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DISTRIBUTION AND SEASONAL VARIATION OF LJUNGAN VIRUS IN BANK VOLES (*MYODES GLAREOLUS*) IN FENNOSCANDIA

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ABSTRACT: Ljungan virus (LV) is a picornavirus originally isolated from Swedish bank voles (*Myodes glareolus*) in 1998. The association of LV with human disease has been debated ever since, but fundamental data on the ecology of the virus are still lacking. Here we present results of the first intensive study on the prevalence of LV in bank voles trapped in Fennoscandia (Sweden and Finland) from 2009–12 as determined by PCR. Using an LV-specific real-time reverse transcriptase PCR, LV was detected in the liver of 73 out of 452 (16.2%) individuals and in 13 out of 17 sampling sites across Sweden and Finland (mean per site prevalence 16%, SE 3%, range 0–50%). We found more infected animals in autumn compared to spring, and lighter and heavier individuals had a higher prevalence than those with intermediate body masses. The result that LV prevalence is also lower in heavier (i.e., older) animals suggests for the first time that LV infection is not persistent in rodents.

Key words: Finland, Parechovirus B, picornavirus, prevalence, rodent-borne virus, real-time reverse transcriptase PCR, Sweden.

INTRODUCTION

Ljungan virus (LV, also known as Parechovirus B), a positive-sense RNA virus belonging to the family *Pircornaviridae*, was first isolated in wild populations of bank voles (*Myodes glareolus*) in Sweden by Niklasson et al. (1998, 1999). Later reports suggested that this virus may be associated with human diseases such as myocarditis and type 1 diabetes, gestational pathologies such as intrauterine fetal death, and fetal central nervous system malformations such as hydrocephaly and anencephaly as well as with sudden infant death syndrome (McDonald 2009; Nilsson et al. 2015). Symptoms typical of some of the above human diseases can be reproduced in laboratory mice (*Mus musculus*) and captive bank voles inoculated with LV (Niklasson et al. 2003a; Samsioe et al. 2006). However, to date the role of LV as a causal agent of human disease remains controversial (e.g., Tapia et al. 2010; Jääskeläinen et al. 2016). The role of wild rodents as reservoirs of LV is also unknown.

Here we report patterns of detection of LV viral RNA in bank voles in Fennoscandia (Sweden and Finland). As bank voles are reservoir hosts of several zoonotic pathogens, such as Puumala hantavirus (Olsson et al. 2010; Voutilainen et al. 2015) and cowpox virus (Pelkonen et al. 2003), we expected LV to be widespread across the region. Although previous studies have detected this virus in bank voles, this PCR-prevalence study of LV covers a larger geographic area and includes a larger number of populations and sample size per population than any similar study thus far (see Results). We decided to start our survey with bank voles because this is the most

common rodent species in Europe (Hansson and Henttonen 1985; Vapalahti et al. 2003).

MATERIALS AND METHODS

Bank vole trapping and tissue sampling

In northern Finland, bank voles were snap-trapped within 4 km of Pallasjärvi (site 1, Fig. 1; Henttonen et al. 1987; Henttonen 2000) in autumn 2011 as part of a larger long-term monitoring program of small mammal cycles in Finland (Korpela et al. 2013). Voles were dissected on-site and tissues were archived at –20 C. Also, as part of a long-term study in Sweden, bank voles were snap-trapped in 1 yr and season (except those from the Umeå site that were trapped in spring and fall from 2009–12) in 16 sites along a north-south transect (Fig. 1 and Table 1). Sites 2 and 6 are described by Magnusson et al. (2013); sites 3, 5, and 9 by Hörnfeldt (2004); site 4 by Rodushkin et al. (2011); and site 15 by Lindström and Hörnfeldt (1994). Trapping was conducted in spring and autumn (from April–June and from September–November, respectively, depending on latitude) using Finnish snap traps (Etutuote Ky, Vaasa, Finland). Each vole was weighed, wrapped individually, kept on ice or frozen in the field, transported, and stored whole at approximately –20 C. All procedures were carried out with permission from the Animal Ethics Committee in Umeå (A 44-08, A 61-11, and A 121-11), the Swedish Board of Agriculture (A 135-12 and Dnr A78-08), and the Swedish Environmental Protection Agency (Dnr 412-2635-05, Dnr 412-4009-10, and Nv 02939-11). In Finland, the bank vole is not protected, and snap trapping does not require ethical permits under the Finnish Act on Animal Experimentation (62/2006) and on a further decision by the Finnish Animal Experiment Board (16 May 2007), as snap-trapping is not classified as an animal experiment.

