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Wild Red Foxes (*Vulpes vulpes*) as Sentinels of Rodent-borne Hantavirus and Lymphocytic Choriomeningitis Virus in the Province of Soria, Northern Spain

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ABSTRACT: Three hundred and fourteen red foxes (*Vulpes vulpes*) in the province of Soria, Spain, were examined for hantavirus and lymphocytic choriomeningitis virus (LCMV) infection (and were likely to have been infected by feeding on infected rodents). Immunofluorescence and western blot assays confirmed 3.5% (11/314) to have antibodies to hantaviruses, and the immune fluorescence assay showed 2.2% (7/314) to have antibodies to LCMV. The serologic status of the animals showed no statistically significant association with sex or age. Although studies on the prevalence of hantaviruses and LCMV normally focus on rodents, our results showed that foxes can provide complementary information in determined areas.

Key words: Animal sentinel, epidemiology, hantaviruses, lymphocytic choriomeningitis virus.

Hantaviruses and arenaviruses have a nearly worldwide distribution, and a study from northern Italy (Tagliapietra et al. 2018) suggests these potential pathogens may pose an emerging public health threat that warrants stronger epidemiologic surveillance. Though largely rodent-borne, hantaviruses are also carried by bats, moles, and shrews (Sabino-Santos et al. 2015). In a Belgian study (Escutenaire et al. 2000), antibodies to Puumala hantavirus (PUUV) were mostly detected in the bank vole (*Myodes glareolus*), but also in the wood mouse (*Apodemus sylvaticus*) and red fox (*Vulpes vulpes*). Unfortunately, these viruses can also infect humans, mainly through the inhalation of aerosols contaminated with rodent excreta.

Arenavirus antibodies are also commonly found in numerous rodent species (Kallio-Kokko et al. 2006). Those against lymphocytic choriomeningitis virus (LCMV) are often detected in mice and sometimes in hamsters. In humans, LCMV infection can cause meningitis, multi-organ failure in transplant recipients, and severe intrauterine developmental defects of the fetus.

Contact between wild and domestic animals indirectly increases the degree of contact between wild animals and humans, and therefore the likelihood of pathogen transmission. Foxes often live in peri-urban and agricultural areas and adapt well to the presence of domestic animals and humans (Fishman 2004). Although it is generally accepted that foxes do not serve as hantavirus and arenavirus reservoirs (Malecki et al. 1998), they may become infected by rodent viruses when feeding on rodents and in this way foxes become indicators of virus circulation within their feeding ranges. In conjunction with rodent-focused studies, studies on foxes might provide information useful for preventing human infection.

The literature contains no information on whether the foxes of central Spain are infected by rodent-borne hantaviruses and arenaviruses. The aim of our work was to determine the prevalence of antibodies to these viruses in foxes in the province of Soria (42°08′20″N to 41°04′15″N and 01°47′45″E to 03°31′45″E).

The foxes we studied (n=314) were donated by licensed hunters (n=290) or found dead (n=24) by forestry agents (usually road kills). Specimens were transported to the laboratory in sealed plastic bags and immediately examined. Specimen collection lasted 4 yr. The sex and age group (juvenile, adult, or old) of each animal was recorded. Permission to study these animals was obtained from the regional government of Castilla y León in compliance with current legislation (protocol no. 06.01.017.006), and in keeping with the

ethical guidelines of the Committee on Animal Experimentation of the University of Alcalá (protocol no. CEI 2011034).

A total of 153 foxes were male (48.7%) and 161 foxes were female (51.3%); 49 (15.6%) of the foxes were juvenile (i.e., with milk teeth; <1 yr old), 182 (57.96%) were adult (i.e., with permanent, unworn teeth; 1–5 yr old), and 83 (26.43%) were old (i.e., with markedly worn teeth; >5 yr old).

Serum samples were examined for antibodies using an in-house indirect immunofluorescence assay following the method of Lledó et al. (2002) for hantaviruses, and that of Lledó et al. (2003) for LCMV. The PUUV strain Cg18/20, Seoul virus strain 80/39, and LCMV strain Armstrong were used as antigens. The first two were propagated in VeroE6 (American Type Culture Collection CRL 1586), and the third in L-929 cells (American Type Culture Collection-CCL 1), and fixed onto spot slides. Rabbit anti-dog (*Canis lupus familiaris*) immunoglobulin G serum (Sigma, St. Louis, Missouri, USA), diluted 1/128 in phosphate-buffered saline containing Evan blue, was used as a fluorescein-labeled conjugate. Sera showing a typical pattern of fluorescence at titers of ≥16 were considered positive. Uninfected Vero E6 cells and L-929 cells were used as negative controls.

