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ABSTRACT

Background: The emergence and re-emergence of zoonotic and vector-borne diseases are increasing in Europe. Prominent rodent-borne zoonotic viruses include Puumala hantavirus (PUUV; the causative agent of nephropathia epidemica, NE), lymphocytic choriomeningitis virus (LCMV), and orthopoxviruses (OPV). In addition, Ljungan virus (LV) is considered a potentially zoonotic virus.

Objective: The aim of this study was to compare clinical picture between acute PUUV patients with and without additional rodent-borne viral infections, to investigate if concurrent infections influence disease severity.

Study design: We evaluated seroprevalence of and seroconversions to LCMV, LV and OPV in 116 patients hospitalized for NE. Clinical and laboratory variables were closely monitored during hospital care.

Results: A total of five LCMV, 15 LV, and one OPV seroconversions occurred. NE patients with LCMV seroconversions were younger, and had lower plasma creatinine concentrations and platelet counts than patients without LCMV seroconversions. No differences occurred in clinical or laboratory findings between patients with and without seroconversions to LV and OPV. We report, for the first time, LCMV seroprevalence in Finland, with 8.5% of NE patients seropositive for this virus. Seroprevalences for LV and OPV were 47.8% and 32.4%, respectively.

Conclusion: Cases with LCMV seroconversions were statistically younger, had milder acute kidney injury and more severe thrombocytopenia than patients without LCMV. However, the low number of seroconversion cases precludes firm conclusions. Concurrent LV or OPV infections do not appear to influence clinical picture for NE patients.

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1. Background

The emergence and re-emergence of zoonotic and vector-borne (including rodent-borne) diseases are increasing in Europe, at least partly due to changes in host distributions [3]. The relevant rodent-borne viruses include Puumala hantavirus (PUUV), orthopoxvirus (OPV), lymphocytic choriomeningitis virus (LCMV), and Ljungan virus (LV) [1,17,14,41,12]. While many studies have investigated the clinical outcomes of human disease following infection with PUUV (e.g. [24,32], LCMV and OPV, the interactive effects of sequential or co-infections remain little studied and poorly understood.

PUUV is the dominant hantavirus species in Finland and Fennoscandia [41], and the causative agent of nephropathia epi-

demica (NE). The main host of PUUV is the bank vole (*Myodes glareolus*), and the virus is predominantly transmitted horizontally to humans via exposure to contaminated vole excreta [13,39,42]. Human infection is characterized by sudden onset of fever, head and backache, vomiting, and abdominal pain often accompanied by visual disturbances [24]. A minority of patients enter clinical shock. Typical laboratory findings are leukocytosis, thrombocytopenia, elevated plasma creatinine and C-reactive protein (CRP) levels, as well as proteinuria and hematuria. Oliguric acute kidney injury (AKI) develops in most hospital-treated patients, and approximately 5% of patients require transient dialysis treatment. The prognosis of AKI is, however, favorable [32]. In Finland, 1000–3500 people are diagnosed annually with NE, generally in early to mid-winter when voles are most likely to enter human dwellings [41].

LCMV is a rodent-borne virus belonging to the *Arenaviridae*, genus *Arenavirus*, and causes the human disease lymphocytic choriomeningitis, which ranges in severity from biphasic flu-like illness to meningitis and encephalitis [20,34]. Intrauterine infections may occur and result in chorioretinitis, hydrocephalus, microcephaly or macrocephaly, mental retardation, and fetal death [16,22,36]. Chronically infected laboratory and companion mice and hamsters, in addition to wild house mice (*Mus musculus*), are considered to be the primary sources of LCMV infection in humans, and acquired via exposure to rodent excreta [9,6,4,1]. The virus has also been detected in other wild rodents, with prevalence in bank voles reported as 3.3% in the Province of Trento in Italy [17] and 7.4% in northern Italy [38]. In Finland, no previous studies have examined the seroprevalence of LCMV among humans; however, a low seroprevalence for LCMV has been noted among human patients in Spain, Netherlands, Italy, Viet Nam, USA and Canada [8,21,10,20,17,25].