A subset of 452 animals captured from 2009–12 (selected as being those most recently trapped and therefore most likely to yield undegraded RNA) were thawed and dissected in October 2013 at the Department of Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences, Umeå, Sweden. Five sites (Vålådalen, Ammarnäs, Vindeln, Grimso, Haparanda) were represented by spring samples (n=155) while other sites by autumn samples (n=297). In order to study seasonal variation in LV prevalence, from the area around Umeå we randomly selected 135 voles trapped in either autumn (n=80) or spring (n=55) during these 4 yr. All individuals were classified into functional groups according to body mass in Sweden, as juveniles (≤13.9 g), subadults (14–18 g), or adults (≥18 g) and by breeding status in Finland (Haukisalmi et al. 1988; Cattadori et al. 2006). In addition, in Finland breeding adults were identified as either young of the year or overwintered ones (Haukisalmi et al. 1988; Prévot-Julliard et al. 1999).

RNA extraction and amplification

From each of the 452 bank voles selected, a 0.5-g piece of liver was dissected out, stored at –20 C, and eventually transported on dry ice to the Fondazione Edmund Mach (Trentino, Italy) for molecular screening. The RNA extraction was performed under a sterile hood using the RNeasy Lipid Tissue MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was reverse-transcribed into cDNA by using the OneStep RT-PCR kit (Qiagen). Each RNA sample was then run in duplicate in an LV-specific real-time reverse transcriptase PCR, following Donoso Mantke et al. (2007), using the forward primer 5′-GCGGTCCCACTCTTCACAG-3′ and the reverse primer 5′-GCCCAGAGGCTAGTGTTAC-CA-3′. In order to obtain enough high-quality RNA for sequencing, a nested-PCR amplification was carried out using the AmpliTaq Gold® 360 DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a touchdown PCR with an initiation of 95 C for 10 min; amplification of 45 cycles at 95 C for 30 s; 62 C for 6 cycles; 61.5 C for 6 cycles; 61 C for 6 cycles; 60.5 C for 6 cycles; 60 C for 21 cycles; elongation at 72 C for 40 s; and a final extension at 72 C for 7 min. The PCR products were visualized in the QIAxcel Advanced System (Qiagen), and putative positive samples were identified by a discrete band at about 187 base pairs (a sample was considered positive if at least one of the two replicates gave the expected PCR product). For all the PCR amplifications, we included negative controls consisting of molecular grade water and positive controls containing LV RNA isolated from the prototype strain 87-012 provided by B. Niklasson, University of Uppsala, Sweden.

We confirmed that the resulting PCR product was from LV by sequencing it. We either directly purified the product with the PCR Purification Combo Kit (Invitrogen, Carlsbad, California, USA) or purified the gel fragment with both the PureLink® Quick Gel Extraction (Invitrogen) and the purification kit mentioned above. Purified DNA was sequenced in both directions using a BigDye® Terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems Division, Foster City, California, USA) following manufacturer's instructions, except that the final volume was 10 μL. The dye-labeled sequencing products were run on an ABI PRISM® 310 Genetic Analyzer

