Department of Virology
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Doctoral Program in Biomedicine
Doctoral School in Health Sciences

DISTRIBUTION AND CLINICAL ASSOCIATIONS OF LJUNGAN VIRUS (PARECHOVIRUS B)

CRISTINA FEVOLA

ACADEMIC DISSERTATION

To be presented for public examination with the permission of
the Faculty of Medicine, University of Helsinki,
in lecture hall LS1, on 11 01 19, at noon

Helsinki 2019

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Cover photo: Cristina Fevola, The Pala group (Italian: *Pale di San Martino*), a mountain range in the Dolomites, in Trentino Alto Adige, Italy.

ISBN 978-951-51-4748-6 (paperback)
ISBN 978-951-51-4749-3 (PDF, available at http://ethesis.helsinki.fi)
Unigrafia Oy, Helsinki, Finland 2019

To you the reader, for being curious.

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

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II. Fevola C, Rossi C, Rosà R, Nordström Å, Ecke F, Magnusson M, Miller AL, Niemimaa J, Olsson GE, Jääskeläinen AJ, Hörnfeldt B, Henttonen H, Hauffe, HC. Distribution and seasonal variation of Ljungan virus in bank voles (*Myodes glareolus*) in Fennoscandia. Journal of Wildlife Diseases. 53:459-471, 2017.

III. Fevola C, Kuivanen S, Smura T, Vaheri A, Kallio-Kokko H, Hauffe HC, Vapalahti O, Jääskeläinen AJ. Seroprevalence of lymphocytic choriomeningitis virus and Ljungan virus in Finnish patients with suspected neurological infections. Journal of Medical Virology, 90:429-435, 2017.

IV. Fevola C, Rossi C, Rosso F, Girardi M, Rosà R, Delucchi L, Rocchini D, Garzon-Lopez CX, Arnoldi D, Bianchi A, Buzan E, Charbonnel N, Collini M, Ďureje Ĺ, Ecke E, Ferrari N, Fischer S, Gillingham E, Hörnfeldt B, Kazimírová M, Konečný A, Maas M, Magnusson M, Miller A, Niemimaa J, Nordström Å, Obiegala A, Olsson G, Pedrini P, Piálek J, Reusken CB, Rizzolli F, Romeo C, Silaghi C, Sironen T, Stanko M, Tagliapietra V, Ulrich RG, Vapalahti O, Voutilainen L, Wauters L, Rizzoli A, Vaheri A, Jääskeläinen AJ, Henttonen H, and Hauffe HC. Broad geographical distribution of Ljungan virus in small mammals in Europe. Vector-borne and Zoonotic diseases. Manuscript, 2018.

LIST OF ABBREVIATIONS

aLRT	approximate log likelihood ratio test
BPP	Bayesian posterior probability
bp	base pair
CNS	central nervous system
CPE	cytopathogenic effect
CPXV	cowpox virus
CSF	cerebrospinal fluid
EDENext	Emerging diseases in a changing European environment
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GLM	general linear model
GLMM	generalized linear mixed model
HFRS	hemorrhagic fever with renal syndrome
HPeV	human parechovirus
HCPS	hantavirus cardiopulmonary syndrome
ICTV	International Committee on Taxonomy of Viruses
IFA	indirect immunofluorescence assay
IgG	immunoglobulin G
IgM	immunoglobulin M
IRES	internal ribosome entry site
IUFD	intrauterine fetal death
LCMV	lymphocytic choriomeningitis virus
LV	Ljungan virus
mRNA	messenger RNA
NE	nephropathia epidemica
OPV	*Orthopoxvirus*
PCR	polymerase chain reaction
PUUV	*Puumala virus*
RT-PCR	reverse transcription PCR
RT-qPCR	reverse transcription quantitative PCR
SIDS	sudden infant death syndrome
T1D	type 1 diabetes
UTR	untranslated region
VPg	viral protein genome-linked

ABSTRACT

Worldwide, infectious diseases are responsible for death of 15 million people annually with a significant impact on public health and economic growth. Many of these diseases are zoonotic, that is, transmitted from wild or domestic animals to humans. The incidence of zoonotic diseases is increasing mainly as a result of more intense and frequent contacts between humans and between humans and other animals. Zoonotic pathogens include viruses, bacteria, fungi, rickettsia, protists, prions and protozoa. Viruses, in particular, are capable of rapidly adapting to new hosts, with rodents as the primary reservoir. "Rodent-borne" viral infection in humans occurs by direct contact with feces, saliva, and urine of infected rodents, or by inhalation of viral particles from aerosolized rodent excrement.

Among rodent-borne viruses, those belonging to the genera *Mammarenavirus*, *Orthohantavirus* and *Orthopoxvirus* are a particular focus of study both in humans and animals, since they represent some of the most widespread rodent-borne disease-causing pathogens. More recently, the interest in parechoviruses has been increasing because some are known to cause diseases in humans, while others are carried by rodents, although the zoonotic potential of rodent-borne parechoviruses has not been established.

Ljungan virus (LV), which belongs to the species *Parechovirus B*, was first isolated from bank voles (*Myodes glareolus*) in Sweden in 1998. It belongs to the *Picornaviridae* family, which includes many viruses that infect humans and other animals. Currently, there is little information about LV host range and epidemiology, but a few reports suggest an association between LV and human disease.

The main aims of this doctoral thesis were 1) to establish the symptoms associated with LV in humans, 2) to investigate the association of LV with human central nervous system (CNS) disease, and 3) to determine the prevalence and distribution of LV in human and other animal populations in Europe. LV-associated symptoms were investigated in two human cohorts. Serum samples from Finnish patients hospitalized for suspected nephropathia epidemica (NE) caused by the *Orthohantavirus* Puumala virus (PUUV) were screened for

the presence of lymphocytic choriomeningitis virus (LCMV, *Arenavirus*), cowpox virus (CPXV, *Orthopoxvirus*) and LV, in order to compare the disease outcomes in these patients and to establish if the co-existence of viruses could lead to an increase in the severity of symptoms. However, no unusual or additional manifestations between PUUV cases and PUUV-LV/LCMV/CPXV cases were detected (I).

To determine if LV (together with the rodent-borne virus LCMV) could be one of the causes of neurological symptoms in Finnish patients with suspected CNS disease, anti-LV and LCMV antibodies were analyzed from serum and cerebrospinal fluid samples. LV- and LCMV-specific nucleic acids were also analyzed from the patient samples. However, no association between LV or LCMV antibodies or nucleic acids and the neurological manifestations in the patient cohort was detected (III).

In order to improve the knowledge of the host and geographical distribution of LV, tissues from multiple rodent and insectivore species from ten European countries were screened for LV nucleic acids (II; IV). We confirmed that LV is widespread geographically, having been detected in at least one host species in nine out of ten countries involved in the study. Seventeen out of 21 species screened were LV PCR-positive, and the virus was detected for the first time in the northern red-backed vole (*Myodes rutilus*) and the tundra vole (*Microtus oeconomus*), as well as in insectivores, including the bicolored white-toothed shrew (*Crocidura leucodon*) and the Valais shrew (*Sorex antinorii*). Results indicated that bank voles are the main rodent host for LV (overall PCR-prevalence: 15.2%). Male and subadult bank voles are significantly more likely to be LV-positive, and the prevalence has a temporal pattern (higher in autumn compared to spring and summer), possibly due to adult bank voles clearing the infection. Interestingly, higher levels of precipitation (rain and snow) at any given time, are associated with a lower LV prevalence six months later.

