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ORIGINAL ARTICLE

Molecular and Serological Study of Rickettsial Infection in Humans, and in Wild and Farm Animals, in the Province of Burgos, Spain

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Abstract

Limited information is available on the presence of rickettsial infection in humans and animal reservoirs in Spain. Exposure to spotted fever group rickettsia in healthy humans and in farm and wild animals in the Province of Burgos, Spain, was examined by serological methods. Rickettsial DNA was also sought by PCR in animal samples. Of 102 human serum samples examined by indirect immunofluorescence assays (IFA), 5.88% were positive for antibodies against *Rickettsia conorii* (titers 1/128–1/512). Significant differences were detected in human seroprevalence with respect to age. In further IFAs, 102 out of 375 (27.2%) serum samples from the wild animals reacted with *R. conorii* antigens (titers 1/64–1/1024); 32 out of 281 (11.38%) samples from farm animals were also positive for *R. conorii* (titers 1/64–1/2048). The prevalence detected among total wild animals was significantly higher than among total farm animals. No rickettsial DNA was found by PCR in any farm or wild animal sample.

Key Words: Epidemiology—Rickettsia—Vector-borne—Reservoir host.

Introduction

In recent years, Europe has experienced an increase in the incidence of certain zoonoses, particularly those transmitted by arthropod vectors, such as rickettsiosis (Blanco and Oteo 2006). The number of methods available for detecting and identifying rickettsia has increased and allowed the detection of new rickettsial pathogens as well as already-known types in new locations. Increasing interest in rickettsial diseases now demands that the epidemiology of their causal agents be reassessed (Richards 2012, Santibáñez et al. 2013).

Recent epidemiological studies undertaken in Spain have detected emerging species of spotted fever group *Rickettsiae* (SFGR) such as *R. slovaca*, *R. massiliae*, *R. raoultii*, *R. felis*, and *R. monacensis* in arthropods (Márquez et al. 2006, Márquez 2008, Lledó et al. 2010). Seroprevalence studies in humans and animals have detected antibodies to *R. slovaca* and *R. felis* as well as reemerging *R. typhi* and *R. conorii*; indeed, some clinical cases by emerging species (like *R. sibirica mongolitimonae)* have also been reported by serological or molecular methods (Bernabeu-Wittel et al. 2006, Lledó et al. 2006, Amusategui et al. 2008, Antón et al. 2008, Nogueras et al. 2013, Ramos et al 2013).

Mediterranean spotted fever (MSF), caused by *R. conorii*, is an endemic rickettsiosis of southern and eastern Europe and parts of Africa and Asia (Brouqui et al. 2007). It is mainly transmitted by the tick *Rhipicephalus sanguineus*. Most clinical cases are therefore noted in rural areas. Our ecological and epidemiological comprehension of MSF has experienced important advances in the last 10 years as the disease has emerged and reemerged in different countries (Brouqui et al. 2007, Rovery and Raoult 2008). However, many questions remain unanswered, including the identities of all the vectors and reservoirs of *R. conorii*, and whether other risk factors exist for the onset of the severe form of MSF (Rovery and Raoult 2008, Schex et al. 2011). Although MSF is regarded as a historically endemic disease in Spain, with the highest incidence usually seen in the summer months and mainly in rural areas, new epidemiological studies are needed to update our knowledge of its distribution and epidemiology.

The aim of the present study was to determine the prevalence of antibodies to SFGR in humans and animals in a region of the Province of Burgos in northern Spain, where human activities commonly involve continued contact with animals. No previous studies have been performed on the distribution and prevalence of *R. conorii* infection in this area.

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Materials and Methods

Study area

This study was performed in the Merindades area of the Province of Burgos in northern Spain (central point 42°55′52″N, 3°29′2″W). Here, mean daily summer temperatures range between 16°C and 20°C, whereas those for winter range between 2°C and 5°C. Rainfall ranges from 900 to 1100 mm/year and is usually high in winter. The area is mainly rural, but recreational activities attracting nonresidents have increased in recent years.