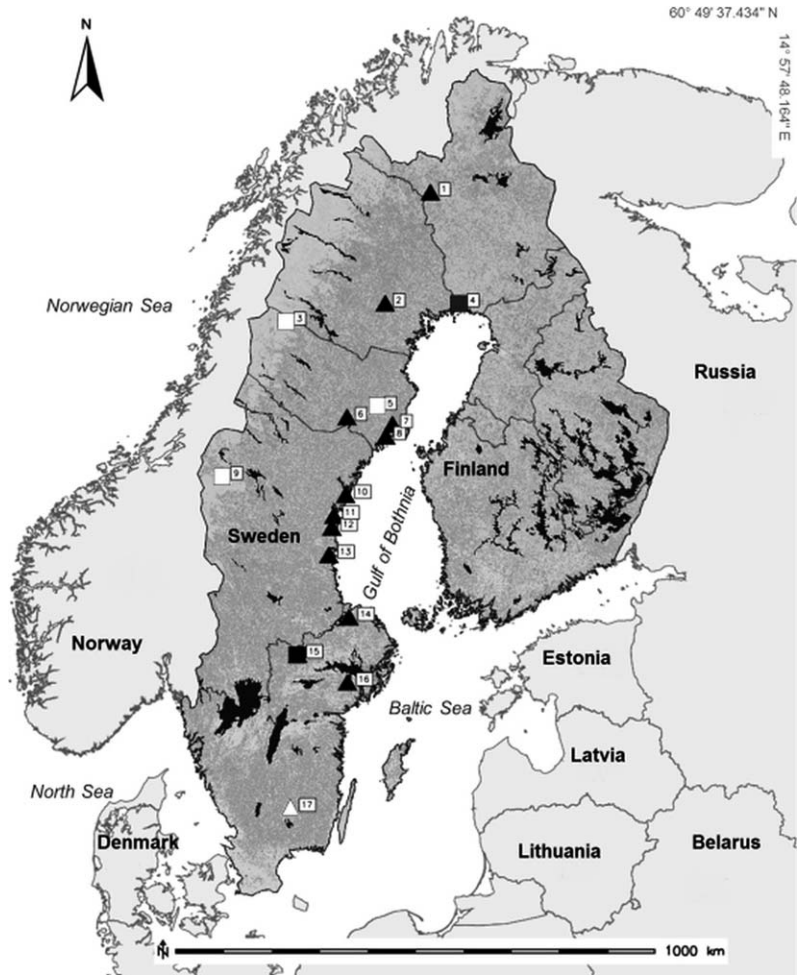

FIGURE 1. Trapping sites in Fennoscandia where bank voles (*Myodes glareolus*) were captured in autumn (triangles) or spring (squares). Only site 7, Umeå, was sampled both in autumn and in spring. Closed symbols=at least one Ljungan virus PCR-positive animal was captured; open symbols=all individuals were Ljungan virus PCR-negative.

(Applied Biosystems). The sequences were edited manually using BioEdit version 7.2.5 (Hall 1999) and assembled using Sequencher version 4.7 DNA sequence analysis software (Gene Codes, Ann Arbor, Michigan, USA). Each nucleotide sequence was subjected to Basic Alignment Search Tool (BLAST) analysis (National Center for Biotechnology Information 2016). The PCR prevalence of LV at each sampling site was calculated as the number of LV-positive samples per total samples collected for that site.

Statistical analysis

We adopted a generalized linear model (GLM) with a binomial error distribution and logit link to assess biologic parameters correlated with LV occurrence (detected by PCR) from all samples collected at the Umeå sampling site. The binary response variable was the presence or absence of LV RNA in vole liver samples while sex, body mass, functional group, year, and trapping season (spring and autumn) were selected as fixed explanatory variables. We also applied the same statistical model to determine differences in the LV occurrence in all the sampling sites trapped in autumn only (because these sites were the most numerous and cannot be directly compared to sites trapped only in spring). In this case, the explanatory variables were sex, body mass, functional group, year, and sampling site. Variance inflation factors and correlation tests (Dormann et al. 2013) showed that functional group and body mass were significantly correlated. In both analyses, starting

TABLE 1. Prevalence with SE of Ljungan virus estimated by real-time reverse transcriptase PCR in bank voles (*Myodes glareolus*) from trapping sites in Fennoscandia.

