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Serological survey in the Finnish human population implies human-to-human transmission of Ljungan virus or antigenically related viruses

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SUMMARY

Ljungan virus (LV) is a picornavirus related to human parechoviruses (HPeV). The virus has been found in bank voles (*Myodes glareolus*) and several other rodent species, and suggested to have zoonotic potential. Thus far, seroepidemiological data on LV infections in humans are scarce. In this study, we aimed to characterize the demographic and geographical distribution of LV-reactive antibodies in Finland, and to investigate its occurrence in patients suspected of having a rodent-borne disease, nephropathia epidemica (NE) caused by Puumala hantavirus (PUUV). Using an immunofluorescence assay (LV strain 145SLG), we screened human sera (n = 1378) and found LV-reactive antibodies in 36% of samples. The probability of possessing LV-reactive antibodies peaked at age of 14 years, suggesting that most infections occur in childhood. The prevalence of LV-reactive antibodies was significantly higher in the urbanized area surrounding Helsinki than in more rural Central Finland. These findings are uncharacteristic of a rodent-borne pathogen, and therefore we consider human-to-human transmission of one or several Ljungan-like viruses as a likely cause for most of the observed antibody responses.

Key words: Parechovirus B, picornaviruses, rodent-borne viruses, virology, zoonoses.

INTRODUCTION

Ljungan virus (LV; currently also known as Parechovirus B; genus *Parechovirus*, family Picornaviridae) [1, 2] is considered a rodent-borne virus, since it has thus far been isolated from the bank vole (*Myodes glareolus*) in Europe [1] and two other vole species of subfamily Arvicolinae in North America [3–5]. LV RNA has been detected in several rodent species of the Muridae family [6–9]. Besides LV, the genus *Parechovirus* includes the human parechoviruses (HPeVs) consisting of 16 currently known genotypes [2]. Several HPeV types are highly prevalent in humans and more than 90% of adults have had HPeV infection [10, 11]. Some HPeVs have also been detected in non-human primates and swine [12–14]. Interestingly, HPeV4 strains detected in humans and swine in Bolivia [13] lacked an RGD motif found in VP1 carboxyl-termini of all other HPeV4s, and had instead a variant carboxyl-terminal end (17 amino acids). This finding resembles characteristics of LV, as the RGD motif is lacking and LV has a variant carboxyl-terminal end (43 amino acids long)

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[15]. Recently, two new species of Parechovirus, the Sebokele virus 1 isolated from the African wood mouse (Hylomyscus sp.) [16], and the ferret parechovirus [17] have been suggested.

LV infection has been suggested to be involved in intrauterine death [18], central nervous system (CNS) malformations [19], sudden infant death syndrome [20] and type 1 diabetes [21, 22], but the associations remain unconfirmed. Thus far, as for virological data seroepidemiological data on LV infections in humans are also scarce: the existing two studies were restricted to children and adolescents in Southern Sweden [21, 22]. In our earlier study, we established and evaluated LV-specific immunofluorescence and neutralization assays, and in a small panel of Finnish human sera detected a surprisingly high prevalence (38%) of neutralizing antibodies to LV [23].

In this study, we aimed to characterize the demographic and geographical variation in the prevalence of LV-reactive antibodies in the Finnish human population. The Finnish population provides an optimal landscape for seroepidemiological studies on LV, since the incidence of another bank vole-borne virus, Puumala hantavirus (PUUV) causing nephropathia epidemica (NE) in humans is highest in Finland among the European Union countries [24]. For this reason, the epidemiology of NE is well known in Finland [25–27]. We hypothesized that if LV was transmitted from bank voles to humans, the seroepidemiological characteristics of LV would resemble those of PUUV, and furthermore, expected to find a positive association between earlier exposure to bank vole excreta (as indicated by the presence of PUUV antibodies) and the presence of LV-reactive antibodies.