Serum samples from the above positive samples were then analyzed by western blotting to confirm the presence of anti-hantavirus immunoglobulin G antibodies. Blotting was performed as described by Hjelle et al. (1997). For this, the recombinant N proteins of PUUV and Seoul virus were expressed using the pET23b vector (Novagen, Madison, Wisconsin, USA) in *Escherichia coli* BL21 (Novagen), and purified in a metal chelation column using a C-terminal polyhistidine moiety. Positive and negative control sera (provided by the European Network for Diagnostics of Imported Viral Diseases) were also examined. Differences in antibody prevalence between the sexes and age classes were analyzed using the chi-square and Fisher exact tests as required. Significance was set at $P<0.05$.

Eleven foxes (3.5%, 11/314) had antibodies against hantaviruses. Antibody prevalence was 5.0% (8/161) in female foxes, and 2.0% (3/153) in males ($P=0.219$). Eight adult animals (4.4%, 8/182) were antibody-positive, one (2.0%, 1/49) was a juvenile, and two (2.4%, 2/83) were old ($P=0.596$). Positive serum sample titers ranged from 32 to 512 (Table 1). Seven foxes had antibodies to LCMV (prevalence 2.2%, 7/314); five (3.1%, 5/161) were females and two (1.3%, 2/153) were males ($P=0.449$); five (2.7%, 5/182) were adults, and two (2.4%, 2/83) were old ($P=1$). Positive serum sample titers ranged from 32 to 64 (Table 1). No significant difference was seen in the prevalence of antibodies to either type of virus with respect to age or sex.

Although much is known about rodent reservoirs, little information exists on how other wild animals may act as hantavirus and LCMV reservoirs. Bats (Order Chiroptera; Family Vespertilionidae), shrews and moles (Order Soricomorpha; Family Soricidae) are potential reservoirs for hantaviruses (Sabino-Santos et al. 2015), but coyotes (*Canis latrans*) in New Mexico, US and northeastern Arizona, US have been shown not to act in this way (Malecki et al. 1998). These canids quickly develop antibodies that clear the infection, preventing it being passed on. Like cats (*Felis catus*) and humans, these animals are probably dead-end hosts.

Few studies on hantavirus infection in mammalian predators of rodents have been performed in Europe, and most of those that have been undertaken have focused on the domestic cat (Nowotny et al. 1994). In Belgium, hantavirus antibodies were detected in the sera of pet dogs and cats (Dobly et al. 2012), and a significantly higher seroprevalence was detected in cats than in dogs in the southern part of the country (the south is more forested and harbors more rodents than the north). In Belgium, an antibody prevalence of 2.4% was reported in foxes (Escutenaire et al. 2000), slightly lower than the overall 3.5% (11/314) that we recorded.

Pet hamsters (*Mesocricetus auratus*) may also have a role in the transmission of LCMV (Reperant et al. 2016); an in-depth analysis of

TABLE 1. Serum from 314 red foxes (*Vulpes vulpes*) in the province of Soria, Spain, found to be positive for immunoglobulin G against lymphocytic choriomeningitis, Puumala, and Seoul viruses.

	Titer		
Positive animals	Lymphocytic choriomeningitis virus	Puumala virus	Seoul virus
1	32	Negative	Negative
2	32	Negative	Negative
3	64	Negative	Negative
4	32	Negative	Negative
5	64	Negative	Negative
6	32	Negative	Negative
7	32	Negative	Negative
8	Negative	64	128
9	Negative	64	128
10	Negative	128	64
11	Negative	32	128
12	Negative	32	128
13	Negative	256	256
14	Negative	128	128
15	Negative	128	32
16	Negative	128	512
17	Negative	ND[a]	128
18	Negative	256	64

[a] ND = not determined.

the risk posed by these companion animals is needed, especially because LCMV has been associated with aseptic meningitis in Spain (De Ory et al. 2009). In southern Spain, LCMV antibodies have been found in 2.9% of patients with meningitis. Additionally, LCMV has been proposed as a noteworthy causal agent of neurologic illness in immunocompetent persons (Pérez-Ruiz et al. 2012). Our results show that red foxes are unlikely to be reservoirs of LCMV (only 2.23% of foxes had antibodies to this virus), but can become infected with the virus.

Red foxes are now common in peri-urban areas (Plumer et al. 2014) where they take waste human food (Mueller et al. 2018). However, if they have preyed on rodents, they might provide another route via which humans can come into contact with hantaviruses and arenaviruses (Baker et al. 2000). Other animals may be sources of contact in other areas. Although antibodies to the studied viruses were detected in some of the foxes examined, more research will be needed to determine whether these animals have a role in the epidemiology of these potential pathogens.

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