In conclusion, LV is widespread geographically and found in many hosts that are reservoirs for rodent-borne zoonotic pathogens. However, the seroprevalence of LV or an LV-like virus in humans is above 40% and higher in younger patients (confirmed in this study and by others) suggesting that LV- or an LV-like virus might be transmitted by an alternative route. Thus far, LV has not been isolated from humans and has not definitively been confirmed as an infectious agent in humans. Despite high seroprevalence found in patient cohorts, LV was not detected in association with human CNS disease, and did not seem to

cause disease symptoms. Therefore, this study adds to the growing body of evidence that LV is unlikely to cause zoonotic or non-zoonotic disease. However, since LV has been associated with other non-CNS symptoms in rodents, whether LV or LV-like viruses are potential human pathogens deserve further investigation.

ABSTRAKTI

Infektiotaudit aiheuttavat vuosittain 15 miljoonan ihmisen kuoleman maailmanlaajuisesti, mikä puolestaan vaikuttaa merkittävästi yleiseen terveystilanteeseen sekä ekonomiseen kasvuun. Infektiotaudeista useat ovat zoonooseja eli tarttuvat ihmisiin mm. villi- tai kotieläimistä. Zoonoosi-insidenssi on nousussa ihmisten sekä ihmisten ja vektoreiden välisten kontaktien lisääntymisen vuoksi. Zoonooseja voivat aiheuttaa niin virukset, bakteerit, sienet kuin myös eukarioottiset ja prokarioottiset organismit. Primaarivarantona toimivat usein jyrsijät ja näihin jyrsijäisäntiin sopeutuneet virukset. Jyrsijävälitteiset virusinfektiot leviävät ihmisiin mm. jyrsijän uloste-, sylki- ja virtsa-kontaktin kautta.

Sekä ihmis- että eläinperäisiä mammarena-, orthohanta- ja orthopoxviruksia on tutkittu laajimmin, koska niitä esiintyy maailmanlaajuisesti ja aiheuttavat lisäksi jyrsijävälitteisiä zoonooseja. Nykyään kiinnostuksen kohteina ovat lisäksi jyrsijöissä esiintyvät parechovirukset. Ihmisvälitteisten parechovirusten tiedetään olevan patogeenisia ja helposti leviäviä mutta vielä ei ole osoitettu jyrsijöiden parechovirusten aiheuttavan zoonooseja.

Parechovirus B –lajiin kuuluva Ljungan virus (LV) löydettiin ensimmäisen kerran vuonna 1998 ruotsalaisesta metsämyyrästä (*Myodes glareolus*). Ljungan virus kuuluu muiden parechovirusten kanssa suureen pikornavirusperheeseen. Eri pikornavirukset infektoivat niin ihmisiä tai kuin eläimiäkin. Ljungan virus on jyrsijävirus mutta muuten tästä viruksesta tiedetään vähän ja esimerkiksi sen isäntäjoukon laajuutta tai epidemiologiaa ei ole tarkkaan selvitetty. Löytyy vain harvoja tutkimuksia, joissa Ljungan viruksen seroprevalenssia ihmisillä on selvitetty tai ehdotettu yhteyttä ihmistauteihin.

Tämän väitöskirjatyön päätavoitteina oli tutkia ja selvittää Ljungan viruksen assosiaatiota ihmistauteihin ja näistä varsinkin keskushermostoinfektioihin, sekä määrittää viruksen prevalenssia ja levinneisyyttä Euroopan laajuisesti ihmisissä ja jyrsijöissä. Ljungan viruksen assosiaatiota ihmistauteihin tutkittiin kahdessa eri potilaskohortissa. Toinen potilaskohortti koostui suomalaisista sairaalahoitoisista myyräkuumepotilaista.

Myyräkuumeen aiheuttaa jyrsijävälitteinen orthohantaviruksiin kuuluva Puumala-virus (PUUV). Ljungan viruksen lisäksi tästä potilaskohortista tutkittiin myös lymfosyyttisen koriomeningiittiviruksen (LCMV) sekä lehmärokkoviruksen (CPXV, *Orthopoxvirus*) aiheuttamia serokonversioita. Tuloksia ja potilaiden oirekuvia verrattiin toisiinsa. Lopputuloksena voitiin todeta, että myyräkuumeen taudinkuvassa ei huomattu eroa riippumatta siitä oliko potilaalla vain PUUV-infektio vai mahdollinen LV/LCMV/CXPV-yhteisinfektio. Toinen potilaskohortti koostui potilaista, joilla epäiltiin keskushermostoinfektiota. Tässä kohortista tutkittiin potilasnaytteistä niin Ljungan viruksen kuin LCMV:n seerumivasta-aineita sekä etsittiin selkäydinnestenäytteistä näiden virusten nukleiinihappoa. Aineistosta ei löydetty yhtään LV- tai LCMV-nukleiinihappopositiivista selkäydinnäytettä.

Ljungan viruksen jyrsijäisäntäkirjoa ja maantieteellistä levinneisyyttä tutkittiin eri jyrsijälajien kudosnäytteiden avulla. Näitä näytteitä kerättiin yhdeksästä eri Euroopan maasta ja näytteistä tutkittiin Ljungan viruksen nukleiinihappoja. Pystyimme osoittamaan että LV on maantieteellisesti erittäin laajalle levinnyt. Jokaisesta tutkitusta yhdeksästä Euroopan maasta pystyttiin osoittamaan Ljungan viruksen nukleiinihappoa ainakin yhdestä jyrsijälajista. Testatuista 21 jyrsijälajista 17 lajissa osoitettiin LV nukeliinihappoa. Ensimmäistä kertaa LV löydettiin myös punamyyrästä (*Myodes rutilus*), lapinmyyrästä (*Microtus oeconomus*), kenttäsupiaisesta (*Crocidura leucodon*) sekä valaisenpäästäisestä (*Sorex antinorii*). Tulosten perusteella metsämyyrä (*Myodes glareolus*) toimii kuitenkin Ljungan viruksen pääisäntänä (nukleiinihappoprevalenssi 15.2%). Urokset sekä nuoret aikuiset metsämyyrät olivat merkittävästi useammin Ljungan virus positiivisia ja temporaalisen prevalenssin mukaan syksyllä esiintyvyys oli korkeampi kuin keväällä tai kesällä. Temporaalinen vaihtelu viittaisi siihen, että aikuisilla metsämyyrillä infektio on mahdollisesti itsestäänrajoittuva. Mielenkiintoista oli, että korkeammat sade- ja lumimäärät (vuodenajasta riippumatta) liittyivät matalampaan LV prevalenssiin seuraavan kuuden kuukauden jälkeen.

Yhteenvetona todettakoon, että LV:n ei voida näiden tutkimusten pohjalta osoittaa olevan zoonoottinen virus vaikkakin LV on laajalle levinnyt maantieteellisesti ja sitä löydetään useasta eri jyrsijälajista, myös sellaisista joiden tiedetään toimivan muiden zoonoottisten patogeenien isäntinä ja vaikka LV:n humaaniseroprevalenssin on todettu olevan 40%:n

luokkaa. Korkea humaaniseropositiivisuus voi kuitenkin viitata, että LV- tai sen kaltaiset virukset voivat ehkä levitä ihmisten keskuudessa. Koska Ljungan viruksen on kuitenkin osoitettu aiheuttavan jyrsijöillä esim. muita kuin keskushermostoon liittyviä taudinkuvia, Ljungan viruksen tai Ljunganin kaltaisten virusten tutkimusta tulisi jatkaa näiden osalta.

