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Ljungan Virus Detected in Bank Voles (*Myodes glareolus*) and Yellow-Necked Mice (*Apodemus flavicollis*) from Northern Italy

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ABSTRACT: Identified in 1998, Ljungan virus (LV; *Picornaviridae*) causes type 1 diabetes–like symptoms and myocarditis in bank voles (*Myodes glareolus*) from Sweden and Denmark, and may be a zoonotic agent of several important diseases (e.g., intrauterine fetal death, type 1 diabetes, Guillain-Barré syndrome, and myocarditis). Using a real-time reverse transcriptase–polymerase chain reaction assay and sequence analysis, we detected LV in bank voles, and for the first time, in yellow-necked mice (*Apodemus flavicollis*) collected during 2006 from a site in northern Italy. The global distribution of LV and its role as a mammalian pathogen deserve further attention.

Key words: *Apodemus flavicollis*, Ljungan virus, *Myodes glareolus*, northern Italy, PCR, type 1 diabetes.

Type 1 diabetes in humans appears to be rapidly increasing worldwide (Devendra et al., 2004). Although an individual's genetic susceptibility is considered to play a major role in the development of the disease, a number of environmental factors have also been identified as potential causes, including viruses that cause type 1 diabetes–like symptoms in native rodents and/or other wild or domestic mammals (Devendra et al., 2004; Ludvigsson, 2006; Yoon and Jun, 2006). Knowledge of the distribution of these viruses among wild and domestic mammal species is crucial to assess their potential importance as human pathogens, identify zoonotic sources of a virus, and lay groundwork for possible vaccine development.

In 1998, Niklasson and colleagues isolated a picornavirus in natural populations of the bank vole (*Myodes glareolus*) in northern Sweden that causes clinically recognizable type 1 diabetes–like symptoms in this species (Niklasson et al., 1998, 1999, 2003a, b). This pathogen, subsequently named the Ljungan virus (LV), has been identified in wild voles in Denmark and the United States, as well as in lemmings and inbred laboratory rats (Johansson et al., 2003; Niklasson et al., 2003a, 2006a, 2007a; Samsioe et al., 2006). In addition, LV has been shown to induce type 2–like diabetes, uterine resorptions, malformations, and neonatal death in CD-1 laboratory house mice (Niklasson et al., 2006b; Samsioe et al., 2006). It has also been noted that the manifestation of various symptoms in LV-infected animals increases with stress (Freimanis et al., 2003; Niklasson et al., 2003a, b, 2006b; Samsioe et al., 2006). Intriguingly, in Sweden it has been shown that the incidence of type 1 diabetes, Guillain-Barré syndrome, and myocarditis in the human population is correlated with rodent population cycles (Niklasson et al., 1998), and children newly diagnosed with type 1 diabetes have significantly increased levels of LV antibodies compared to controls (Niklasson et al., 2003a). More recently, this virus has also been associated with intrauterine fetal deaths in humans (Niklasson et al., 2007b), and it is resistant to extreme pH, oxidizing environments, and detergents, although it is sensitive to heat (Ekström et al., 2007). Hence, it has been hypothesized that the

bank vole as well as other small rodents could act as reservoirs and/or vectors of LV that may be a zoonotic agent of several widespread and economically important diseases, including type 1 diabetes (Niklasson et al., 1998; 2003a). This paper shows that both the geographic and species range of LV is wider than previously reported.

As part of a larger project investigating various zoonoses, rodents were trapped approximately every 6 wk from May to October 2006 using 50 Longworth (Penlon Limited, Abingdon, UK) and 50 Ugglan (Grahnab AB, Hillerstorp, Sweden) live traps filled with nesting material and baited with grain; traps were placed at the locality of Prasöta (municipality of Mazzo di Valtellina; 46°15′45.35″N, 10°16′50.08″E; 1,115 m above sea level; Province of Sondrio, Lombardy, Italy). Fifty traps were placed along the edge of an alpine meadow–coniferous forest and 50 along a forestry road flanked by drystone walls. One trap was placed every 10 m in the evening and checked for three consecutive mornings. Each morning, captured animals were brought live to the laboratory where they were sedated with isofluorane and euthanized. Immediately following death, urine glucose levels were measured using Keto-Diastix (Bayer Diagnostics, Munich, Germany) when possible; various blood and organ samples were taken and sex, age, and breeding status were noted. Whole animals were frozen at −20 C for later dissection and then all tissues were stored at −80 C. Because a number of animals proved positive for glycosuria (a characteristic symptom of type 1 diabetes), liver samples of 20 yellow-necked mice (*Apodemus flavicollis*) and 20 bank voles (*M. glareolus*) were sent on ice to the laboratories of Apodemus AB, Stockholm, Sweden (www.apodemus.se) for analysis. Animals were chosen on the basis of whether urine tests were available, so samples from throughout the trapping season were used. Species identifications for each individual of *Apodemus* were confirmed in the genetics laboratory of the Edmund Mach Foundation, S. Michele all'Adige, Italy (www.fondazioneedmundmach.eu) using the method of Michaux et al. (2001).