METHODS

Altogether, we selected 1378 serum samples, representing 17 of the 20 Finnish hospital districts for the study (Table 1). The sera were acquired from archived sample panels of the Department of Virology and Immunology, Helsinki University Central Hospital, collected during 2006–2014. Most samples (n = 1155) were acquired without any prior selection from a panel of patients with suspected acute NE and tested for PUUV IgG and/or IgM antibodies. However, children, adolescents and women were underrepresented in this panel, and therefore the dataset was supplemented with two other groups: sera from pregnant women ($n = 88$) and patients aged <20 years ($n = 135$) with suspected acute CNS infection. The sera from pregnant women had been previously tested for syphilis and antibodies against hepatitis B and HIV, and the sera from CNS patients tested for antibodies to herpes simplex viruses (HSV) -1 and -2, varicella-zoster virus (VZV), human herpesvirus-6 (HHV-6), *Mycoplasma pneumoniae*, influenza viruses A and B, parainfluenza viruses, enteroviruses, and adenoviruses.

The sera were diluted in 1:20 phosphate-buffered saline and tested for the presence of LV-reactive IgG antibodies using an immunofluorescence assay (IFA) (LV strain 145SLG) described previously [23].

The distribution of the studied samples was not equal across different sexes, age groups, hospital districts, and patient groups (Table 1), so that the effects these factors on the likelihood on LV antibody positivity were likely to confound each other. As such, we used a multivariable analysis for statistical inference. Since the effect of age on the likelihood of LV-IFA positivity was not expected to follow any particular pattern, we used generalized additive models (GAMs), where age was included as a smooth term and sex, hospital district and patient group were included as factors. Using a similar model, we further studied whether a history of exposure to bank vole excreta (as indicated by the presence of PUUV antibodies) increased the likelihood of being positive for LV IgG antibodies by IFA. In this analysis, only data from patients suspected of having NE and of known PUUV antibody status (i.e. tested for both IgM and IgG antibodies $n = 1129$) were used. The presence of either PUUV IgM, IgG, or both was interpreted as PUUV antibody positivity and therefore, an earlier exposure to bank vole excreta was assumed. In addition to age-smooth term, sex and hospital district, PUUV antibody status was included as an explanatory factor. Binomial error distributions and a logit link function were used in both analyses. The analyses were run with R statistical software [28] using the 'gam' function of the gamm4 package [29]. Statistical inference was made from the full models to avoid any confounding effects between explanatory variables. Pregnant women were considered to represent a healthy background population, and therefore the group was selected as the baseline level for model predictions.

RESULTS

In total, we found 36% (497/1378) of sera to be positive by LV-IFA. The observed group-wise seroprevalences

Table 1. *Numbers tested (percent positive in parentheses) by Ljungan virus immunofluorescence assay*

	Entire dataset	NE suspects	Pregnant women	CNS symptoms
Sex				
Female	623 (35)	467 (30)	88 (45)	68 (54)
Male	755 (37)	688 (35)		67 (60)
Age group (years)				
0–9	61 (48)	6 (17)		55 (51)
10–19	86 (69)	54 (61)		32 (81)
20–29	164 (43)	110 (40)	31 (45)	23 (52)
30–39	219 (41)	143 (38)	54 (48)	22 (41)
40–49	187 (33)	181 (33)	3 (0)	3 (67)
50–59	237 (35)	237 (35)		
60–69	239 (25)	239 (25)		
70–79	117 (25)	117 (25)		
80–89	54 (20)	54 (20)		
90–99	14 (29)	14 (29)		
Hospital district				
Helsinki & Uusimaa	616 (42)	422 (38)	80 (44)	114 (55)
Pirkanmaa	229 (32)	229 (32)		
Central Finland	184 (24)	181 (23)		3 (100)
Lapland	144 (31)	144 (31)		
Kymenlaakso	29 (34)	24 (38)		5 (20)
Western Bothnia	29 (55)	28 (57)		1 (0)
North Karelia	29 (24)	28 (21)		1 (100)
Central Bothnia	22 (45)	22 (45)		
Southern Savonia	18 (17)	18 (17)		
South Karelia	18 (44)	9 (22)	8 (63)	1 (100)
Tavastia Proper	14 (21)	14 (21)		
Vaasa	14 (50)	9 (22)		5 (100)
Päijänne-Tavastia	13 (31)	13 (31)		
Eastern Savonia	8 (50)	8 (50)		
Åland	8 (50)	4 (50)		4 (50)
Northern Savonia	2 (0)	2 (0)		
Northern Bothnia	1 (100)	—		1 (100)
PUUV antibody status				
Negative	506 (37)	506 (37)		
Positive	623 (30)	623 (30)		
Unknown	249 (49)	26 (23)	88 (45)	135 (57)
Total	1378 (36)	1155 (33)	88 (45)	135 (57)

