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Ljungan Virus and an Adenovirus in Italian Squirrel Populations

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ABSTRACT: We report Ljungan virus infection in Eurasian red squirrels (*Sciurus vulgaris*) for the first time, and extend the known distribution of adenoviruses in both native red squirrels and alien gray squirrels (*Sciurus carolinensis*) to southern Europe.

The introduction of alien species is one of the major causes of infectious disease emergence in wildlife, representing a threat not only to biodiversity conservation, but also to domesticated animals and humans (Daszak et al. 2000). Alien species can introduce new pathogens, alter the epidemiology of local pathogens, become reservoir hosts, and increase disease risk for native species (Prenter et al. 2004; Dunn 2009).

As part of a broader project to study the role of infectious diseases and parasites in the competition between alien and native species, we investigated Ljungan virus (LV) and adenovirus infections in introduced North American eastern gray squirrels (*Sciurus carolinensis*) and native Eurasian red squirrels (*Sciurus vulgaris*) in northern Italy. More specifically, we investigated whether arboreal sciurids are involved in LV circulation, and if adenovirus infection in squirrels is present in Italy.

Ljungan virus was isolated in 1999 (Niklasson et al. 1999), and has subsequently been detected in many species of small rodents, especially voles (Arvicolinae; Johansson et al. 2003; Hauffe et al. 2010; Salisbury et al. 2013). In northern Italy, this virus has been reported in a small sample of bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*) with 50% and 10% prevalence, respectively (Hauffe et al. 2010). Although its zoonotic potential is still debated, LV has been associated with type 1 diabetes (T1D), myocarditis, and several gestational diseases in humans (McDonald 2009; Blixt et al. 2013). Experimentally infected laboratory mice develop signs of these same diseases, and LV-infected wild voles can also develop T1D-like syndromes (McDonald 2009; Blixt et al. 2013).

Outbreaks of enteric adenovirus infections associated with gastrointestinal disease and mortality have been described in both free-living and captive red squirrels in Germany and the UK (e.g., Martínez-Jiménez et al. 2011; Peters et al. 2011), where subclinical adenovirus infections among introduced gray squirrels have also been reported (Everest et al. 2009).

We analyzed 232 gray squirrels from five populations, culled as part of a control program (2011–12), and 77 road-killed red squirrels for adenoviruses. All the specimens were collected in Piedmont and Lombardy regions (between 44°35′55″ and 46°35′55″N; 7°37′41″ and 10°32′08″W). For adenovirus analysis, sample sets of both squirrel species were heterogeneous for sex, age class, and season of collection. For LV analysis, we used a subset of adult squirrels collected in autumn (49 gray squirrels and nine red squirrels), to maximize the chance of finding the virus, because infection is assumed to be correlated with small mammal (host) density and, therefore, probability of exposure (HCH and others, unpubl.).

For LV screening, total RNA was extracted from liver (stored at −80 C) using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). We performed

a One-Step reverse transcriptase-PCR (Qiagen) in duplicate, using primers described by Donoso Mantke et al. (2007). Amplicons were purified using the PureLink Quick gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, California, USA) and directly sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA) on an ABI 3130 sequencer. Sequences (189 base pairs) were checked using the basic local alignment search tool (NCBI 2013). For adenovirus screening, nucleic acid was extracted from spleen tissue (stored at −20 C), using the manufacturer's recommendations for the QIAamp DNA Mini kit (Qiagen). We performed nested PCR using primers described by Everest et al. (2012). To confirm the specificity of the primers, amplicons were recovered from agarose gels and purified using the Qiaquick Gel extraction kit (Qiagen), and then used as templates in direct dye-termination sequence reactions (Big Dye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems).

Two red squirrels (22%), but no gray squirrels, were infected with LV. An adenovirus was detected in 12 (16%) red squirrels and two (0.9%) gray squirrels. No cases of coinfection were detected.

To our knowledge this is the first record of LV in the Eurasian red squirrel, indicating that the infection is not limited to small, ground-dwelling rodents, and extending the potential host-spectrum of LV to arboreal mammals. Little is known about the circulation of LV in the environment; however, one of our sequences was identical to a widespread haplotype found in several rodent species across Europe, and the other was identical to a haplotype carried by bank voles in Lombardy (Hauffe et al. 2010), suggesting that squirrels might play an active part in both intra- and interspecific LV circulation; further ecologic and phylogenetic analyses are underway to confirm this.

Poor preservation of road kills did not allow us to identify clinical signs of infection in adenovirus-positive red squirrels, whereas infected, freshly killed, gray squirrels did not show any abnormalities at postmortem examination. All the adenovirus-infected red squirrels were collected in areas where the alien species is not present. Moreover, the two positive gray squirrels lived in areas where the native species is still present or was present until recently. Our findings extend adenovirus distribution in red and gray squirrels to southern Europe, but the gray squirrel does not appear, from these results, to be the source of adenovirus infection in the native species. Our results are consistent with recent findings by Everest et al. (2013) suggesting that the infection could be maintained by the native species or by other sympatric woodland rodents such as wood mice (*Apodemus sylvaticus*).

Despite the limitations of this study (in particular, potential biases linked to convenience sampling and small sample sizes), we show for the first time that red squirrels can be infected with LV, and that one or more adenoviruses are present in southern Europe. We cannot determine whether squirrels are reservoir hosts of these infections, or whether these are results of spillover from other small mammal species (e.g., voles) in the same ecosystem. Transmission of infectious diseases in arboreal sciurids is still poorly understood; their role in disease emergence could be underestimated and further research to disclose their epidemiologic significance is needed.

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