1 LITERATURE REVIEW

1.1 Infectious diseases and rodent-borne viruses

Infectious diseases represent an emerging global health issue and are responsible for about 25% of human deaths on a global scale (Table 1; Daszak et al. 2000). Three quarters of human infectious diseases are zoonotic, i.e. diseases transmitted from mosquitoes, ticks and wild mammals to humans (Taylor et al. 2001; Jones et al. 2008; Smith et al. 2014). About 200 zoonoses have been described thus far; some of them have been noted for centuries, others, on the contrary, have been identified only in the recent past (World Health Organization, 2017). Zoonoses are increasingly the focus of national and international health authorities. The reasons for concern are essentially two: on one hand, several zoonoses classified among emerging or re-emerging pathologies are considered possible major causes of new pandemics (e.g. severe acute respiratory syndrome; Morse et al. 2012); on the other hand, the prevention, control and treatment of zoonotic diseases entail significant and increasing economic costs. Even when they do not have serious effects on human health, zoonoses can have significant social consequences (alarm situations, collective panic) and economic consequences such as mass slaughter of livestock, collapse in consumption and exports (e.g. foot-and-mouth disease; Otte et al. 2004).

Zoonoses can be classified using different criteria. One classification, based on the nature of the etiological agent, distinguishes zoonotic diseases as viral, bacterial, mycotic and parasitic. The biological diversity found in viruses is far superior to that shown overall in bacterial, plant and animal organisms; in fact, viruses are able to parasitize all groups of known living organisms (Nasir et al. 2012). Knowledge of this diversity is the key to understanding the interaction of viruses with their hosts (Alcami and Koszinowski 2000).

Viruses have patterns of host infection that can be categorized as either acute or persistent. An acute infection (such as shown by Laine et al. 2015) is temporary in that the host's immune response quickly (within one month) eliminates the continuation of the infection in the same host by preventing replication of the virus. These viruses must find a new host

during the limited period of replication, in order to continue the infectious cycle. Persistent viral infection can be defined as that state that follows an initial period of productive infection and antiviral response of the host, in which the virus maintains the ability to replicate continuously or periodically in the same host, over months (McMahon 2014). In addition, there is no complete clearance of the virus by the host's immune system, and the replicative activity of the virus can be partially or totally suppressed for prolonged periods, even though the reactivation capacity is maintained ("latency"; Villarreal et al. 2000).

The study of zoonotic pathogens is complicated by the fact that they are almost always asymptomatic in non-human reservoir hosts, and the infection is identifiable only with a careful monitoring and surveillance plan. Small mammals, particularly rodents, host a great variety of viral pathogens. Humans can acquire the pathogen through direct contact with mammalian hosts (bites, blood, excreta; e.g. rabies virus), or indirectly through an arthropod intermediate host (e.g. tick-borne encephalitis virus). Viruses that are transmitted directly by rodents to humans are referred to as 'rodent-borne viruses'.

Rodents belong to the phylum *Chordata*, in the class *Mammalia* and order *Rodentia* (Korth 1994). They represent very suitable pathogen hosts since they are found in every continent except Antarctica (Meerburg 2015). There are about 1500 recognized extant species, many of which are opportunistic. They are mainly highly fecund with a rapid reproductive rate, becoming sexually mature after only a few weeks or months after birth (Sengupta 2013). A number of species live in and around houses and livestock buildings, feeding on crops and stored food, or contaminating them with their excreta, and making them unsuitable for consumption.

Within rodent reservoir species, transmission may occur vertically from mother to offspring (Lipsitch et al. 1996), or horizontally as a result of direct contact with infected animals in the same burrow, during aggressive encounters, or through sexual transmission (Hinson et al. 2004). For instance, the Argentinian (formerly Junin) mammarenavirus in the drylands in vesper mouse (*Calomys musculinus*) and the Seoul orthohantavirus in the Norway rat (*Rattus norvegicus)* are primarily transmitted through bite and scratch wounds (Hinson et al. 2004; Mills et al. 1997). For many viruses, the route by which animals pass the infection to each other and to other host species, including humans, remains elusive,

although many rodent-borne pathogens appear to be transmitted indirectly through secreta and excreta (saliva, urine, feces). What we do know is that transmission of infection between rodents also depends on population density and behavior, which in turn vary with availability of food and environmental conditions, such as climate and landscape configuration (Olsson et al. 2003). Consequently, the transmission of rodent-borne pathogens can be indirectly affected by ecological factors (Gubler et al. 2001).

The potentially pathogenic microorganisms carried by rodents are numerous, but not all are transmitted to or infect humans. Among rodent-borne viruses, those of the genera *Mammarenavirus*, *Orthohantavirus*, and *Orthopoxvirus* are a particular focus of study both in humans and animals, since they represent some of the most widespread rodent-borne disease-causing pathogens (Wilson and Peters 2014; Oldal et al. 2015; Bergstedt Oscarsson et al. 2016). There are also viruses that cause a variety of illnesses, ranging from asymptomatic infection to severe illness and death in humans, known to infect rodents, but their transmission from rodents to humans has not been proven. For instance, members of the *Parechovirus* genus occur worldwide, show genetic heterogeneity and different types are associated with different clinical diseases (Harvala and Simmonds 2009; Abedi et al. 2018); closely related species are also found in rodents, but the role of rodents as vectors of parechoviruses is unestablished.

Table 1: *Overview of viral pathogens that are transmitted from rodents to humans, disease associations, host species, and geographic distribution (https://www.cdc.gov/).*

Viral agent	**Associated human diseases**	**Rodent host species**	**Role of the rodent species***	**Geographic distribution**
Mammarenaviruses	South American arenaviruses	Cane rat (*Zygodontomys brevicauda*), drylands vesper mouse, (*Calomys musculinus*), large vesper mouse (*C. callosus*)	Carrier	South America: Argentina, Bolivia, Venezuela and Brazil
Lassa virus (*Mammarenavirus*)	Lassa fever	Natal multimammate mouse *(Mastomys natalensis)*	Carrier	Western Africa
Lymphocytic choriomeningitis virus (*Mammarenavirus*)	Lymphocytic choriomeningitis	House mouse (*Mus musculus*)	Reservoir	Worldwide
New-World orthohantaviruses	Hantavirus cardiopulmonary syndrome	Deer mouse (*Peromyscus maniculatus*), cotton rat (*Sigmodon hispidus*), rice rat (*Oryzomys palustris*), white-footed mouse (*P. leucopus*)	Carrier	North and South America
Old-World orthohantaviruses	Hemorrhagic fever with renal syndrome	Striped field mouse (*Apodemus agrarius*), brown or Norway rat (*Rattus norvegicus*), bank vole (*Myodes glareolus*), yellow-necked field mouse (*Apodemus flavicollis*)	Carrier	Eastern Asia, Russia, Korea, Scandinavia, western Europe, and the Balkans
California encephalitis orthobunyavirus (*Orthobunyavirus*)	La Crosse encephalitis	Eastern chipmunks (*Tamias striatus*), eastern gray squirrels (*Sciurus carolinensis*)	Reservoir	North America
Colorado tick fever virus (*Coltivirus*)	Colorado tick fever	Golden-mantled ground squirrel (S*permophilus lateralis*), least chipmunk (*Tamias minimus*), Columbian ground squirrel (*Citellus columbianus columbianus*), yellow pine chipmunk (*Eutamias amoenus*), porcupine (*Erethizon dorsatum*), deer mouse, busy tailed wood rat (*Neotoma cinerea*)	Reservoir	Canada and the western United States
Omsk hemorrhagic fever virus (*Flavivirus*)	Omsk hemorrhagic fever	Muskrat (*Ondatra zibethica*), water vole (*Arvicola terrestris*), narrow-skulled voles (*Microtus gregalis*)	Carrier/Reservoir	Western Siberia

