *R. slovaca* and had seroconverted to IgG. Three *Dermacentor marginatus* ticks were obtained from these patients and were found to harbour *R. slovaca*.

KEY WORDS: ***Rickettsia slovaca***, zoonoses, emerging disease, epidemiology, ticks, Spain.

INTRODUCTION

The spotted fever group (SFG) of rickettsial species contains more than 20 genetically and antigenically related members (1). *Rickettsia slovaca*, a member of this group (2), was first isolated in 1968 from a *Dermacentor marginatus* tick in Slovakia (3). The first human *R. slovaca* infection was reported in 1980 (4). *Dermacentor marginatus,* the bacterium's main vector, is found throughout Europe and Central Asia

as far as the western border of China (2). Others species of *Dermacentor* ticks, such as *D. reticulatus*, may also act as vectors (5).

Infection with *R. slovaca* is manifested as a local reaction (transient skin inflammation) following a tick bite, usually on the scalp, and the lymph nodes may be large and painful in the region of the bite, a condition known as tick–borne lymphadenopathy (TIBOLA). Low-grade fever, rash, fatigue, dizziness, headache, sweating, myalgia, arthralgia and loss of appetite may also be apparent (6). Sequelae such as persistent asthenia and localized alopecia have been reported in some cases (7).

The aim of this study was to determine the seroprevalence of *R. slovaca* infection in the northern Spanish provinces of Palencia and Burgos, an area with medium sized urban populations and numerous, isolated villages whose main activities are agriculture and cattle raising.

PATIENTS AND METHODS

Serum specimens: Sero-epidemiological studies were performed in 1996-2003 using serum samples from subjects who attended healthcare centres for reasons unrelated to infectious diseases (n=200, 100 from each province; overall 92 males, 108 females; age range 0-95 years), and from patients who had been bitten by ticks and therefore at risk of rickettsial infection (346 samples from 183 patients; 92 males, 91 females; age range 3-83 years).

The age and sex of all subjects were recorded. The occupation, area of residence (rural <10,000 inhabitants or urban >10,000 inhabitants) and the manifestation of symptoms were also recorded for those who had been bitten by ticks.

All serum samples were maintained at -20°C until analyses. All were initially tested for *Borrelia burgdorferi* to rule out cross reactivity.

The survey was performed with the knowledge and consent of all subjects, and in compliance with the ethical standards of the Human Experimentation Committee of the University of Alcalá de Henares and the Helsinki Declaration of 1975 (as revised in 1983).

Immunofluorescence: Sera were tested for *R. slovaca* antibodies by the indirect immunofluorescence assay (IFA) described by Phillip et al., 1976 (8). *Rickettsia slovaca* strain 246 CDC provided the necessary antigens. These bacteria were propagated in Vero E6 cells (ATCC CRL 1586) and fixed on spot slides. The fluorescein-labelled conjugates used were rabbit anti-human IgG and IgM sera (Sigma, St Louis, MO) diluted 1/128 in PBS containing Evan's blue. Briefly, two-fold dilutions of each serum sample were added to the antigen spots and incubated in a humidity chamber for 30 min at 37ºC. After washing, the conjugate was added to each. The slides were then incubated for 30 min, washed, and examined using an Olympus fluorescence microscope BH2 (10x40). Positive and negative control sera were also examined. Sera showing a typical pattern of fluorescence at titres of ≥1:80 to IgG and of ≥1:20 to IgM were deemed positive (9).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western-blotting

Sera with titres of IgM ≥1:20 and/or showing seroconversion to IgG in IFA were tested by Western blotting against *R. slovaca* strain 246 CDC. Purified antigens were separated by SDS-PAGE as described by Laemmli et al. (10). Briefly, these were applied to 3-8% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred electrophoretically to nitrocellulose membranes. As described previously (11), reactive antibodies in sera diluted 1:100 in PBS were assayed with alkaline phosphatase conjugated goat anti-human IgG and anti-human IgM (Sigma) and substrate (nitroblue

tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate of Sigma). The presence of (at least) 118, 125 and 130 kD bands (specific protein antigens-SPAs) was deemed to indicate a positive result (12).

