Arch. Virol. May 12, 2013. DOI: 10.1007/s00705-013-1731-6

Ljungan virus is endemic in rodents in the UK

Anne-Marie Salisbury[1], Michael Begon[3], Winifred Dove[1], Bo Niklasson[2] and James P. Stewart[1*]

[1]Department of Infection Biology, University of Liverpool, Liverpool L3 5RF, UK
[2]Apodemus AB, Grevgatan 38, Stockholm, Sweden
[3]Ecology and Evolution of Infectious Disease Group, University of Liverpool, Liverpool, L69 7ZJ, UK

[*]**Corresponding author:** James P. Stewart, Department of Infection Biology, University of Liverpool, Liverpool Science Park IC2, 146 Brownlow Hill, Liverpool L3 5RF, UK. Tel: +44 151 795 0221. E-mail: j.p.stewart@liv.ac.uk.

Running title: Ljungan virus in UK rodents

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SUMMARY

Ljungan virus is a recently-identified member of the *Picornaviridae* that was isolated from bank voles in Sweden. It has been shown to cause type 1 diabetes–like symptoms and myocarditis in bank voles (*Myodes glareolus*) and it has been suggested that it has zoonotic potential. Here we showed for the first time that Ljungan virus was prevalent (20 – 27% positive by PCR) in four species of UK rodent (*Myodes glareolus* [bank vole], *Apodemus sylvaticus* [wood mice], *Microtus agrestis* [field vole] and *Mus musculus* [house mice]). Sequence analysis showed that Ljungan virus of genotypes 1 and 2 were found, although genotype 1 was more prevalent and more frequently associated with brain tissue. This study highlights the prevalence of Ljungan virus in the UK and the need for confirmation of its zoonotic potential.

Main Text

Ljungan virus (LjV) is a member of the *Parechovirus* genus within the *Picornaviridae* [22]. Human parechovirus (HPeV), the other species in the genus, is commonly found in children with diarrhoea and gastroenteritis [8]. The initial isolation of LjV was from bank voles (*Myodes glareolus*) in Sweden [15, 16]. It was found that the incidence of human myocarditis, diabetes and Guillain-Barré syndrome in Sweden varied with the 3-4 year abundance cycles of the bank vole and it was hypothesized that bank voles were the reservoir and/or vector of an infectious agent causing these diseases [15]. Subsequently, LjV was isolated from Swedish diabetic bank voles with viral antigen and picornavirus-like particles being detected in the destroyed pancreatic beta cells [16, 17]. LjV has been isolated in other species of wild voles and lemmings in northern Sweden [18], and detected in wild voles or mice in Denmark, the USA and Italy [9, 12]. In addition to the study of wild rodents, it has been shown that type 2 diabetes-like disease can be LjV-induced in a mouse model [19]. The detection of LjV antigens by immunohistochemistry in foetal tissue samples in cases of human intrauterine foetal death [20] indicates that other disease-associations may be found. However, the association with fetal and infant morbidity and mortality in humans is controversial and is still to be proven [14]. More information is needed to assess the potential zoonotic role of LjV.

To date, five LjV strains have been isolated, representing 4 genotypes. Three LjV strains (87-012, 174F and 145SL) were originally isolated from Swedish bank voles. Sequence analyses showed that the 87-012 and 174F strains form genotype 1 (gt1), while the 145SL strain represents a second genotype (gt2) [13]. Two novel strains of LjV (M1146 and 64-7855) representing genotypes 3 and 4 respectively have subsequently been isolated in the USA. Strain M1146 was isolated from a montane vole (*Microtus montanus*) in Oregon, USA [12] and strain 64-7855 from a southern red-backed vole (*Myodes gapperi*) in the north-eastern USA [23, 24].