Human serum samples

A total of 102 samples of human serum (male/female ratio 83.3/16.7%; age 55 ± 11.4 year, range 24–88 years) were collected from visitors to health centers run by the Spanish National Institute of Health over 2006–2007. (Each subject had either attended a health care center for a reason unrelated to infectious disease and were volunteers.) All serum samples were maintained at −20°C until analysis. The following information was recorded for each donor: Age, sex, occupation, area of residence, travel history, and history of contact with animals and arthropod bites. All subjects gave their informed consent to be included in the study in compliance with the ethical standards of the Human Experimentation Committee of the University of Alcalá de Henares and the Helsinki Declaration of 1964 (as revised in 2004).

Animal serum samples

Blood, serum, and tissue (lung, kidney, liver, and brain [the latter only for rodents]) samples from 375 wild animals belonging to 19 species (Table 1) were collected between June, ◀T1

Table 1. Wild Animals Studied: Prevalence Results for Serum, Blood, and tTssue Samples

Species examined	*Serum samples studied/ prevalence*	*Blood samples studied/ positives*	*Tissue samples studied/ positives*
Arvicola terrestris (Water vole)	2/0%	—	—
Apodemus flavicollis (Yellow-necked mouse)	9/33.3%	—	9/0
Apodemus sylvaticus (Wood mouse)	24/33.3%	—	24/0
Canis lupus (Wolf)	3/0%	2/0	—
Genetta genetta (Genet)	2/50%	1/0	—
Lepus europaeus (European hare)	16/18.7%	12/0	1/0
Martes foina (Beech marten)	2/0%	1/0	2/0
Martes martes (Pine marten)	2/0%	—	1/0
Meles meles (Badger)	7/28.57%	2/0	2/0
Mus musculus (House mouse)	22/4.5%	22/0	—
Mustela erminea (Stoat)	1/0%	—	—
Mustela nivalis (Least weasel)	2/0%	—	1/0
Mustela putorius (Polecat)	2/0%	—	1/0
Myodes glareolus (Bank vole)	5/20%	—	6/0
Neovison vison (American mink)	121/54.5%	—	25/0
Sciurus vulgaris (Red squirrel)	4/25%	2/0	3/0
Sus scrofa (European wild boar)	103/14.5%	38/0	1/0
Talpa occidentalis (Iberian mole)	3/33.3%	1/0	3/0
Vulpes vulpes (Fox)	45/0%	3/0	3/0
Total	**375/27.2%**	**84/0**	**82/0**

Table 2. Farm Animals Studied: Prevalence Results for Serum and Blood Samples

Mammal species	*Serum samples studied/prevalence*	*Blood samples studied/positive*
Cattle	105/21.9%	31/0
Horses	23/8.7%	9/0
Pigs	50/14%	—
Sheep	103/0%	31/0
Total	**281/11.38%**	**71/0**

2006, and September, 2007. Wild animals were captured alive or found dead (victims of road accidents). Blood and serum samples from farm animals (cattle, horses, pigs, and sheep, all in extensive or semiextensive farming systems) were collected over 2006 and 2007 (Table 2). All samples were stored at −20°C until analyses. Permission to take and study these animal samples was obtained from the regional government of Castilla y León in compliance with current legislation and in keeping with the ethical guidelines of the Committee on Animal Experimentation of the University of Alcalá de Henares.

Serology

Sera were tested by the indirect immunofluorescence assay (IFA), as described by Phillip et al. (1976). The rickettsia providing the antigen was *R. conorii* (strain Malish 7). Rickettsia were propagated in Vero E6 cells (ATCC CRL 1586) and fixed on spot slides. The fluorescein-labeled conjugate used was adapted according to the species studied. All antibodies (immunoglobulin G [IgG]) were from Sigma (St. Louis, MO) (Table 3). Positive and negative control sera were included (provided by Dr. Fatima Alves, Centro de Estudos de Vectores e Doenças Infecciosas, Portugal-CEVDI). Titers of ≥1/128 were considered positive for humans, and ≥1/64 were considered positive for animals.