NE, Nephropathia epidemica; CNS, central nervous system; PUUV, Puumala virus.

(by age group, sex, hospital district, patient group) are reported in Table 1. Individual age had a statistically significant effect on the probability of being LV-IFA positive (Table 2). In the analysis of the entire dataset, the predicted likelihood of LV-IFA positivity increased from 33% [95% confidence interval (CI) 12–55] in infants (<1 year) to 60% (95% CI 45–75) by age 14 years (Fig. 1). Thereafter the likelihood decreased steadily, being 22% (95% CI 0–48) at age 96 years. Males were significantly more often seropositive than females (Table 2); according to the predictions from the entire dataset model, 48% (95% CI 33–63) of average-aged men (46 years) and 40% (95% CI 27–53) of women were likely to be LV seropositive.

The prevalence of LV-reactive antibodies was significantly lower in the hospital district of Central Finland than in the Helsinki & Uusimaa district (Table 1, Fig. 1); the predicted probability for an average-aged woman of the background population to be LV-seropositive in Central Finland was 25% (95% CI 13–38), and in Helsinki & Uusimaa it was 40% (95% CI 27–53). Compared to the healthy background population (pregnant women), patients suspected of having NE or an acute CNS infection had no significantly different probabilities of being positive

Table 2. *Parameter coefficients (on logit scale) of generalized additive models analysing the probability of being Ljungan virus-immunofluorescence assay positive for the entire dataset and for NE suspects data*

	Entire dataset			NE suspects		
Parameter	Estimate (S.E.)	*z*	*P*	Estimate (S.E.)	*z*	*P*
Intercept*	−0·42 (0·24)	−1·9	0·060	−0·65 (0·14)	−4·6	<0·001
Male sex	**0·34 (0·13)**	**2·7**	**0·007**	**0·35 (0·14)**	**2·6**	**0·010**
Age†	—	—	**<0·001**	—		**<0·001**
PUUV antibodies	—	—	—	−0·11 (0·15)	−0·8	0·436
Hospital district						
Pirkanmaa	−0·30 (0·18)	−1·5	0·125	−0·29 (0·18)	−1·6	0·108
Central Finland	**−0·67 (0·20)**	**−3·3**	**0·001**	**−0·70 (0·22)**	**−3·2**	**0·002**
Lapland	−0·28 (0·21)	−1·3	0·186	−0·28 (0·22)	−1·3	0·203
Kymenlaakso	−0·26 (0·41)	−0·6	0·522	−0·06 (0·44)	−0·1	0·888
Western Bothnia	0·74 (0·40)	1·9	0·063	**0·86 (0·41)**	**2·1**	**0·034**
North Karelia	−0·59 (0·45)	−1·3	0·191	−0·66 (0·48)	−1·4	0·174
Central Bothnia	0·17 (0·45)	0·4	0·709	0·21 (0·46)	0·5	0·651
South Karelia	0·15 (0·49)	0·3	0·763	−0·70 (0·82)	−0·9	0·389
Southern Savonia	−1·06 (0·65)	−1·6	0·101	−1·13 (0·65)	−1·7	0·081
Tavastia Proper	−0·82 (0·67)	−1·2	0·217	−0·87 (0·67)	−1·3	0·190
Päijänne-Tavastia	−0·46 (0·62)	−0·7	0·460	−0·46 (0·62)	−0·7	0·459
Vaasa	−0·48 (0·82)	0·9	0·389	−0·55 (0·81)	−0·7	0·494
Åland	0·08 (0·73)	0·1	0·909	0·27 (1·02)	0·3	0·793
Eastern Savonia	0·83 (0·74)	1·1	0·260	0·85 (0·73)	1·2	0·243
Northern Savonia	−136·7 (>1000)	0·0	>0·999	−136·6 (>1000)	0·0	>0·999
Northern Bothnia	136·2 (>1000)	0·0	>0·999			
Sample panel						
NE suspects	−0·22 (0·27)	−0·8	0·406			
CNS symptoms	0·22 (0·34)	0·7	0·515			