Powassan virus (*Flavivirus*)	Powassan virus disease	Red squirrels (*Tamiasciurus hudsonicus*), chipmunks (*Tamias amoenus*), groundhogs (*Marmota monax*), white-footed mice	Reservoir	North America and Russian Far East
Tick-borne encephalitis virus (*Flavivirus*)	Tick-borne encephalitis	Bank vole, field mouse (*Apodemus agrarius*), red voles (*Myodes rutilus Schreber*)	Reservoir	Russia, China, eastern, central and western Europe, Scandinavia
West Nile virus (*Flavivirus*)	West Nile virus disease	Fox squirrels (*Sciurus niger*), eastern gray squirrels (*S. carolinensis*), western gray squirrels (*S. griseus*), eastern chipmunks (*T. striatus*)	Reservoir	United States

*Reservoir: rodents harbor pathogens and thus serve as potential sources of disease outbreaks, but always via an intermediate host.
Carrier: rodents pass a pathogenic agent from a vertebrate host to another. Carriers show almost no symptoms of a disease.

Humans can come into contact with rodents and acquire infections in multiple ways; depending on their occupation and the area where they are resident or spend their free time (Vaheri et al. 2013). Activities that may bring people in contact with contaminated rodent excreta include forestry work, clearing out a woodshed and sweeping the floor, cleaning up excreta when entering a holiday cabin, and some farming practices (Rose et al. 2003; Tagliapietra et al. 2018). This is because the risk of inhalation of aerosolized, virus-contaminated urine, feces, or saliva in outdoor areas and in poorly aired living spaces is high. Reducing contact with rodents and their excreta is the main solution for preventing virus transmission. For example, sealing living areas against rodent infestation, keeping indoor areas clean, emptying trash bins regularly, using traps or poisons to eliminate rodents, and wearing a mask and gloves while sweeping excreta-contaminated areas, all help to reduce rodent infestation and risk of exposure (Vaheri et al. 2013).

Infections with a rodent-borne virus may lead in some cases to long-term effects in humans, with outcomes that range from mild, temporary symptoms to severe and even fatal disease (Hjelle and Torres-Perez 1976). Mortality is higher where treatment is unavailable, especially in developing countries, or when a patient's immune response is weak, as may happen in the young, elderly, pregnant or immunocompromised. The role or potential role of rodents in the transmission of viral pathogens to humans makes the ecology of rodent populations of particular importance for human health and risk assessment. For many years, public health employees have been collaborating with ecologists to determine the factors leading to infections in rodent hosts and humans, and how to prevent them (Burroughs and Knobler 2002). However, little is known about the prevalence of some of these viruses in rodent hosts or humans, the transmission of these viruses from rodents to humans, or the effect of co-infection of several rodent-borne or potential of rodent-borne viruses on disease symptoms in humans.

1.1.1 Mammarenaviruses (arenaviruses)

The *Mammarenavirus* genus belongs to the *Arenaviridae* family whose members are generally associated with rodent-borne zoonotic diseases. Thus far, 40 species of mammarenaviruses have been recognized (Gonzalez et al. 2007; ICTV Virus Taxonomy, http://www.ictvonline.org/) (Table 2), and classified into two groups according to

antigenic properties (Wulff et al. 1978). Old World viruses include lymphocytic choriomeningitis virus (LCMV); Lassa and Mopeia viruses, infecting rodents in the Eastern hemisphere. Instead, the New World mammarenaviruses, Tacaribe and Argentinian viruses, have been found in rodents in the Western hemisphere (Bowen et al. 1996).

Each mammarenavirus strain is associated with a specific rodent host that acts as a natural reservoir for the virus. Their genetic material consists of a bi-segmented negative single-stranded RNA, and it is divided into two subgenomic segments, L (large, about 7.5 kb) and S (small, about 3.5 kb) (Figure 1). Both segments consist of two non overlapping open reading frames (ORFs), with different polarity, which use an ambisense coding strategy to direct the synthesis of two polypeptides opposite in orientation. The S RNA encodes for the structural components, while the L segment for the non-structural components (Emonet et al. 2011). The diameter of the enveloped particles ranges from 50 to 300 nm with a surface layer 8–10 nm in lenght (Salvato et al. 2011). Mammarenaviruses replicate with minimal perturbation of the host cells. The virus enters the cell through endosomes; the gene transcription occurs in the cytoplasm of the host cell after viral uncoating and genome release. The transcription starts at the 3' end of the two RNA segments (S and L). The nucleoprotein (NP) and the RNA dependent RNA polymerase (L polymerase) are translated from mRNAs with antigenomic sense polarity, which is transcribed directly from the viral RNAs. The ORFs for both the viral glycoprotein precursor and the finger Z protein are located, correspondingly, at the 5′ end of the S and L genome segments, and are translated from mRNA transcribed from the complementary RNAs. Newly synthesized vRNAs are packaged into infectious virions by interaction of the viral Z protein. Newly synthesized and assembled virions bud through the plasma membrane of infected cells without causing cell lysis, through a process mediated by the Z protein.

Mammarenavirus infections are currently confirmed using two diagnostic laboratory methods. Detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) allows a rapid and sensitive diagnosis. Antibodies against virus particles can be detected by an indirect immunofluorescence assay (IFA), or IgG- and IgM-ELISA (enzyme-linked immunosorbent assay).