DNA extraction and PCR

Total DNA was extracted from wild animal blood and tissues and from farm animal blood using the COBAS AmpliPrep® system (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's recommendations. DNA of *Rickettsia* genera was detected by PCR-amplification of: (1) The citrate synthase gene *gltA*, using primers RpCs 1258 and RpCs 877, which amplify a 381-bp fragment; (2) the gene coding for protein 190-kDa *OmpA* using the primers Rr 190.70p and Rr 190.602n, which amplify a 532-bp fragment; and (3) the gene *ompB*, using the primers 120–M59′ and 120-807, which amplify a 833-bp fragment. The first two amplifications were performed following the protocol of Regnery et al. (1991); the third was performed following that of Roux and Raoult (2000). To prevent DNA contamination and the carryover of amplified products, sterile tools were used at all times in each step of the analysis (DNA extraction, preparation of the reaction mixture, and amplification and analysis of the PCR products). Each step was performed in separate work areas. A negative control (Milli-Q water) was included in all amplifications.

Table 3. The Fluorescein-Labeled Conjugate Used According to Human and Animal Species Studied

Human and animals	*Fluorescein-labeled conjugate*
Human	Rabbit anti-human IgG
Canidae	Rabbit anti-dog IgG
Felidae and *Mustelidae*	Goat anti-cat IgG
Rat, squirrel, and mole	Rabbit anti-rat IgG
Mouses	Rabbit anti-mouse IgG
Leporidae	Anti-rabbit IgG
Suidae	Anti-pig IgG
Horses	Goat anti-horse IgG
Cattle	Goat anti-bovine IgG
Sheep	Rabbit anti-sheep IgG

IgG, immunoglobulin G.

Data analyses

The chi-squared or Fisher exact test was used to compare differences in proportions employing two-way tables with a StatView® software in Apple™ format. Significance was set at $p \leq 0.05$.

Results

IgG antibodies to *R. conorii* were found in six human serum samples (total 5.8%; females 5.8%, males 5.8%). The age of the seropositive subjects ranged from 68 to 82 years (76 ± 4.8 years). No significant differences in seroprevalence were seen with respect to sex, but significantly more positive results were recorded among the those over 60 years ($p \leq 0.001$; $\chi^2 = 7.35$). The IgG antibody titers for seropositive subjects ranged between 1/128 and 1/512 (Table 4). All seropositive human subjects had contact with animals; three had occupational risks. One of the subjects referred to a history of arthropod bites.

A total of 102 sera with antibodies specific to *R. conorii* were detected among the wild animals (prevalence 27.2%), while 32 were detected among the farm animals (prevalence 11.47%; $p \leq 0.001$ compared to the wild animals; $\chi^2 = 24.7$). Titers for these positive animals ranged from 1/64 to 1/2048 (Table 4). Among the wild animals, mink showed the highest prevalence ($p \leq 0.001$ compared to the remaining species; $\chi^2 = 67.46$) (Table 1). Among the farm animals, cattle showed the highest prevalence ($P \leq 0.001$ compared to the remaining farm species; $\chi^2 = 18.37$) (Table 2).

No rickettsial DNA was detected by PCR in any animal sample.

Discussion

Defining the vector species of a disease is of the utmost importance for its control, yet in some cases it remains unclear which rickettsial species are carried by which arthropod vectors, nor is it certain which mammalian species serve as their reservoirs (Schex et al. 2011). In many cases, the natural epidemiological cycle is well known, but in recent years there have been changes in these classic epidemiological cycles (Lledó et al. 2003). After many years of falling prevalence, there would now seem to be a reemergence of rickettsial disease in Spain, perhaps associated with changes in recreational habits that bring people into closer contact with the reservoir hosts and arthropod vectors.