Ljungan virus (LV) (also referred to as parechovirus B, *Picornaviridae*) was first isolated in wild populations of bank voles by Niklasson and colleagues in Sweden [26,27]. LV has been associated with several human diseases [26,28–31], and causes symptoms in laboratory mice [28], but LV has yet to be isolated from humans or proven a causative agent of disease. High seroprevalence (36%, 38%) to LV has recently been detected in humans in Finland [15], indicating a fairly high exposure to LV and/or to an antigenically-related virus in early childhood.

Cowpox virus (CPXV) is an OPV in the *Poxviridae* family causing the majority of OPV infections in Eurasia in both humans and rodents [11]. Wild rodents are considered the main reservoir hosts [5], although human transmission often occurs from companion animals such as rats and via intermediate domestic pets such as cats [40]. In wild rodents, the highest seroprevalence has been recorded in bank voles, wood mice (*Apodemus sylvaticus*), and field voles (*Microtus agrestis*) [2,7,18,12]. Human OPV infection is rare and usually produces a localized lesion on the fingers, hands, or face [33]. In Finland, the sera collected at a Finnish Veterinary meeting in 2001 showed that every person more than 50 years old had OPV antibodies (100% seroprevalence), as measured by immunofluorescence assay (CPXV-IFA) most probably due to the national smallpox vaccinations that ended in 1980 [33]. Due to these vaccinations, people born before year 1980 may still have antibodies against OPV although the immunity is waning.

2. Objectives

The primary aim of this study was to compare clinical outcomes of acute NE patients caused by PUUV with and without additional rodent-borne viral infections. We tested the co-occurrence of LCMV-, LV- and OPV-reactive antibodies and seroconversions in patients hospitalized with acute NE at Tampere University Hospital in Finland. We are not aware of any previous studies on sequential

or co-infections of these viruses in humans, or the clinical implications.

3. Study design

3.1. Patient samples

A total of 534 serum samples were collected from 2000 to 2009 from 116 patients hospitalized for NE at Tampere University Hospital (Finland) with serologically confirmed acute PUUV infection. None of the patients died during the study. Between two and seven serum samples were collected from each patient during hospitalization. Additional serum samples were collected during the convalescent phase, i.e. at 15 days, 6 months and 12 months post-hospital admittance. All samples were stored at −20 °C until further analysis. PUUV infections were confirmed with diagnostic serological assays and routine laboratory analyses were carried out using standard methods at the Pirkanmaa Hospital District Laboratory Centre (Finland). A detailed medical history was obtained and physical examination was performed during hospital care. The following clinical and laboratory variables were recorded: presence of shock, need for dialysis treatment, duration of hospital stay, weight gain during hospital treatment, the highest and lowest hematocrit, the highest blood leukocyte and the lowest platelet count, and the highest plasma creatinine and CRP-level.

All patients provided written informed consent before participation, and the study was approved by the Ethics Committee of Tampere University Hospital (Finland).

3.2. Immunofluorescence assay (IFA) and PCR

Serum samples from acute NE patients were analysed by IFA tests for the presence of LCMV, LV and OPV reactive IgG antibodies based on previously described and validated methods [33,17,14], using Vero E6 cells infected with LCMV (strain Armstrong), and Vero cells infected with or LV (strain LV 145 SLG; Johansson et al., 2003 or CPXV (strain CPXV/FIN/T2000, human isolate; [33]. Sera were diluted 1:20 in phosphate-buffered saline (PBS) for initial screening, added to IFA-slides, and incubated in a moist chamber at 37 °C for 30 min. For human sera, the slides were washed three times with PBS and once with distilled water, and incubated at 37 °C for 30 min with the secondary antibody anti-human immunoglobulin G (IgG) FITC conjugate, diluted 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA). After the second incubation, the IFA slides were washed again as above and dried. Cover slips were added with mounting medium and the slides were examined under a fluorescence microscope (Leica Microsystems, Espoo, Finland) with a FITC filter. For each virus, all the samples were tested in the same assay, on the same day; positive and negative controls were included. The LCMV, LV and OPV all had separate positive controls.