The aims of this study were to assess the prelavence, host specificity and genome types of LjV present in rodents in the UK

In total 209 small rodents of four species (*Apodemus sylvaticus* [wood mouse], *Myodes glareolus* [bank vole], *Microtus agrestis* [field vole] and *Mus musculus* [house mouse]) were trapped at several locations within and close to Kielder Forest in northern England (55^{o} 13' N, 2^{o} 33' W). To analyse the frequency and types of LjV present in the rodent populations, animals were sacrificed using isofluorane anaesthesia. Brain and blood samples were immediately removed and stored at -80°C. RNA was extracted from the brain samples using RNeasy mini kit (Qiagen) and from the blood samples using QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. Analysis of the RNAs by RT-PCR assay was performed according to the protocol described previously [10, 11] using primers specific for the 5'UTR of the virus genome [5]. PCR products were analyzed using agarose gel electrophoresis and potential LjV-specific products (187bp) were confirmed and analyzed further by direct sequencing. PCR products were excised from agarose gels and purified using QIAquick gel extraction kit (Qiagen, Germany) before being sequenced by the dideoxynucleotide termination method (Cogenics Inc.) using the same primers as for PCR. Sequence data were analysed using the GCG suite of programs [4]. Control PCR reactions were performed using RNA extracted from tissue-cultured LjV strains 87-012 (gt1) and 145SL (gt2). The sensitivity of the assay was such that one copy of viral RNA could be detected in a background of 1 µg of negative cellular RNA as determined by limiting dilution.

The results (Table 1) showed that 51 out of 209 (24.4 %) rodents were positive for LjV. Significantly more were positive for LjV in the brain (20.1 %) than in the blood (6.7 %) ($P < 0.05$, Fisher's exact test). Only five of the animals were positive in both the brain and the blood. The frequency of LjV in different species varied from 19.7 % to 27.0 % but there was no statistical significance in this variation (Fisher's exact test).

To analyse the viruses present in more detail, sequences generated from the all the LjV-positive samples above were aligned and analysed for phylogenetic relatedness. The phylogenetic tool available at www.phylogeny.fr was used to perform this analysis [3]. Sequences of 147 bp in length generated from PCR products and corresponding to nt 303 – 449 of LjV strain 87-012 (Genbank EF202833) were aligned using MUSCLE [6], alignments

were curated using Gblocks [2] and then analysed for phylogenetic relatedness using maximum likelihood [PhyML [1, 7]]. The final consensus tree generated after bootstrap analysis was drawn using mswordtree [21] and is shown in Fig. 1. Variability between strains was seen in 28 out of 147 nucleotide positions. The sequences reliably and consistently grouped into strain 87-012 (gt1)-like and strain 145SL (gt2)-like (Fig. 1 and Table 2). The proportion of animals positive for LjV gt1 (71.1 %) was significantly greater than those positive for LjV gt2 (28.8 %) ($P < 0.05$; Fisher's exact test). The proportion of animals positive for gt1 was >70 % in all species apart from *M. Musculus* where gt2 was more prevalent (Table 2). However, due to the sample size, this was not statistically significant. Only one animal, a field vole, was positive for both strains, gt1 in the brain and gt2 in the blood. Interestingly, analysis of the distribution of genotypes between brain and blood (Fig. 2) showed that gt1 was far more prevalent in the brain than gt2 ($P < 0.05$; Fisher's exact test), whereas the two genotypes were of approximately equal prevalence in the blood.

This study significantly extends the known geographic distribution of LjV to England. LjV has previously been found in voles in Scandanavia, Italy and the United States. Our results show that LjV is present in a lower proportion of bank voles in England (27.0 %) than that found in Italy and northern Sweden [9] (B. Niklasson, unpublished data). The reasons for the difference in frequency are likely not related to the assay, as all the studies use the same PCR primers. However, the level of PCR-positive bank voles indicates that LjV is still endemic in this species in the UK. In addition, we demonstrated that LjV is present at a similar frequency in another three free-living rodent species, *A. sylvaticus*, *M. agrestis* and *M. musculus*. Analysis showed that there was variation in the sequences analysed with the majority (71.1 %) of the viruses being gt1 (87-012)-like and the rest gt2 (145SL)-like. This indicates that, as in Sweden, these two genotypes are the predominant ones circulating in the UK. Of the two organs analysed, viral RNA was found principally in the brain and this was overwhelmingly gt1. This indicates the ability of the virus to persist in neurological tissue in wild rodents and also that LjV gt1 may have a tendency to be more neurotropic than gt2 during a natural infection.