The present serological results confirm the presence of antibodies to *R. conorii* in humans and most of the animal

Table 4. Serum Titers for Anti-*R. conorii* Antibodies (IgG)

Groups	*Titer 1/64*	*Titer 1/128*	*Titer 1/256*	*Titer 1/512*	*Titer 1/1024*	*Titer 1/2048*
Human samples		2 (1.9%)	3 (2.9%)	1 (0.9%)	—	—
Wild animal samples	62 (16.5%)	24 (6.4%)	8 (2.1%)	4 (1%)	1 (0.2%)	—
Farm animal samples	3 (1%)	4 (1.4%)	3 (1%)	7 (2.5%)	9 (3.2%)	6 (2.3%)

IgG, immunoglobulin G.

species examined in the study area. However, because of serological cross-reactivity between rickettsiae of the spotted fevers group (Santibañez et al. 2006), we consider the results as preliminary, while another assay can be used to validate IFA results by future studies using more antigens and serological methods that are more specific, such as western blot. Therefore, it is possible that some of the seropositive cases observed in the present study were (or had been) infected with SFG rickettsiae other than *R. conorii.*

Seroprevalence in humans (5.8%) was much lower than that recorded in the 1980s for the Spanish provinces of Seville (26.3%) (García and Najera 1984) and Salamanca (73.5%) (Herrero et al. 1989), but is reminiscent of more recent figures reported for the provinces of Soria (5%) (Saz et al. 1993), León (1%) (Rojo-Vázquez 1997), and the Spanish northwest (8%) (Segura-Porta et al. 1998) and south (8.7%) (Bernabeu-Wittel et al. 2006). Compared to Portugal (7.6%) (Bacellar et al. 1991), Croatia (43.7%) (Punda-Polic et al. 2003), Greece (7.9%) (Daniel et al. 2002), southern France (18%) (Raoult et al. 1987), Slovakia (32%) (Sekeyova et al. 2012), and Israel (10%) (Harrus et al. 2007), the present figure is low.

The present seroprevalence results were also similar to those reported in the above studies with respect to age, sex, and contact with animals and ticks. In the present work, seroprevalence was significantly higher in those over 60 years old, as reported for the Spanish province of Albacete (Bartolomé et al. 2005). Several clinical studies (Bartolomé et al. 2005, Aliaga et al. 2009, Baltadzhiev et al. 2012) have reported local reemergence of the disease in Spain and Bulgaria; adults over 40 years of age have been those most affected, with a peak seen in patients over 60 years, many of whom have suffered severe illness. The present study, and those mentioned above, all seem to suggest that MSF continues to be most common among people living or working in rural areas, as suggested by Segura-Porta et al. (1998) and Podsiadly et al. (2010), although other authors have found higher prevalence figures in urban and suburban areas than in semirural areas (Raoult et al. 1987, Bolaños-Riveros et al. 2011).

Of the 19 species of wild animals studied, 11 had members seropositive for *R. conorii.* In general, the prevalences detected in the wild species were significantly higher than those recorded for farm species, as reported by other authors (Jilintai et al. 2008). The prevalence of seropositive wild animals ranged from 4.5% for the house mouse *Mus musculus* to 54.5% for the American mink (*Neovison vison*). (This is the first study to report antibodies to *R. conorii* in *N. vison* in Spain.) American mink found their way into the wild in Europe after escaping from captivity. They are known to approach farms, and they may therefore play an important role in both the maintenance of *R. conorii* in nature and its transmission to farm animals.