Samples were recorded as positive or negative in IFA based on the pattern and amount of fluorescence in comparison to the positive and negative controls [14]. All positive samples were positive for titer 20 or more. Seroconversions were detected if the first sample after hospitalization was seronegative for IgG against the target virus, but next samples were IgG seropositives.

In an attempt to identify virus species in OPV-seroconverted samples, we used previously described methods [35]. For arenaviruses, one-step RT-PCR was carried out using Invitrogen SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and RiViGene primers: two forward primers: LVL3359_Yplus(5-AGAATCAGTGAAAGGGAAAGCAAY) and LVL3359G_Yplus (5′-AGAATTAGTGAAAGGGAGAGTAAT); and two reverse primers: LVL3754D_Rminus (5′-CACATCATTGGTCCCCATTTACTGTGR) and

Table 1
Number of positive serum samples to LCMV, LV and OPV, and seroconversions during the study. Seroprevalence and seroconversion to each virus from the 116 NE patients is included in brackets.

	Anti-LCMV IgG	Anti-LV IgG	Anti-OPV IgG
Positives	10 (8.5%)	55 (47.8%)	41 (32.4%)
Seroconversions[a]	5 (4.3%)	15 (12.9%)	1 (0.9%)

LCMV, lymphocytic choriomeningitis virus; LV, Ljungan virus; OPV, orthopox virus.
[a] Due to sampling frequency, the exact date of seroconversion is not available, but in most cases, it occurred within one month of hospitalization.

LVL3754A_Rminus (5′-CACATCATTGGTCCCCATTTACTATGR). The PCR amplification protocol consisted of an initiation of 50° for 30 min and 94° for 2 min; amplification of 45 cycles at 94° for 20 s; 55° for 30 s; elongation at 72° for 60 s; and a final extension at 72° for 5 min. For LV, the same Invitrogen kit was adopted, with MetaBion reverse primer pan-parechovirus (PEV) RV (5′-GTAACASWWGCCTCTGGG) and forward primer PEV FW (5′GTACCTYCWGGCCATCCTTG). The LV protocol was similar to that for arenavirus, except that 45 cycles were performed with an annealing temperature of 55°. For arenaviruses and PEVs, the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) was used for nucleic acid extraction according to manufacturer's instructions.

3.3. Statistical analysis

Generalised linear mixed models, with a binary distribution and logit link function, were constructed to evaluate the effects of individual characteristics (i.e. sex, age) and sequential or co-infections on IgG seropositivity to LV, OPV and LCMV. Sex (dichotomous variable), age (categorical), place of residence (dichotomous) and seropositivity to the other two virus species were included as explanatory variables. Year of PUUV diagnosis was set as a random factor to account for potential between-year variation.

Due to the low overall number of seroconversions for OPV, statistical analyses were not applicable but descriptive results are provided.

4. Results

In addition to acute PUUV infection, the highest number of seroconversion cases occurred for LV (15/116; 12.9%; Table 1). For LV, 10 out of the 15 cases occurred in male patients, between 31 and 69 years-old; while the 5 female patients were between 28 and 63 years old. Nine patients were urban and six were rural residents. A total of five LCMV seroconversion events occurred during the study (4.3% cases), comprising four females and one male aged between 22 and 40-years old, three with rural and two with urban places of residence. A single OPV seroconversion case (0.9%) was observed in a 31 year-old male from a rural area. No multiple seroconversions in the same patient were detected. All sera samples collected at the time of seroconversion were negative for arenavirus, PEV and OPV.

No differences were seen in clinical or laboratory findings of patients with or without seroconversions to LV (Table 2). NE patients with LCMV seroconversions were younger, had lower plasma creatinine concentrations and lower platelet counts than patients without LCMV seroconversions. The one male patient who seroconverted to OPV stayed four days in hospital. During the hospital stay his highest creatinine value was 256 μmol/l, the lowest platelet count 36×10^9, and the highest leukocyte count 16.1×10^9. All patients with seroconversions to LV, LCMV or OPV had a typical clinical course of NE. No atypical central nervous system (CNS)-related symptoms were observed.