Analysis of ticks for rickettsial DNA: Three engorged ticks removed from the scalp of three patients that developed acute infections were placed in a vial containing gauze moistened with physiological saline and were sent to the Department of Parasitology of the University of Salamanca for species identification and PCR analysis to look for rickettsial DNA. After identification, each tick was first disinfected by immersion in 70% alcohol, rinsed in sterile water, and dried on sterile filter paper. Then, DNA was extracted in 5% Chelex-100 (13 In searching for *Rickettsia* spp., we routinely proceeded as described (14): All DNA samples were first tested for a fragment of the rickettsial *glt*A gene (15), and then, in the *glt*A-positive samples, a fragment of the rickettsial *omp*A gene (16) was amplified, sequenced, and compared with gene databases (GenBank) for identification. To prevent DNA contamination and the carryover of amplified products, sterile tools were used at all times and each step of the analysis (extracting DNA, preparing the reaction mixture, and amplifying and analyzing the PCR product) was carried out in separated work areas. Two negative controls (Milli-Q water and DNA from laboratory-reared noninfected ticks) were included in the amplification trial. These controls were not amplified.

Statistical analysis: Data were compared using the χ^2 or Fisher exact test. Significance was set at $P<0.05$.

RESULTS

IgG antibodies to *R. slovaca* were found in six of the serum samples collected from the general population (seroprevalence 3.33%; 2/108 females [seroprevalence 1.8%] and 4/92 males [seroprevalence 4.3%]). The mean age of those positive was 50 years (standard deviation 22 years; range 19-87 years). Seroprevalence in the general Burgos population was 5% (5/100) and 1% (1/100) in that of Palencia (not significantly different). No significant differences were found in seroprevalence with respect to sex or age. Positive serum sample titres ranged from 1:80 - 1:640 (Table 1).

IFA detected 31 bitten patients (16.93%; 20 males [seroprevalence 21.73%] and 11 females [seroprevalence 12%]) with significant IgG titres against *R. slovaca.* Seropositive subjects were aged between 9 and 83 years (mean 46.7 years, standard deviation 18.6 years). Seroprevalence in Burgos was 9% (2/22) and in Palencia 18% (29/161). The highest seroprevalence, 32.4%, was found in farmers and/or stock-breeders; among the remaining occupations seroprevalence was only 13% ($P < 0.01$; $\chi^2 = 7.9$). In addition, people over 60 years of age were more likely to be seropositive (29.7%) compared to all other ages (13.6%) ($p < 0.05$; $\chi^2 = 5.39$). The inhabitants of rural areas showed a seroprevalence of 19.25% (26/135); the seroprevalence among urban dwellers was 10.4% (5/48). No significant differences were found with respect to sex, Province or area of residence. Positive serum titres ranged from 1:80 to 1:1280 (Table 1). No positive serum samples were reactive to *B. burgdorferi.*

Statistical differences were found in seroprevalence between the two studied groups ($p<0.001$; $\chi^2 = 21.27$). Five patients (2.7%; 2 females, 3 males) of the tick-bitten group were positive for IgM and showed seroconversion to IgG in IFA. Western blot analysis confirmed these patients to be seropositive for *R. slovaca*. All had been bitten in winter.

The mean age of these subjects was 51 years (range, 28-74 years). All five were from Palencia and lived in rural areas; three were shepherdesses. Three patients developed a scalp lesion and had enlarged cervical and occipital and painful lymph node. Only one patient had a fever. None showed a rash. The other two, in whom the tick had attached to the abdomen or leg, showed a skin lesion at the bite site, but no rash. One had fever and lymphadenopathy. All five patients were treated with doxycycline.

The three ticks removed from the patients that developed acute infections were adult female *Dermacentor marginatus*. Successful amplifications of the *glt*A and *omp*A rickettsial gene fragments were obtained from all ticks. Sequencing and comparison of the three *omp*A amplicons enabled us to identify *R. slovaca* (GenBank accession no. U433808) in every tick.