Sampling site[a]	Nearest location	No. *Myodes glareolus* screened	No. PCR-positive samples	Prevalence (±SE) of Ljungan virus in *M. glareolus* (%)	Year of trapping	Latitude/longitude
1	Pallasjärvi	16	4	25.0 (±10.8)	2011	68°01′46″N, 24°12′16″E
2	Harads	20	1	5.0 (±4.9)	2010	65°40′54″N, 21°3′16″E
3[b]	Ammarnäs	20	0	0	2011	65°51′47″N, 16°23′54″E
4[b]	Haparanda	20	3	15.0 (±8.0)	2011	65°48′47″N, 24°7′11″E
5[b]	Vindeln	20	0	0	2011	64°18′12″N, 19°39′13″E
6	Fredrika	20	6	30.0 (±10.2)	2010	63°56′25″N, 17°25′56″E
7[b]	Umeå	135	27	20.0[c] (±3.4)	2009–12	63°53′22″N, 20°43′6″E
8	Hörnefors	20	1	5.0 (±4.9)	2011	63°33′47″N, 19°47′17″E
9[b]	Vålådalen	20	0	0	2011	62°35′49″N, 12°32′8″E
10	Härnösand	20	4	20.0 (±8.9)	2011	62°29′33″N, 17°48′55″E
11	Njurunda	20	2	10.0 (±6.7)	2011	62°15′14″N, 17°29′41″E
12	Gnarp	20	10	50.0 (±11.2)	2012	61°51′13″N, 17°12′8″E
13	Enånger	20	6	30.0 (±10.2)	2012	58°42′38″N, 10°31′20″E
14	Tierp	20	5	25.0 (±9.7)	2011	60°34′3″N, 17°48′40″E
15[b]	Grimsö	20	3	15.0 (±8.0)	2011	59°41′14″N, 15°22′57″E
16	Gnesta	20	1	5.0 (±4.9)	2012	58°56′11″N, 17°11′40″E
17	Växjö	21	0	0	2012	57°06′33″N, 14°55′50″E
Total		452	73	16.2 (±1.7)		

[a] See Figure 1.

[b] Sites sampled in spring (all others sampled in autumn except Umeå, for which 80 samples were collected in the autumn and 55 in spring).

[c] For autumn samples only, prevalence was 30% while spring prevalence was 5.5%.

from the full model which included all noncollinear explanatory variables, we carried out a stepwise model selection based on Akaike information criterion (Akaike 1974) and the model with the lowest Akaike information criterion was then selected as best model. All the statistical analyses were performed using R software using the R package stats (R Development Core Team 2016) and the function corvif (Zuur et al. 2009).

RESULTS

Following its discovery, LV and LV antibodies were reported from rodent species from several countries (see Supplementary Material Table S1). However, most of these studies are sporadic both geographically and temporally, with small sample sizes. We detected LV in the livers of 73 out of 452 (16.2%) individuals. Rodents that were LV-positive were found in 13 out of 17 sampling sites in Fennoscandia including 22.5% of the autumn and 5.8% of the spring samples (Fig. 1 and Table 1). The mean prevalence per site was 16% (SE 3%), ranging from 0–50% overall and from 0–50% in autumn and 0–20% in spring (Table 1). Individuals that were LV-positive had a body mass ranging from 11.8–25.5 g (n=318) while there were no positives among the lightest and heaviest animals; that is, individuals with a body mass of 6–12 g (n=17) or 26–39 g (n=69), respectively. Among the 73 LV-positive individuals, 34 were female and 38 were male (sex was not determined for one LV-positive animal). The GLM analysis showed a significantly higher LV prevalence in males than in females in the samples from around Umeå (P=0.032; Table 2), with the same trend for the samples from all sites sampled in autumn (P=0.053; Table 3). In the overall dataset, LV PCR prevalence in adult voles was 11.7% (31/233), 22% in subadults (21/76), and 10% in juveniles (5/46). The GLM results for Umeå and for sites sampled in autumn indicated that infection probability showed a significant concave-down relationship with body mass; that is, the body mass effect was significant and

TABLE 2. Coefficient estimate and significance of explanatory variables remaining in the best selected models for predicting Ljungan virus occurrence in bank voles (*Myodes glareolus*) in Umeå (Sweden), based on spring and autumn data from 2009–12. Reference values were autumn for 'season' and females for 'sex.'