Of the serum samples tested, 8.5% were positive to LCMV IgG antibodies (10/116), 47.8% were positive to LV IgG antibodies (55/116), and 32.4% were positive to OPV IgG antibodies (41/116).

Statistical analysis showed that sex, age and place of residence had no effect on LV seropositivity, or the presence of LCMV and OPV antibodies (Table 3; Supplementary Table S1 in the online version, at http://dx.doi.org/10.1016/j.jcv.2016.10.002). Similarly, none of the evaluated individual patient characteristics affected LCMV seropositivity (Table 3; Supplementary Table S1 in the online version, at http://dx.doi.org/10.1016/j.jcv.2016.10.002). However, males were significantly more likely to be OPV seropositive than females (Table 3), and the effect of age approached significance due to a trend of higher seroprevalence with increasing age, except for the oldest age group (61–77 year olds).

Place of residence and the co-presence of antibodies against the other two viruses had no effect on OPV seropositivity (Table 3; Supplementary Table S1 in the online version, at http://dx.doi.org/10.1016/j.jcv.2016.10.002).

5. Discussion

In this study of 116 NE patients, LCMV seroprevalence was 8.5%. These are the first data on LCMV seroprevalence in Finland, and higher than that reported from other countries such as Spain (1.7%), the Netherlands (2.9%), and Italy (2.5% [17]. This higher value is likely the result of increased rodent exposure, since the sample pool was derived from patients hospitalized as a result of another rodent-borne virus (PUUV). Although LCMV may cause aseptic meningitis and encephalitis in some cases, no particular CNS-related symptoms were identified in our study. LCMV seroconverted patients were younger than NE patients without seroconversions and had less severe AKI and more severe thrombocytopenia. Only five LCMV seroconversions were detected, thereby precluding firm conclusions but highlighting the need for larger studies.

At present, there are no specific symptoms recognized for human LV infection and we did not encounter any unusual or additional symptoms in NE patients that seroconverted to LV during hospitalization. In total, 47.8% of patient samples were LV seropositive, higher than reported previously in Finland (38% [14]; 36% [15]. However, [15] reported that the seroprevalence of LV in the age group of 30–39 years was 41%; similarly, in this study most of the LV-seropositive acute NE patients were in the 31–40 years age group.

Only one OPV seroconversion was detected during the study. The patient had no skin lesions or any other common symptoms for OPV. The seroprevalence was as high as 32.4% for OPV, probably partly due to previous smallpox vaccinations. Similar studies for OPV have shown that high seroprevalences have been also detected elsewhere (Brazil, 27.9% [23]; Republic of Congo, 56.9% [19].

For PUUV, LCMV and OPV incubation times can vary, making it difficult to infer whether exposure to LCMV and OPV virus occurred at the same time as PUUV. Nevertheless, the relatively high number of seroconversions during the first month of hospitalization suggest that at least some are likely to have occurred during the PUUV exposure event, and most within a short interval. It has still not been confirmed whether LV causes clinical disease in humans, and therefore the incubation time is also unknown; however, we did not note any specific symptoms that may have indicated possible LV infection with a clinical outcome. No LV, LCMV or OPV were detected using PCR and no viremia was detected. However, the samples were originally collected for serology tests and not optimally stored for these methods.

Our results demonstrate that LCMV, LV and OPV antibodies were common in patients hospitalized with acute NE in Finland. Even though 21 seroconversions occurred during hospitalization suggesting simultaneous or sequential infections, viral nucleic acid was not detected nor were differences in clinical outcomes identified for

Table 2
Median (range) values for clinical variables of patients. P-values refer to the comparison between median values of each clinical variable in the groups of seroconverted serum samples and the non-seroconverted ones.