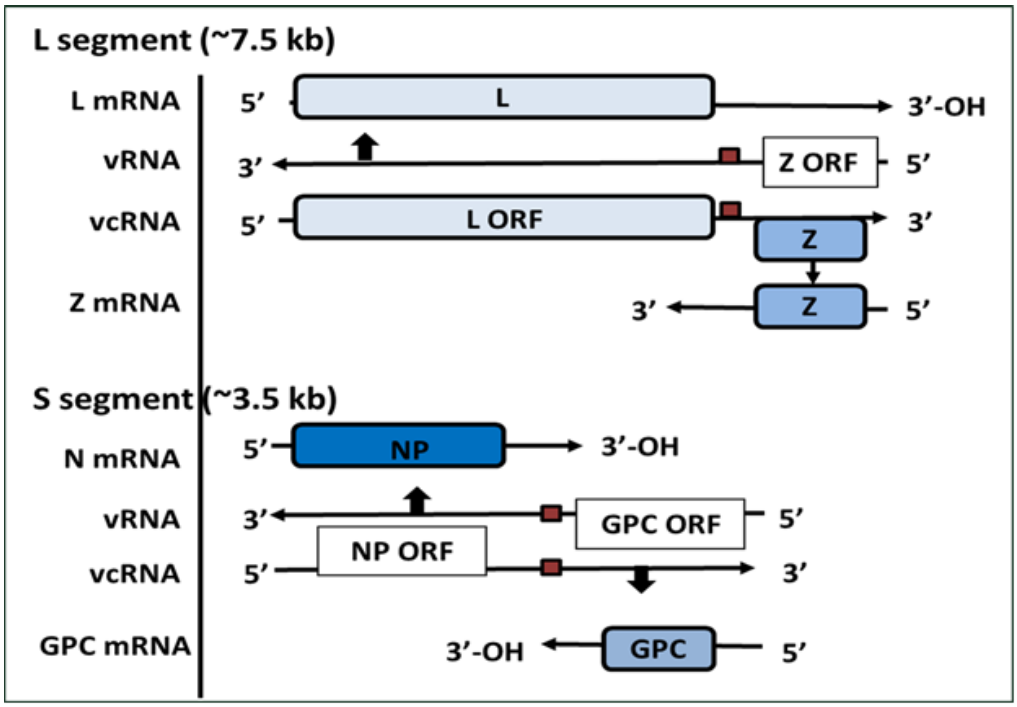

Figure 1. *Schematic representation of the mammarenavirus genome. The L (large) segment encodes the viral RNA dependent RNA polymerase (RdRp, or L polymerase), and a small zinc-binding protein (Z), whereas the S (small) RNA encodes the viral glycoprotein precursor, GPC, and the nucleoprotein, NP. vRNA, viral RNA; cRNA, complementary RNA; ORF, open reading frame.*

Humans become infected with mammarenaviruses by inadvertently inhaling aerosolized rodent excreta and secreta (aerosol transmission), by contact with food contaminated with rodent droppings (oral transmission), or by direct contact of abraded skin with infected rodent droppings. Infected humans are generally asymptomatic or show mild flu-like symptoms, but the viruses can also cause aseptic meningitis, encephalitis and congenital abnormalities, especially in immunocompromised individuals. In the USA, there is a 5% seroprevalence in human population (Peters 2006). Infected rodents do not show signs of illness despite being long term or chronic carriers of the virus (Tagliapietra et al. 2009).

LCMV was the first virus in this family to be recognized as causal agent of a rodent-borne disease, causing a meningitis outbreak in 1933 in California (Wynns and Hawley 1939). Historically, LCMV was classified as an Old-World mammarenavirus, but currently it is found in both Eastern and Western hemispheres. It is also the only mammarenavirus reported in Europe; however, data on its incidence and epidemiology are lacking. In humans, antibodies against LCMV have been found in Spain (Lledó et al. 2003), the Netherlands (Elbers et al. 1999) and Italy (Kallio-Kokko et al. 2006). In 2006, a preliminary study showed an overall antibody prevalence of 5.6% in wild rodents caught in the province of Trento (Northern Italy): 6.1% in the yellow-necked mouse (*Apodemus flavicollis*), 3.3% in the bank vole (*M. glareolus*) and 14.3% in the common vole (*Microtus arvalis*). Another study showed that 2.5% of forestry workers in Italy had anti-LCMV antibodies (Kallio-Kokko et al. 2006). A follow-up study conducted in northern Italy determined the seroprevalence of LCMV in small mammals in that area (Tagliapietra et al. 2009): LCMV was found again in the most common and widespread species of wild rodents in the area (*A. flavicollis, M. glareolus* and *M. arvalis*), with an overall prevalence of 6.8%. The long-term dynamics of LCMV in a population of yellow-necked mice in the Province of Trento (Italy) was also studied from 2000 until 2006. The intensive monitoring of LCMV in that population showed a positive correlation between the virus seroprevalence and the density of rodents. Body weight and sex of the animals were also correlated with the antibody prevalence, suggesting that the horizontal transmission of LCMV occurs especially among older and heavier males, most likely due to their aggressive interactions.

Table 2. *Arenaviridae: genera and species approved to date, as of July 2017 (http://www.ictvonline.org/), disease(s) associations (if any), host species, and the year of taxonomic assignment.*

Genus	Species	Associated diseases	Host	Year of approval
Mammarenavirus	Allpahuayo mammarenavirus	To be determined	Rodents	2004
	Argentinian mammarenavirus	Argentine hemorrhagic fever	Human, rodents	1958
	Bear Canyon mammarenavirus	To be determined	Rodents	2004
	Brazilian mammarenavirus	Brazilian hemorrhagic fever	Human	1995
	Cali mammarenavirus	To be determined	Human, rodents	1975
	Chapare mammarenavirus	Chapare hemorrhagic fever	Human	2009
	Cupixi mammarenavirus	To be determined	Rodents	2004
	Flexal mammarenavirus	To be determined	Rodents	1991
	Gairo mammarenavirus	To be determined	Rodents	2015
	Guanarito mammarenavirus	Venezuelan hemorrhagic fever	Human, rodents	1995
	Ippy mammarenavirus	To be determined	Rodents	1991
	Lassa mammarenavirus	Lassa hemorrhagic fever	Human, rodents	1979
	Latino mammarenavirus	To be determined	Rodents	1975
	Loei River mammarenavirus	To be determined	Rodents	2016
	Lujo mammarenavirus	Viral hemorrhagic fever	Human	2009
	Luna mammarenavirus	To be determined	Rodents	2012
	Lunk mammarenavirus	To be determined	Rodents	2014
	Lymphocytic choriomeningitis mammarenavirus	Lymphocytic choriomeningitis	Human, rodents	1971
	Machupo mammarenavirus	Bolivian hemorrhagic fever	Monkeys, human, rodents	1971
	Mariental mammarenavirus	To be determined	Rodents	2015
	Merino Walk mammarenavirus	To be determined	Rodents	2014
	Mobala mammarenavirus	To be determined	Rodents	1991
	Mopeia mammarenavirus	To be determined	Rodents	1991
	Okahandja mammarenavirus	To be determined	Rodents	2015
	Oliveros mammarenavirus	To be determined	Rodents	1999
	Paraguayan mammarenavirus	To be determined	Rodents	1971
	Pirital mammarenavirus	To be determined	Rodents	1999
	Ryukyu mammarenavirus	To be determined	Rodents	2017
	Serra do Navio mammarenavirus	To be determined	Rodents	1971
	Solwezi mammarenavirus	To be determined	Rodents	2016
	Souris mammarenavirus	To be determined	Rodents	2017

	Tacaribe mammarenavirus	To be determined	Bats, rodents	1971
	Tamiami mammarenavirus	To be determined	Rodents	1971
	Wenzhou mammarenavirus	To be determined	Rodents	2015
	Whitewater Arroyo mammarenavirus	Hemorrhagic fever with liver failure	Human, rodents	1999
Reptarenavirus	California reptarenavirus	To be determined	Snakes	2014
	Giessen reptarenavirus	To be determined	Snakes	2017
	Golden reptarenavirus	To be determined	Snakes	2014
	Ordinary reptarenavirus	To be determined	Snakes	2017
	Rotterdam reptarenavirus	To be determined	Snakes	2014