DISCUSSION

The list of newly discovered rickettsiae has grown rapidly in recent years (7) and several new tick-borne diseases have been identified, including TIBOLA, the causal agent of which is *R. slovaca* (17).

Dermacentor ticks harbouring *R. slovaca* have been detected in France (12), Germany (18), Austria (19), Slovakia (20), Switzerland (21), Armenia (22), Portugal (23), Croatia (24) and Italy (25). Since the first report of a human infection in 1980, clinical *R. slovaca* infections have been confirmed in France and Hungary (7).

In Spain, only a few *R. slovaca* infections have been reported (26) even though *D. marginatus* and *D. reticulatus* are widely distributed. Recently, *R. slovaca* has been detected in *D. marginatus* in both northern and southern Spain (27). The results of a study performed in central Spain showed *D. marginatus* to be the tick that most commonly bit humans (28).

The present study is the first stage of a project whose aim it is to determine the seroprevalence of *R. slovaca* in Spain. To our knowledge no reports exist on the prevalence of antibodies to *R. slovaca* in other European countries, although there are many on *R. conorii*. The seropositivity rate detected in the present study is lower than that reported in seroepidemiological studies of *R. conorii* performed in the Spanish provinces of Salamanca 73.5% (29) and Seville 26.3% (30), and in Croatia 43.7% (31), Greece 46.1% (32), and southern France 18% (33). They are also similar to those found in the above studies with respect to age, sex and contact with ticks. Consistent with these findings, *R. slovaca* infection to Man appears to be more limited in Spain and other Mediterranean countries than *R. conorii* infection. Their epidemiological features (with respect to distribution among the population) may be very similar, but they have different tick vectors and reservoirs.

Dermacentor marginatus appears to be a rural tick species (34), the larvae and nymphs parasitizing small mammals (rodents) and the adults feeding mainly on sheep, goats and cattle (35). The highest percentages of seropositive patients were observed in rural areas and among farmers and/or stock-breeders. These findings can be attributed to more frequent exposure to cattle, etc.

The serological data and the clinical and epidemiological features of the cases of acute infection studied indicate the probable aetiological agent to be *R. slovaca*. Three patients showed the characteristic symptoms of a scalp lesion and cervical and occipital lymph node enlargement. It is important to note that these three patients had ticks still attached when they sought medical help. These were identified as *D. marginatus* and all of them were carriers of *R. slovaca*. In addition, these acute cases occurred during the colder months of the year, as seen in others studies (7, 26). However, no greater risk of

infection by *R. slovaca* was seen in children and women as reported by some authors (7).

In summary, this study shows that *R. slovaca* infection seems to be widely distributed in the study area. However, in the Mediterranean regions where *R. conorii* is endemic and the number of cases is rising (29, 36), the usual method used in epidemiological serosurveys and diagnostic procedures is IFA, and cross-reactivity problems with other SFG rickettsia are common when the antibody titre is low (12). TIBOLA might therefore be commonly misdiagnosed as Mediterranean spotted fever, and its seroprevalence underestimated (33). The fact that this new rickettsial strain may occur in the same regions where *R. conorii* is endemic is of particular epidemiological importance. There is a need to increase the awareness of rickettsioses among doctors to avoid misdiagnosis and delays in specific antibiotic therapy. Molecular studies are planned to further confirm the prevalence of *R. slovaca* in Spain.

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TABLE 1- Titres for antibodies (IgG) to *Rickettsia slovaca*

Groups	IFA[a] titre					Sample
	1:80	**1:160**	**1:320**	**1:640**	**1:1280**	
General population	2 (1%)	1 (0.5%)	2 (1%)	1 (0.5%)	-	200
Tick-bitten patients	16 (8.7%)	3 (1.6%)	3 (1.6%)	2 (1%)	7 (3.8%)	183

[a] Indirect immunofluorescence assay