A real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assay of a portion of the 5′ untranslated region of the LV genome was used to identify and quantify LV RNA following Donoso Mantke et al. (2007). Total RNA was extracted from frozen liver samples using the TRIzol Plus RNA Purification System (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. One extraction was performed for each liver sample and the RNA was run in triplicate in the real-time RT-PCR. Because LV may be present in very low copy numbers in some tissues (Niklasson, unpubl. data), a sample was considered positive if one out of three PCR replicates was positive. In order to verify the amplification of LV, the PCR products from five positive *M. glareolus* samples were run on a 2% agarose gel; a discrete band of 187 bp was detected, excised, and sent to Geneart AG, Regensburg, Germany, for gel purification and sequencing using the same primers as in the real-time RT-PCR. A nucleotide megaBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the one unique complete sequence was performed and percent of identity between this sequence and previously published similar sequences was estimated.

Ljungan virus was detected in the liver from 10/20 *M. glareolus* (50%) and 2/20 *A. flavicollis* (10%) with copy numbers per gram of tissue ranging from 10 to 50,000 (Table 1). The two PCR-positive *A. flavicollis* included one adult male (trapped in May) and one pregnant female (trapped in August). Polymerase chain reaction–positive *M. glareolus* were found throughout the trapping season in both adult and subadult males, and in subadult and adult females (both pregnant and not pregnant). Interestingly, out of four individuals with glucosuria, only one male *M. glareolus*

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TABLE 1. The number of bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*) from the Province of Sondrio, Lombardy, Italy tested for LV[a] and glucosuria.

	No. of animals (males/females):					
Species	Real-time RT-PCR[a] –tested	LV-positive	Tested for glucosuria	Glucosuria-positive	LV- and glucosuria-positive	LV-positive and glucosuria-negative
Myodes glareolus	20 (8/12)	50% (5/5)	15 (8/7)	7% (1/0)	7% (1/0)	47% (4/3)
Apodemus flavicollis	20 (14/6)	10% (1/1)	10 (7/3)	30% (2/1)	0	0

[a] LV = Ljungan virus; RT-PCR = reverse transcriptase–polymerase chain reaction.

(which also had the highest overall level of glucosuria, approximately 2,000 mg/dl) proved positive for LV (Table 1); the three *A. flavicollis* individuals that were glucosuria-positive were LV-negative; and eight glucosuria-negative *M. glareolus* were LV-positive. Additional focused studies are needed to clarify the issues of intrinsically low viral copy number, the "false negatives" in rodent glucosuria testing from field specimens, and the possible primary organ sites of infectivity by the virus.

From five amplicons, two unique sequences of 147 bp differing by 1 bp (AG transition) were generated, but only one had no missing nucleotides (present in samples from two individuals). The other two sequences were identical to these except where five to eight nucleotides were missing. A nucleotide megaBLAST suggested that our one complete 147-bp sequence is most similar to LV strain 174F (accession number: AF327921; Johansson et al., 2002), and confirms that the PCR assay developed by Donoso Mantke et al. (2007) is LV-specific. Of the six LV strains extracted from *M. glareolus* available in public databases, the five originating from Medelpad County in Sweden are similar, but not identical to our sequence (accession numbers [percent of identity]: AF327921 [87], EF202833 [86], AF327920 [86], AF327922 [85], AF020541 [85]); however, one from the United States (AF538689) is more divergent (ca. 70% identity depending on alignment parameters). Further studies of much longer sequences from various parts of the LV genome, and from multiple strains and host species are necessary for an accurate estimate of between-strain and between-site genetic variation.

This short communication significantly extends the known geographic distribution of LV to southern Europe, previously only reported from northern European countries and the United States. Our results suggest that in northern Italy, LV is present in a proportion of bank voles similar to that found in northern Sweden (Niklasson, unpubl. data). In addition, we have shown that LV is also present in another native rodent species, *A. flavicollis*. Like the bank vole, the yellow-necked mouse is extremely common and widespread, with a range covering much of western and central Europe (Michaux et al., 2005). This species has already been shown to be a host for a number of zoonotic viruses, such as Dobrava virus, lymphocytic choriomeningitis (arena) virus, tick-borne encephalitis virus, and cowpox virus (Chantrey et al., 1999; Rizzoli et al., 2004; Kallio-Kokko et al., 2006). However, given the low prevalence of LV in this species, it remains to be shown whether *A. flavicollis* maintains LV on its own, or only in the presence of infected bank voles (see, e.g., Chantrey et al., 1999).

We speculate that as interest in LV increases, this virus will prove to have a worldwide distribution, as well as a broad species range. We are currently testing other mammal species for LV as well as exploring its distribution in various tissues, sequence variation, and role as a pathogen for both animals and humans.

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