1.1.2 Orthohantaviruses (hantaviruses)

In 2017, the *Bunyaviridae* family was reclassified by the ICTV as *Bunyavirales*, a complete new order of viruses. The viruses belonging to the *Bunyaviridae* family were reassigned to nine new families: *Hantaviridae, Feraviridae, Fimoviridae, Jonviridae, Nairoviridae, Peribunyaviridae, Phasmaviridae, Phenuiviridae,* and *Tospoviridae.* Members of the *Hantaviridae* family are the emerging pathogens of the highest public interest. The only genus in the *Hantaviridae* family is *Orthohantavirus*, which includes 41 species (Table 3). The genus consists of negative-sense, single-stranded enveloped RNA viruses, with a diameter of 80 to 110 nm. The genome consists of three segments named S (small), M (medium), and L (large) (Figure 2). The L RNA encodes the RNA-dependent RNA polymerase (RdRp, or L protein) that functions as the viral replicase and helicase, and is associated with the ribonucleocapsids. The M RNA encodes the glycoproteins G_n and G_c, associated with the lipid membrane, interacting with the receptors on host cell surfaces. The S RNA encodes the nucleocapsid (N) protein, which encapsulates the viral RNA (vRNA), and regulates the replication and transcription phases. The N protein together with the vRNA form the ribonucleocapsids, which in turn are used as the template by viral L protein for the synthesis of viral mRNA and replication of the viral genome (Cheng et al. 2014).

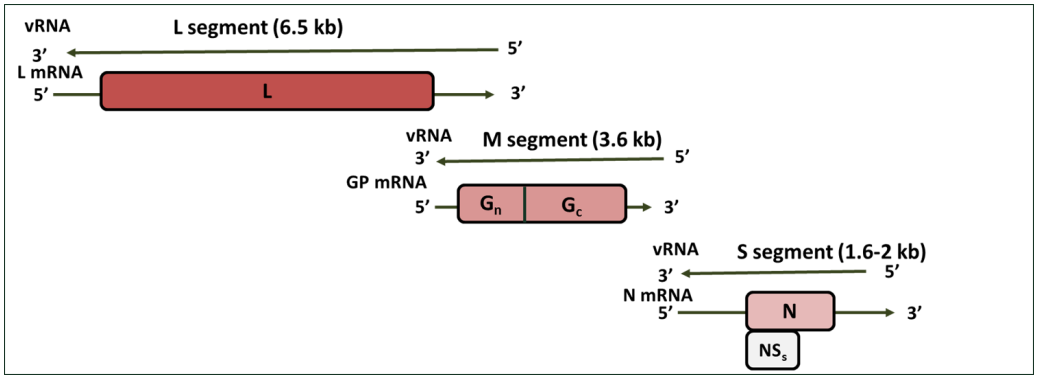

Figure 2. *Schematic representation of the orthohantaviruses genome. The L (large) segment encodes the viral RNA dependent RNA polymerase (L polymerase), the M (medium) segment encodes two glycoproteins Gn and Gc, whereas the S (small) RNA encodes the nucleoprotein, N. vRNA, viral RNA; GP, glycoprotein; NS, nonstructural protein.*

Orthohantaviruses are also classified as New-World and Old-World. New-World orthohantaviruses were first described in 1993 in the Four Corners region, in the south-western USA (Nichol et al. 1993). They cause hantavirus cardiopulmonary syndrome (HCPS) in humans. Infection may lead to acute respiratory failure with a mortality rate of about 40%. Old-World orthohantaviruses are found mainly in Europe and Asia, and are known to cause hemorrhagic fever with renal syndrome (HFRS), with a mortality rate of 0.1-15% (Goeijenbier et al. 2013, Vaheri et al. 2013). Symptoms of HFRS are fever, myalgia, and abdominal pain, vomiting and back pain, followed by face reddening and rashes, then renal symptomatology. The severity of the clinical picture ranges from asymptomatic to fatal (Vaheri et al. 2008). The most common form of HFRS in Europe is represented by the nephropathia epidemica (NE) caused by Puumala virus (PUUV), hosted by bank voles. The clinical picture of PUUV infection varies, but is mostly characterized by fever, headache, muscle pain, nausea and vomiting. The acute phase includes anemia, leukocytosis, thrombocytopenia, elevated liver enzyme and increase in the serum creatinine and C-reactive protein levels, as well as renal involvement with transient proteinuria, hematuria, and oliguric acute kidney injuries followed by polyuria.

In general, PUUV infections occur in northern and central Europe, in the Balkans and in Russia, within the distribution area of *M. glareolus*, which represents the most widely distributed reservoir of rodent-borne disease in Europe. More than 9000 cases of HRFS are reported annually from these areas, but only a relatively low number of cases require hospitalization (Avsic-Zupanc et al. 1994). PUUV infections are particularly common in Finland, where 1000-3000 cases of PUUV infections are detected annually, with an overall seroprevalence of 5% (Vapalahti et al. 2003). The most recent surveillance data on PUUV infection conducted in Finland covered the period of 1995-2008. Makary and colleagues (2010) reported an average annual PUUV incidence rate of 31 cases/100000 population, with a higher incidence in males than in females, and the highest incidence in groups of people aged 35-49 and 50-64. In Finland, the highest rate of hospitalization due to PUUV infection as primary diagnosis is Kymenlaakso (Makary et al. 2010). However, the true incidence of PUUV infections in Finland may be underestimated, since the data are based only on the cases reported by physicians after confirmed laboratory testing (Brummer-Korvenkontio et al. 1999; Vapalahti et al. 1999; Vaheri et al. 2008).

Humans represent accidental hosts of orthohantaviruses, and become infected through direct contact (in case of skin lesions) or through inhalation of infected rodent excreta (Reusken and Heyman 2013). *Hantaviridae* represent the only family of viruses within the *Bunyavirales* order not associated with an arthropod vector (Elliott 1997). Each strain is usually closely associated with a single species of rodent or insectivore, the result of co-evolution between the virus and the host. Therefore, it is not surprising that the ecology and geographical distribution of orthohantaviruses are correlated with the spread of their natural reservoir (Plyusnin and Morzunov 2001; Klempa 2009). The reservoirs remain chronically infected by the virus throughout their life cycle (Klein and Calisher 2007). Dogs, cats, rabbits and guinea pigs, which have been in contact with infected rodents, have been found seropositive to orthohantaviruses, but their role as disease agents in these domestic species does not seem to be significant (Escutenaire and Pastoret 2000).

Acute PUUV infection is routinely diagnosed using serological tests, i.e. by detection of IgG and IgM antibodies using IFA or ELISA. In addition, both nested RT-PCR and reverse transcriptase real-time quantitative PCR (RT-qPCR) can be used to detect orthohantavirus infections in both humans and rodent hosts, but they are not commonly used in routine diagnosis. Test results can be confirmed by immunoblotting, Sanger sequencing and/or virus isolation (Vaheri et al. 2008).

Table 3. Hantaviridae genus and species approved to date, as of July 2017 (http://www.ictvonline.org/), disease(s) associations (if any), host species, and the year taxonomic assignment.