Variable	Estimate	SE	Z-Value[a]	Pr(>|Z|)[b]
(Intercept)	−26.186	8.063	−3.248	1.16e-03**
Season_Spring	−3.696	0.961	−3.847	1.2e-04***
Body mass	2.523	0.821	3.072	2.13e-03**
(Body mass)^2	−0.067	0.021	−2.933	3.36e-03**
Sex_Males	1.499	0.696	2.150	0.032*

[a] Estimate to SE ratio.

[b] Two-tailed *P* values for Z statistics; significance level: ***$P<0.001$; **$P<0.01$; *$P<0.05$.

positive while (body mass)^2 was significant and negative ($P<0.01$; Tables 2, 3). This trend indicated that, after an initial increase, LV prevalence reached a peak for an intermediate value of body mass and then decreased. For the sampling area around Umeå, seasonal variation in LV prevalence was observed, both overall and in adults only (Fig. 2), and the GLM results confirmed that LV PCR-prevalence was significantly higher in autumn than in spring ($P<0.001$; Table 2) in both these categories.

DISCUSSION

We studied the PCR-prevalence of LV in its main rodent host in Fennoscandia, where the virus was first discovered. Our results from 452 animals and 17 sampling sites show that LV is present in 13 (76%) of the sites we studied and distributed from northern to southern Fennoscandia. However, the range of PCR prevalence is striking: 0–50% among study sites (Table 1). The LV has previously been reported from a limited number of voles trapped in different locations in northern and central Sweden (Niklasson et al. 1999; Niklasson et al. 2003b) and in central Finland (Jääskeläinen et al. 2013), but our study significantly extends the knowledge of the geographic distribution of this virus within Fennoscandia. Comparisons of our study to those in other countries are problematic because the latter are based on sampling in

TABLE 3. Coefficient estimate and significance of explanatory variables remaining in the best selected models for predicting Ljungan virus occurrence in bank voles in sites sampled in autumn in Fennoscandia (sites where all individuals were Ljungan virus PCR-negative were omitted from the analysis). Reference levels were females for 'sex' and Harads for 'site.'

Variable	Estimate	SE	Z-value[a]	Pr(>|Z|)[b]
(Intercept)	−17.498	4.787	−3.655	2.57e-04***
Sex_Males	0.833	0.430	1.936	0.053
Body mass	1.432	0.479	2.990	2.79e-03**
(Body mass)^2	−0.035	0.012	−2.844	4.46e-03**
Site_Fredrika	1.917	1.155	1.660	0.097
Site_Umeå	2.572	1.158	2.221	0.026*
Site_Hörnefors	0.291	1.558	0.187	0.852
Site_Härnösand	0.912	1.272	0.717	0.474
Site_Njurunda	1.199	1.394	0.861	0.389
Site_Tierp	2.795	1.333	2.097	0.036*
Site_Gnesta	−0.345	1.486	−0.235	0.814

[a] Estimate to SE ratio.

[b] Two-tailed *P* values for Z statistics; significance level: ***$P<0.001$; **$P<0.01$; *$P<0.05$.

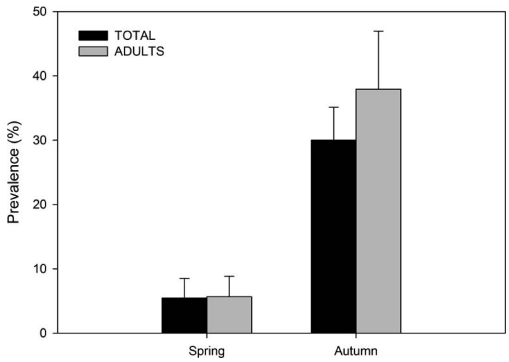

FIGURE 2. Seasonal variation in Ljungan virus PCR prevalence in bank voles trapped in the area around Umeå (Sweden) based on pooled data from 2009–12 for spring (for total population: n=55, positive animals=3; for adults only: n=53, positive animals=3) and autumn (for total population: n=80, positive animals=24; for adults only: n=29, positive animals=11).

single populations; however, the prevalences they reported fell within the range of our study (Table S1): Italy 50% (Hauffe et al. 2010), UK 27% (Salisbury et al. 2014), and Germany 8% (Kallies 2012).