This work supports the hypothesis that LjV has a worldwide distribution, and that it has a broad species range.

ACKNOWLEDGEMENTS

This work was funded by a National Environment Research Council grant (NE/E008038/1) to MB and JPS.

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Table 1. Number of each species positive for Ljungan virus

Species	Number of animals	Number of animals Positive (%)	Number of positives		
			Brain (%)	Blood (%)	Brain + Blood (%)
A. sylvaticus	66	13 (19.7)	10 (15.2)	5 (7.6)	2 (3.0)
M. glareolus	37	10 (27.0)	8 (21.6)	2 (5.4)	0 (0.0)
M. agrestis	83	22 (26.5)	20 (24.1)	5 (6.0)	2 (3.6)
M. musculus	23	6 (26.1)	4 (17.4)	2 (8.7)	0(0.0)
Total	209	51(24.4)	42(20.1)	14(6.7)	5(2.4)

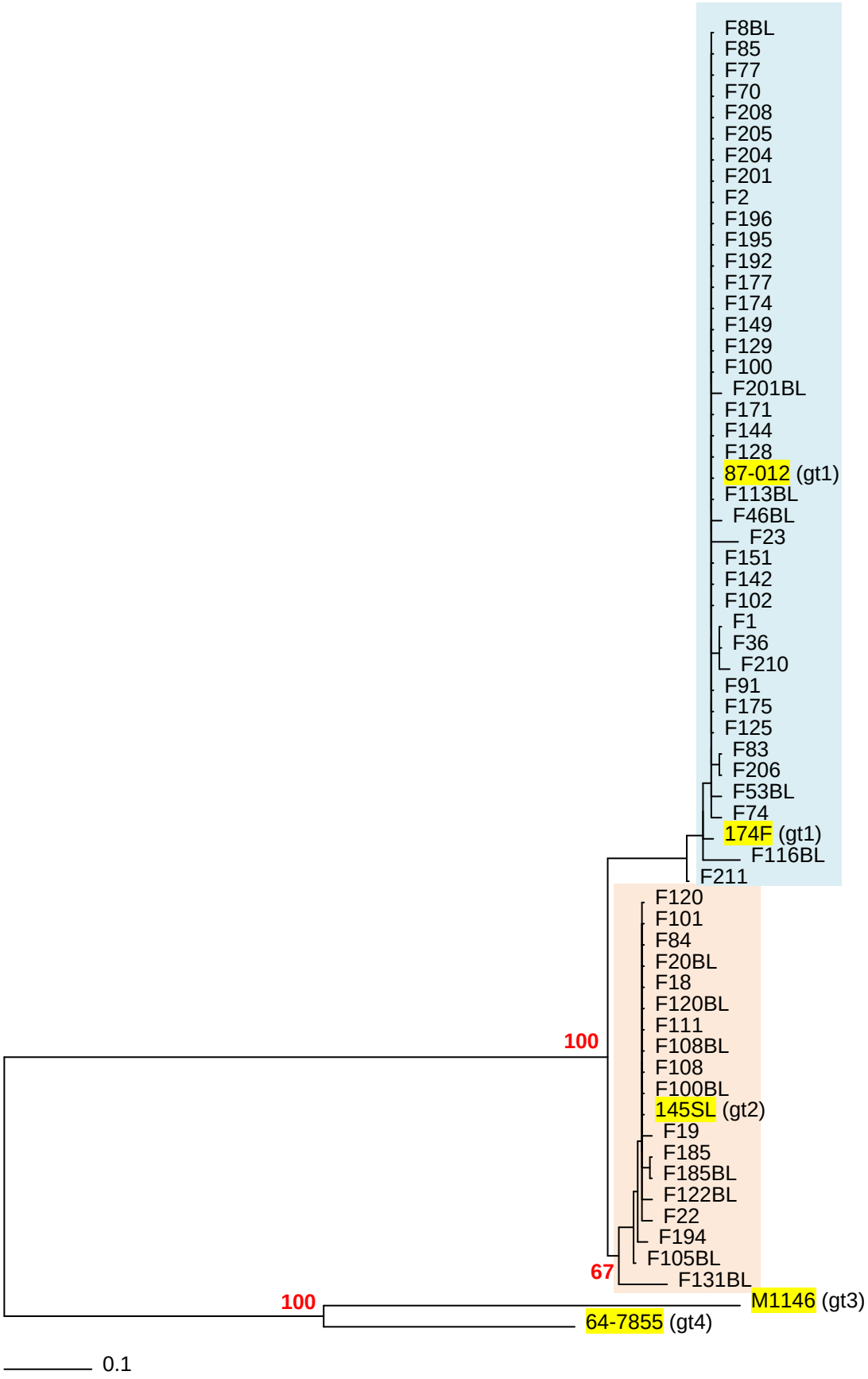
Table 2. Number of each species positive for Ljungan virus genotype 1 or genotype 2