Clinical variables	LV seroconverted patients (N = 15) Median (range)	LCMV seroconverted patients (N = 5) Median (range)	Patients without seroconversions (N = 95) Median (range)
Age (years)	40 (28–69)	30 (22–40)	43 (22–77)
p-values	0.71	**0.01** (p < 0.05)	
Days in hospital	6 (3–15)	5 (2–6)	6 (2–25)
p-values	0.54	0.19	
Creatinine max (μmol/L)	200 (68–917)	76 (61–102)	163 (51–1499)
p-values	0.64	**0.01** (p < 0.05)	
Platelets min (10⁹/L)	70 (31–181)	34 (31–45)	61 (9–238)
p-values	0.10	**0.04** (p < 0.05)	
Leukocytes max (10⁹/L)	9.9 (6.3–17.6)	10.05 (6.9–14.4)	10.1 (3.9–31.2)
p-values	0.61	0.69	
CRP max (mg/L)	75.5 (16.7–266.8)	105.6 (72.7–178.9)	82.5 (15.9–269.2)
p-values	0.85	0.29	
Hematocrit min	0.33 (0.31–0.42)	0.37 (0.32–0.41)	0.36 (0.25–0.44)
p-values	0.57	0.77	
Hematocrit max	0.44 (0.35–0.55)	0.44 (0.40–0.46)	0.44 (0.33–3.9)
p-values	0.36	0.91	
Change in weight (kg)	2.8 (0–9.9)	1.2 (0–2.6)	2 (0–12.0)
p-values	0.84	0.24	

LV, Ljungan virus; LCMV, lymphocytic choriomeningitis virus; CRP, C-reactive protein; max, maximum; min, minimum. Statistical methods used: Medians and ranges were given for clinical variables. Findings in NE-patients with and without seroconversions with LV or LCMV. Wilcoxon test, *t*-test; Rstudio [37], p < 0.05 is considered statistically different.

Table 3
Coefficient estimate and significance of explanatory variables for explaining LV, LCMV and OPV seroprevalence in NE patients. Statistically significant effects are shown in bold. Reference levels were female sex, 20–30 year olds and city residence. For OPV seroprevalence, reference level for age was >35 year old.

Response	Source of variation	Num. df	Denom. df	F	P
LV seroprevalence	sex	1	90	0.78	0.38
	age	4	90	0.62	0.65
	home location	1	90	0.38	0.54
	OPV antibodies	1	90	0.00	0.98
	LCMV antibodies	1	90	0.31	0.58
LCMV seroprevalence	sex	1	92	3.04	0.08
	age	2	92	0.62	0.54
	home location	1	92	0.10	0.75
	OPV antibodies	1	92	0.04	0.83
	LV antibodies	1	92	0.07	0.79
OPV seroprevalence	**sex**	**1**	**90**	**6.21**	**0.015**
	age	4	90	2.39	0.056
	home location	1	90	2.36	0.13
	LV antibodies	1	90	0.04	0.85
	LCMV antibodies	1	90	0.21	0.64

LV, Ljungan virus; NE, nephropathia epidemica; OPV, orthopox virus; LCMV, lymphocyticic choriomeningitis virus; Num. df, numerator degrees of freedom; Denom. df, denominator degrees of freedom; F, F-value; P, P-value. For the analysis of seropositivity to LV and OPV, age was categorised into five groups: 20–30, 31–40, 41–50, 51–60, and 61–77 years of age. To test age effects on LCMV seropositivity (10 positive samples), the age was categorised into three groups: less than 35, 36–50, and more than 51 years. For place of residence, we defined 'urban' as the inner city and suburbs of Tampere, and all other areas as 'rural'. The analyses were conducted using the program SAS, version 9.3 (SAS Institute, 2011).

these patients. In five LCMV seroconversions we found some statistical differences between age, creatinine value and platelet counts. However, there is no convincing evidence that NE is more severe with parallel LCMV infection. Together this information indicates that sequential or co-infection with other rodent-borne viruses, while possible in up to about one in five NE patients, is probably not a major contributor to NE disease severity.

Conflict of interests

None declared.

Ethical approval

The study was approved by the Ethics Committee of Tampere University Hospital (Nos. 99256 and R04180).

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