There may be several reasons for the seasonal variation in LV prevalence, both overall and in adult animals (Fig. 2). We found a higher prevalence of LV in bank voles in autumn, when populations are primarily composed of nonbreeding subadults of the year, compared to that in the following spring when populations consist mainly of overwintered adult individuals (Prévot-Julliard et al. 1999; Yoccoz et al. 2001). However, any hypothesis must be based on knowledge of viral kinetics in the host; for example, it is known that hantaviruses cause chronic infections in rodent hosts and that antibodies are detectable for life (Vaheri et al. 2013). If this is true of LV, then a lower PCR prevalence in spring suggests that LV had an effect on rodent mortality. Little is known for LV in this respect; however, bank voles may clear LV infection such that (at least in longitudinal field monitoring of wild rodents) LV antibodies disappear or decrease to an undetectable level. Thus, the lower spring prevalence we detected could be due to clearance of infection. More studies on the kinetics of LV and its impact on host fitness and survival are needed in order to resolve this question. However, host social behavior could also play an important role in seasonal variation of LV infection. During the reproductive season, lasting from approximately March to September (Prévot-Julliard et al. 1999; Crespin et al. 2002), bank voles are known to have a high contact rate, resulting in wounding due to bites and scratches during aggressive encounters (Escutenaire et al. 2000) which may promote the spread of pathogens (Vapalahti et al. 2003), although the transmission mode and route have not yet been determined for LV. Seasonal fluctuation in PCR prevalence partially explains the large range in prevalence between sites, as sites trapped in spring have a 0–20% prevalence (with 3/6 sites with no LV-positive samples) and autumn sites a 0–50% prevalence (but for which only one out of 12 sites showed 0% prevalence). However, factors associated with geographic location may also play a role (e.g., 4/6 spring sites were inland compared to 3/12 autumn sites; Fig. 1). Future studies on LV distribution in voles in these areas should be conducted in both seasons for each site.

Correlation tests employed here show that functional group and body mass are significantly correlated. In addition, we show that body mass is also correlated with the prevalence of the virus in the autumn in the studied vole populations (Table 3), so that lighter and heavier animals show lower LV prevalence than do individuals with intermediate body mass values. For lighter individuals, this result is not unexpected because low prevalence of many pathogens in juvenile rodents is common, mainly because they have not yet had time to be infected, they have limited contact with individuals outside their mother's home range (Verhagen et al. 1986), and they may be protected from infection by maternal antibodies as demonstrated for hantavirus (Kallio et al. 2010; Voutilainen et al. 2016). Interestingly, the result that LV prevalence is also lower in heavier (i.e., older) animals suggests for the first time that LV infection is not persistent in rodents. This result is consistent with parechovirus epidemiology: serologic studies

show that human parechoviruses do not cause persistent infection in immunocompetent humans and that humans appear to face primary infection by the age of two, followed by human parechovirus reinfection boosting the antibody response (e.g., Tauriainen et al. 2007; Tapia et al. 2008).

In our investigation, the sex of the host significantly influenced the prevalence of the virus in autumn and in voles around Umeå, in agreement with several studies on hantavirus seroprevalence in bank voles showing that breeding males have a higher prevalence compared to breeding females (Kallio et al. 2010; Voutilainen 2013). In contrast, several serologic studies on bank voles for orthopoxvirus and hantaviruses (e.g., Augot et al. 2006; Essbauer et al. 2009) indicated that no sex differences existed. As concluded by Voutilainen (2013), sex may affect viral prevalence in a complex way and should be further investigated.

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SUPPLEMENTARY MATERIAL

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