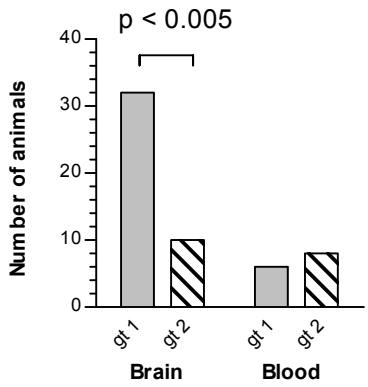
Species	Number of LjV-positive animals	Number of animals gt1-Positive (%)	Number of animals gt2-positive (%)
A. sylvaticus	13	10 (76.9)	3 (23.1)
M. glareolus	10	8 (80.0)	2 (20.0)
M. agrestis	22	17 (73.9)	6 (26.1)
M. musculus	6	2 (33.3)	4 (66.7)
Total	51	37 (71.2)	15 (28.8)

FIGURE LEGENDS

Fig. 1. Phylogenetic analysis of LjV sequences. PCR products corresponding to a portion of the 5'-UTR of Ljungan virus were amplified from RNA extracted from either the blood or brains of free-living rodents. The DNA sequences of these products were then determined. Sequences of 147 bp in length corresponding to nt 303 – 449 of LjV strain 87-012 (Genbank EF202833) were aligned using MUSCLE [6], alignments were curated using Gblocks [2] and then analysed for phylogenetic relatedness using maximum likelihood [PhyML [1, 7]]. The final consensus tree generated after bootstrap analysis was drawn using mswordtree [21]. The branch length is proportional to the number of substitutions per site and the branch support values resulting from 100 bootstrapped data sets are shown in red. Values <50 are omitted for simplicity. Sequences from free-living rodents are numbered with the prefix F and sequences derived from blood were given the suffix BL. The corresponding sequence from five known isolates of LjV were also included in the analysis (highlighted yellow) and annotated with their relevant genotype. Sequences were assigned as either genotype 1 (gt1) (shaded light blue) or genotype 2 (gt2) (shaded pink) based on phylogenetic relatedness to the reference strains 87-012 (gt1) and 145SL (gt2).

Fig. 2. Ljungan virus genotype distribution in brain and blood. PCR products corresponding to a portion of the 5'-UTR of Ljungan virus were amplified from either the blood or brains of free-living rodents as indicated. The DNA sequences of these products were then determined, assigned as either genotype 1 (gt1) or genotype 2 (gt2) based on similarity to the reference strains 87-012 (gt1) and 145SL (gt2) as shown in Fig.1. Statistical analysis was performed using Fisher's exact test.


F8BL
F85
F77
F70
F208
F205
F204
F201
F2
F196
F195
F192
F177
F174
F149
F129
F100
F201BL
F171
F144
F128
87-012 (gt1)
F113BL
F46BL
F23
F151
F142
F102
F1
F36
F210
F91
F175
F125
F83
F206
F53BL
F74
174F (gt1)
F116BL
F211
F120
F101
F84
F20BL
F18
F120BL
F111
F108BL
F108
F100BL
145SL (gt2)
F19
F185
F185BL
F122BL
F22
F194
F105BL
F131BL
M1146 (gt3)
64-7855 (gt4)
100
67
100
0.1


p < 0.005
Number of animals
40
30
20
10
0
gt 1
gt 2
gt 1
gt 2
Brain
Blood