Genus	Species	Associated diseases	Host	Year of approval
Orthohantavirus	Amga orthohantavirus	To be determined	Rodents	2016
	Andes orthohantavirus	Hantavirus cardiopulmonary syndrome	Human, rodents	1999
	Asama orthohantavirus	To be determined	Rodents	2016
	Asikkala orthohantavirus	To be determined	Rodents	2016
	Bayou orthohantavirus	Hantavirus pulmonary syndrome	Human, rodents	1999
	Black Creek Canal orthohantavirus	Hantavirus pulmonary syndrome	Human, rodents	1999
	Bowe orthohantavirus	To be determined	Shrews	2016
	Bruges orthohantavirus	To be determined	Moles	2016
	Cano Delgadito orthohantavirus	To be determined	Rodents	1999
	Cao Bang orthohantavirus	To be determined	Rodents	2016
	Choclo orthohantavirus	Hantavirus pulmonary syndrome	Human, rodents	2016
	Dabieshan orthohantavirus	To be determined	Rodents	2016
	Dobrava-Belgrade orthohantavirus	Hantavirus hemorrhagic fever with renal syndrome	Human, rodents	1999
	El Moro Canyon orthohantavirus	Hantavirus pulmonary syndrome	Human, rodents	1999
	Fugong orthohantavirus	To be determined	Rodents	2016
	Fusong orthohantavirus	To be determined	Rodents	2016
	Hantaan orthohantavirus	Hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome	Human, rodents	1987
	Imjin orthohantavirus	To be determined	Rodents	2016
	Jeju orthohantavirus	To be determined	Shrews	2016
	Kenkeme orthohantavirus	To be determined	Shrews	2016
	Khabarovsk orthohantavirus	To be determined	Rodents	1999
	Laguna Negra orthohantavirus	To be determined	Rodents	1999
	Laibin orthohantavirus	To be determined	Rodents	2016
	Longquan orthohantavirus	To be determined	Rodents	2016
	Luxi orthohantavirus	To be determined	Rodents	2016
	Maporal orthohantavirus	To be determined	Rodents	2016
	Montano orthohantavirus	To be determined	Rodents	2016
	Necocli orthohantavirus	To be determined	Rodents	2016
	Nova orthohantavirus	To be determined	Moles	2016

	Oxbow orthohantavirus	To be determined	Shrews	2016
	Prospect Hill orthohantavirus	To be determined	Rodents	1987
	Puumala orthohantavirus	Haemorrhagic fever with renal syndrome	Human, rodents	1987
	Quezon orthohantavirus	To be determined	Bats	2016
	Rockport orthohantavirus	To be determined	Moles	2016
	Sangassou orthohantavirus	To be determined	Rodents	2012
	Seoul orthohantavirus	Hantavirus hemorrhagic fever with renal syndrome	Human, monkeys, rodents	1987
	Sin Nombre orthohantavirus	Hantavirus cardiopulmonary syndrome	Human, rodents	1999
	Thailand orthohantavirus	To be determined	Rodents	1995
	Thottapalayam orthohantavirus	To be determined	Shrews	1991
	Tula orthohantavirus	Hemorrhagic fever with renal syndrome	Human, rodents	1999
	Yakeshi orthohantavirus	To be determined	Rodents	2016

1.1.3 Orthopoxviruses

The *Orthopoxvirus* (OPV) is a genus within *Poxviridae*, a vast family of large DNA viruses (200-400 nanometers in diameter), which include some of the most deadly viruses known to humans (e.g. variola virus, which causes smallpox; Table 4).

The genome of poxviruses consists of a linear, double-stranded DNA that ranges in size from 130 to 360 kb and has a highly conserved organization and gene content. These viruses encode numerous proteins involved in DNA replication and mRNA synthesis (Moss 2001). The central portion contains genes for replication, conserved within species, while the two flanking portions carry factors responsible for virulence and species-specificity. The extremities of the genome, called the inverted terminal repeats, consist of sequences repeated in tandem covalently-closed by two hairpin loops (or terminal loops) (Mercer et al. 1987) (Figure 3). Poxviruses are the only viruses to have an RNA polymerase very similar to the one of eukaryotic cells, which allows them to replicate in the cytoplasm of the host cells without the need to interact with the nucleus (McFadden 2005). Poxvirus replication occurs with the help of enzymes encoded and expressed by the virus during its replicative cycle. Viral polymerases play a central role in both viral genome replication and transcription, while the translation of proteins happens in the viral translation apparatus.

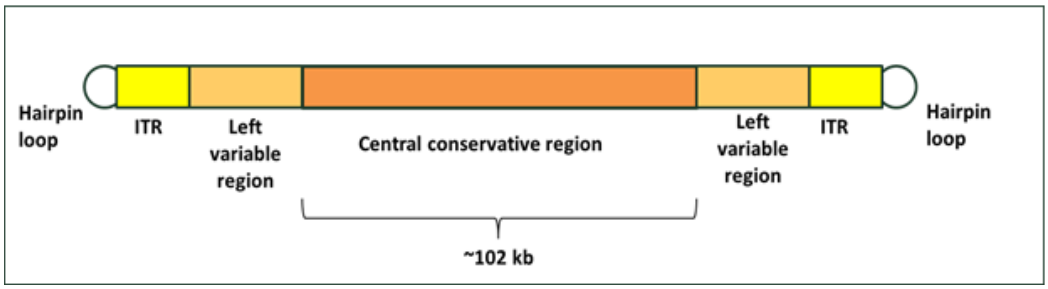

Figure 3. *Schematic representation of the poxvirus genome. ITR, inverted terminal repeat.*

Transmission of poxvirus infections from animals to humans occurs mainly through abrasions, cuts or bites. Only three genera of poxviruses include zoonotic viruses for humans: *Parapoxvirus* (PPV), *Yatapoxvirus* (YPV) and *Orthopoxvirus* (OPV) (Lewis-Jones 2004). All the viral species belonging to the PPV genus (*Bovine papular stomatitis virus, Orf virus, Parapoxvirus of red deer in New Zealand* and *Pseudocowpox virus*) are transmissible to humans from domestic and wild ruminants, and are therefore zoonotic agents, although humans appear to represent accidental hosts. These viruses cause mainly occupational zoonoses, which affect particular categories of workers who come in direct contact with infected animals (Essbauer et al. 2010). For *Orf virus* or *Pseudocowpox virus* infection, transmission to humans can also occur via fomites (Haig and Mercer 1998). Instead, infection caused from the YPV species *Tanapox virus* occurs in Africa and is transmitted to humans by direct contact with non-human primates, which also act as a reservoir of the virus (Lewis-Jones 2004), although a role of insect vectors has also been hypothesized (Downie et al. 1971).

Infections caused by viruses in the OPV genus are the most studied. These viruses cause skin lesions, often associated with lymphadenopathy and febrile symptoms (Lewis-Jones 2004; Pelkonen et al. 2003). After several weeks, vesicles and pustules appear, followed by scabs and scars (Baxby and Bennett 1997). Complications are mostly related to secondary bacterial infections or immunodeficiency of the infected patient (Tan et al. 1991). Of the OPV species, *Monkeypox virus* causes an endemic infection in Africa, in particular in Zaire and Congo, and mainly in children. It can be transmitted zoonotically but also by inhalation and, currently, this is the only zoonotic poxvirus to be transmitted also from human to human (Di Giulio et al. 2004). The *Variola virus* is the only member of the OPV genus for which humans are the only receptive host. Finally, the *Vaccinia virus* infects humans usually through cattle (Damaso et al. 2000), and the vaccinia-like viruses such as *Buffalopox virus*, *Cantagalo virus* and *Araçatuba virus* cause a high number of infections in humans, and are indeed considered emerging zoonosis being transmitted from cattle to humans (Kolhapure et al. 1997; Damaso et al. 2000; de Souza Trindade et al. 2003; Nagasse-Sugahara et al. 2004).