NE, Nephropathia epidemica; S.E., standard error of parameter estimate; *z*, model test statistic; PUUV, Puumala hantavirus; CNS, central nervous system.

* The intercept represents an average-aged female from Helsinki & Uusimaa hospital district, a healthy background population (pregnant women's group; 'entire data set' model) and a PUUV antibody negative patient ('NE suspects' model).

† The smooth terms of age had 6·4 and 1 estimated degrees of freedom in the 'entire dataset' and 'NE suspects' models, respectively.

by LV-IFA (Table 2). In the CNS panel, 21/115 patients had clinically significant findings: 10 were considered to have acute or recently acquired *M. pneumoniae* infection, four had reactivated HHV-6, one acute HHV-6 infection, four VZV infections, one acute HSV-2 infection, and one enterovirus infection (Table 3). Six out of 10 *M. pneumoniae* cases were LV seropositive, 4/5 HHV-6 cases, 2/4 VZV cases and the only HSV-2 case was positive for LV IgG. The acute enterovirus infection was negative for LV IgG (Table 3).

In the model for suspected NE patients alone, the presence of PUUV antibodies was not significantly associated with LV-reactive antibodies (Table 2). Of suspected NE cases, as in the entire dataset, males were more likely to be LV-IFA positive than females, and the prevalence of LV-reactive antibodies was lower in Central Finland than in the Helsinki & Uusimaa hospital district (Table 1). When analysing only NE suspected patients, the likelihood of positivity decreased linearly by age, in contrast to the pattern seen in Fig. 1. for the entire dataset. Significant microbial findings among patients with CNS symptoms and their co-occurrence with LV-reactive antibodies are reported in Table 3.

DISCUSSION

The high overall prevalence (36%) of LV-reactive antibodies in a population of 1378 Finns corroborated the results of our earlier pilot study done in conjunction by establishing and evaluating the LV antibody tests, where 14/37 Finns (38%) were found positive for LV antibodies in both IFA and neutralization tests [23].