All of the wild small mammal species examined had antibodies to *R. conorii,* with the wood mouse *Apodemus sylvaticus* returning the highest seroprevalence. Even in places as distant as Japan, the prevalence of antibodies against SFGR in mice (mainly wood mice) is high (Morita et al. 1990, Okabayashi et al. 1996). In California too, a seroprevalence for SFGR of 14% has been reported for rodents (Adjemian et al. 2008). However, in a recent molecular study conducted in the Basque Country (northern Spain), all of the small mammals examined were negative for SFGR (Barandika et al. 2007), although in Germany SFGR DNA was detected in seven rodents out of 124 studied (Schex et al. 2011).

Some authors report other wild animals may play a host reservoir role for SFGR, such as wild boar (*Sus scrofa*) (Ortuño et al. 2007, Selmi et al. 2009), rabbit (Ruiz-Beltran et al. 1992), deer (Inokuma et al. 2008, Jilintai et al. 2008), and jackal (Waner et al. 1999). In the present work, antibodies were detected in wild boar, European hare, and several carnivorous animals. However, unlike that reported by Boretti et al. (2009), no infections were detected in foxes.

The domestic animals most studied to date have been pet dogs and cats; indeed, the dog is the main domestic animal reservoir of *R. conorii* worldwide. In the present study, however, farm animals were studied (which in rural areas have much contact with humans), which may maintain tick populations and serve as pathogen reservoirs (Ruiz-Fons et al. 2006). As a group, the farm animals had higher titers than humans or wild animals. The seroprevalence detected in cattle (21.9%) was comparable to that reported for seroprevalence to SFGR from Croatia (27.3%) (Punda-Polic et al. 1995) but higher than that observed in Kenya (16.3%), Sri Lanka (15%) (Kovácová et al. 1996), and Japan (9.6%) (Jilintai et al. 2008). In a study performed in Salamanca (Spain), a seroprevalence figure of 78.1% was reported for cattle (Herrero et al. 1989). The seroprevalence recorded in the literature for *R. conorii* in horses is very variable, ranging from 0% in Sicily (Torina et al. 2007) to 100% in the Spanish province of Salamanca (Herrero et al. 1989) (although here only three horses were studied). For other SFGR species outside Europe, high prevalences have been reported in Brazil (Horta et al. 2004) and Panama (65%) (Bermúdez et al. 2011).

In the present work, a seroprevalence of 0% was recorded for sheep. This was somewhat unexpected because other authors have reported values of 38.9% in Salamanca (Herrero et al. 1989), 9.4% in Croatia (Punda-Polic et al. 1995), and 15% in Kenya (Mutai et al. 2013) for antibodies against *R. conorii* and other SFGR species. However, in Sicily, Torina et al. (2007) also found no seropositive sheep.

The literature contains little information on seroprevalence in pigs. In the studies that have been performed, the number

of specimens analyzed has been very low, and the results were highly variable (Herrero et al. 1989, Torina et al. 2007). Studies on larger numbers of animals are required to examine the role of this animal in SFGR infection. In the present study, a prevalence of 14% was recorded.

Finally, PCR detected no *Rickettsia* spp. DNA in any of the animal samples tested, even though in some animal species the prevalence of antibodies to *R. conorii* and antibody titers, were high. Other authors have reported similar situations, finding no bacterial DNA in tissue or blood samples even though the donor animals had high antibody titers (Boretti et al. 2009, Ortuño et al. 2012).

Conclusions

In summary, the present results expand our knowledge regarding the seroprevalence of SFGR in the Province of Burgos and shed light on the epidemiology of tick-borne rickettsial disease. Some infections of SFGR have a re-emergence of cases involving serious disease. This may due to a change in natural epidemiological cycles, with other wild and peridomestic reservoirs becoming available. The large number of seropositive animals (wild and farm) detected suggests that human contact with animals is a risk factor for the acquisition of *R. conorii* infection or other species of SFGR. Further research should attempt to determine the role of different species studied in the epidemiology and ecology of these infections and involve other areas of the country.

Author Disclosure Statement

No competing financial interests exist.

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