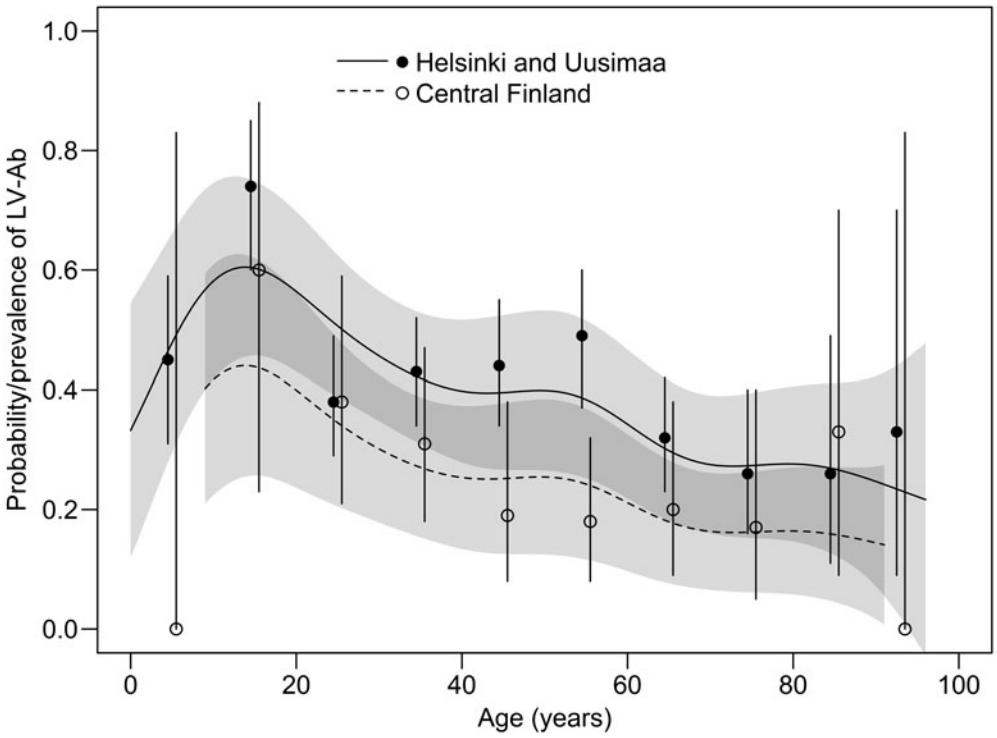

Fig. 1. Ljungan virus-reactive antibodies (LV-Ab) in relation to age in Helsinki & Uusimaa and Central Finland hospital districts. The lines represent the probabilities predicted by the generalized additive model and the grey areas indicate 95% confidence intervals for predicted values, the shading being darker where intervals overlap. The values are calculated for a female representing a healthy background population (pregnant women). The dots represent the observed proportions in each age group and vertical bars indicate their 95% confidence intervals.

Here we found the probability of possessing LV-reactive antibodies to be positively associated with male sex and living in the Helsinki & Uusimaa district, the most urbanized area in Finland, and to peak in adolescents aged 14 years, declining thereafter. The analysis of suspected NE cases alone did not show a similar increase of antibody prevalence before adolescence, but this was probably due to a low number ($n = 24$) of NE suspects aged <14 years. The presence of LV-reactive antibodies was neither associated with being suspected of or having contracted NE, or being suspected of having a CNS infection.

Overall, our findings on LV-reactive antibodies markedly differ from the epidemiological characteristics of PUUV, the causative agent of NE. First, we found the prevalence of LV-reactive antibodies to peak at adolescence, implying that most infections/contacts take place in childhood. By contrast, the risk for contracting NE is highest between ages 37 and 59 years [27] and the prevalence of PUUV antibodies increases until old age [25]. We also observed a decline in the prevalence of LV-reactive antibodies from adolescence to old age, which could be due to waning immunity against the virus in question. However, it seems unlikely that contacts with such a prevalent virus would become notably scarcer with increasing age. Second, our observations of the geographical distribution of LV-reactive antibodies contrast with those of NE. We found LV-reactive antibodies to be significantly more prevalent in Helsinki & Uusimaa than in Central Finland, where NE incidence is nearly six times higher than in Helsinki & Uusimaa [26]. The geographical distribution of NE incidence is considered to reflect the differences in contact rate between bank voles and humans: Helsinki & Uusimaa district is more urbanized, less forested, and has less prominent bank vole population fluctuations than the central and northern parts of the country [30]. Therefore, a similar distribution in human incidence could be anticipated for any bank vole-borne pathogen, including LV. Third, we found LV-reactive antibodies to be much more prevalent in the human population than PUUV antibodies: in the Finnish human population, the overall seroprevalence of PUUV antibodies has been estimated to be 5% [25]. By contrast, in bank voles, LV antibodies have been

Table 3. *Significant microbial findings of patients with suspected CNS infection*

Acute or recently acquired infection	LV IgG positive	LV IgG negative	Total	Information
M. pneumoniae	6	4	10	One *M. pneumoniae* DNA-positive sputum; 8-year-old male treated at ICU (LV IgG positive)
HHV-6 reactivation	3	1	4	Four adolescent patients that were all LV IgG positive; one LV-negative child
HHV-6 acute	1	0	1	2-year-old male; exanthema subitum
VZV	2	2	4	One facial paresis (VZV DNA-positive CSF); one CNS infection (VZV DNA-positive CSF); two chickenpox with minor CNS symptoms
HHV-2	1	0	1	CNS infection (HSV-2 DNA-positive CSF)
Enterovirus	0	1	1	CNS infection (9-year-old male; EV RNA-positive CSF)
Total	13	8	21	

LV, Ljungan virus; ICU, intensive care unit; HHV-6, human herpesvirus 6; VZV, varicella-zoster virus; HSV-2, herpes simplex virus 2; EV, enterovirus; CNS, central nervous system; CSF, cerebrospinal fluid.

reported to be somewhat less prevalent (18%) [23] than PUUV antibodies (20–25%) [31, 32] in the same vole population and time period in autumn. The only result in line with the reported epidemiological associations of NE was the higher antibody prevalence in males [25]. However, male-biased infection is common for pathogens of humans [33] and other vertebrates [34], and is thought to be mediated by physiological and/or behavioural differences between sexes.

These points, together with our finding that LV-reactive antibodies were not more common among suspected NE patients who had been exposed to bank vole excreta (indicated by the presence of PUUV antibodies) compared to those without any knowledge of exposure, lead us to two scenarios that are not necessarily mutually exclusive: (i) LV is not host-specific to bank voles or to rodents generally, but also circulates among humans, or/and (ii) the antibodies observed in this study are not solely, or at all, caused by the currently known LV, but by one or several antigenically similar viruses. The first scenario, i.e. host promiscuity of LV, seems less plausible: although LV RNA has been detected in several rodent species, and HPeVs, the human-borne closest relatives of LV may cross species or even mammalian order boundaries [12–14], we consider it rather unlikely that a rodent-borne LV could circulate in humans effectively enough as to reach the high seroprevalence observed here.

Indeed, the scenario that the observed antibody responses are due to an infection by another, antigenically related to LV, seems more likely. Although HPeVs are highly prevalent in the Finnish population [11, 35], we do not consider cross-reactions to currently known HPeVs to be a likely cause of the observed LV-reactive antibodies, since antibodies against the five most prevalent HPeV types in Finland (HPeV 1, 2, 4–6) [11], did not cross-react with LV [23]. The recent discoveries of new parechoviruses in rodents [15], and mustelids [16], Ljungan-like sequences in swine [36] and non-human primates [14], as well as the continuously widening array of HPeV types also found in non-humans [12–14, 37], imply that the host spectrum and genetic diversity of parechoviruses may be much wider than initially thought. Therefore we consider it likely that most, if not all, of the antibody responses observed here were induced by one or several thus far unknown Ljungan-like viruses that circulate in humans.

Whatever the causative agent of the LV-reactive antibodies observed in this study was, its disease association is probably minor: for the seroconversion to reach 60% in 14-year-old adolescents, more than 2700 infections would need to take place yearly in Finnish children aged 0–14 years. However, closely related HPeVs are known to cause severe infections in infants [10, 38, 39] and therefore, investigations are warranted to identify the causative agent(s) of antibodies observed here and to search for its/their possible disease associations.

It cannot be ruled out that a proportion of the antibodies observed in this study are due to actual LV infections. But as we cannot conclude how large this

proportion is, any conclusions about the disease associations of LV cannot be drawn on the basis of this study.

To conclude, we believe that one or several Ljungan-like viruses, with probably minor disease associations, are circulating in the Finnish human population. However, as closely related HPeVs may cause serious infections in children, investigations to identify these unknown agents are warranted. In near future, next-generation sequencing methods and broad-spectrum PCRs will hopefully shed more light on the host spectrum, genetic diversity and pathogenic potential of Ljungan and related viruses.

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DECLARATION OF INTEREST

None.

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