Among the main OPVs responsible for zoonoses, the Cowpox virus (CPXV) is the most known member of the genus, since it is distributed throughout western Europe and

presents by far the widest host spectrum among the poxviruses, infecting humans, cattle, horses, dogs, cats and in particular small rodents, such as *M. glareolus*, *A. flavicollis* (Essbauer et al. 2010), and the Norway rat (Wolfs et al. 2002). Rarely, the infection is transmitted to humans from cattle, and more frequently from cats (Baxby et al. 1994), particularly in Great Britain where CPVX infections of the domestic cat have become the most frequently diagnosed CPVX infection (Bennett et al. 1986). A recent genomic study of many CPXV isolates suggests that CPXV is polyphyletic and further studies are needed to determine if the five clades identified by Mauldin et al. (2017) can be considered species.

The diagnosis of zoonotic poxvirus infections in humans is initially based on clinical and anamnestic data, followed by laboratory investigations such as electron microscopy, and PCR, which allows the identification of the viral species (Lewis-Jones 2004). There is evidence of cross-protection between virus species, but not genera. Robinson and Mercer (1988) also confirmed the immunological diversity between OPV and PPV in lambs by showing that there is no cross-protection between *Orf virus* (PPV genus) and *Vaccinia virus* (OPV genus).

Table 4. *Poxviridae: subfamilies, genera and species approved to date, as for July 2017 (http://www.ictvonline.org/), disease(s) associations (if any), host species, and the year of assignment to taxonomy.*

Subfamily	Genus	Species	Associated diseases	Host	Year of approval
Chordopoxvirinae	Avipoxvirus	Canarypox virus	Canarypox	Birds	1971
		Fowlpox virus	Fowlpox	Chickens, turkeys	1971
		Juncopox virus	To be determined	Birds	1971
		Mynahpox virus	To be determined	Birds	1995
		Pigeonpox virus	Pigeonpox	Birds	1971
		Psittacinepox virus	To be determined	Parrots	1971
		Quailpox virus	To be determined	Quails	1976
		Sparrowpox virus	To be determined	Birds	1971
		Starlingpox virus	To be determined	Birds	1971
		Turkeypox virus	Turkeypox	Turkeys	1971
	Capripoxvirus	Goatpox virus	Goatpox	Goats	1971
		Lumpy skin disease virus	Lumpy skin disease	Cattle	1971
		Sheeppox virus	Sheeppox	Sheeps	1971
	Centapoxvirus	Yokapox virus	To be determined	Rodents	2016
	Cervidpoxvirus	Mule deerpox virus	To be determined	Deers	2008
	Crocodylidpoxvirus	Nile crocodilepox virus	Nodular skin lesions in young animals	Crocodiles	2011
	Leporipoxvirus	Hare fibroma virus	Hare fibroma	Hares	1971
		Myxoma virus	Myxomatosis	Rabbits	1971
		Rabbit fibroma virus	Rabbit fibroma	Rabbits	1971
		Squirrel fibroma virus	Squirrelpox	Squirrels	1971
	Molluscipoxvirus	Molluscum contagiosum virus	Molluscum contagiosum	Human	1971
	Orthopoxvirus	Camelpox virus	Camelpox	Camels, human	1971
		Cowpox virus*	Cowpox	Monkeys, human, dogs, domestic cats, wild cats, elephants, horses, tapirs, rhinoceros, cattle, rodents, red pandas, okapis, mongooses	1971
		Ectromelia virus	Mousepox	Rodents, human	1971
		Monkeypox virus	Monkeypox	Human, monkeys, rodents	1971
		Raccoonpox virus	To be determined	Racoons	1979
		Skunkpox virus	To be determined	Monkeys, skunks	2011
		Taterapox virus	To be determined	Rodents	1991

		Vaccinia virus	Smallpox	Human, monkeys, cows, horses, rodents	1971
		Variola virus	Smallpox	Human	1971
		Volepox virus	To be determined	Monkeys, rodents	1995
	Parapoxvirus	Bovine papular stomatitis virus	Bovine papular stomatitis	Human, cattle	1971
		Orf virus	Orf disease	Human, sheep, goats, camels	1971
		Parapoxvirus of red deer in New Zealand	Skin infection	Human, red deers	1995
		Pseudocowpox virus	Pseudocowpox	Human, cattle	1971
	Suipoxvirus	Swinepox virus	Swinepox	Pigs	1971
	Unassigned	Pteropox virus	To be determined	Bats	2016
		Squirrelpox virus	Squirrelpox	Squirrels	1999
	Yatapoxvirus	Tanapox virus	Tanapox	Human, monkeys	1971
		Yaba monkey tumor virus	Yaba money tumor	Human, monkeys	1976
Entomopoxvirinae	Alphaentomopoxvirus	Anomala cuprea entomopoxvirus	To be determined	Cupreus chafer	1976
		Aphodius tasmaniae entomopoxvirus	To be determined	Blackheaded pasture cockchafer	1976
		Demodema bonariensis entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Dermolepida albohirtum entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Figulus sublaevis entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Geotrupes sylvaticus entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Melolontha melolontha entomopoxvirus	To be determined	Common cockchafer	1976
	Betaentomopoxvirus	Acrobasis zelleri entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Adoxophyes honmai entomopoxvirus	To be determined	Summer fruit tortrix	2014
		Amsacta moorei entomopoxvirus	To be determined	Asian Gypsy Moth, saltmarsh caterpillar, tiger moths	1976
		Arphia conspersa entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1991

		Choristoneura biennis entomopoxvirus	To be determined	Moths	1976
		Choristoneura conflicta entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Choristoneura diversuma entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Choristoneura fumiferana entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1999
		Choristoneura rosaceana entomopoxvirus	To be determined	Oblique banded leaf roller	2014
		Chorizagrotis auxiliaris entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Heliothis armigera entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1999
		Locusta migratoria entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1991
		Mythimna separata entomopoxvirus	To be determined	Northern armywor	2014
		Oedaleus senegalensis entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1991
		Operophtera brumata entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1976
		Schistocerca gregaria entomopoxvirus	To be determined	Butterflies, moths, grasshoppers, locusts and crickets	1991
	Gammaentomopoxvirus	Aedes aegypti entomopoxvirus	To be determined	Flies	1976
		Camptochironomus tentans entomopoxvirus	To be determined	Flies	1976
		Chironomus attenuatus entomopoxvirus	To be determined	Flies	1976
		Chironomus luridus entomopoxvirus	To be determined	Flies	1976
		Chironomus plumosus entomopoxvirus	To be determined	Flies	1976
		Goeldichironomus holoprasinus entomopoxvirus	To be determined	Chironomids	1976

	Unassigned	Diachasmimorpha entomopoxvirus	To be determined	Longtailed fruit fly parasite	2005
		Melanoplus sanguinipes entomopoxvirus	To be determined	Migratory locust, desert locust, grasshoppers	1976

*polyphyletic, therefore, currently under review.

1.2 Picornaviruses

Family *Picornaviridae* includes positive-sense, single-stranded RNA viruses with monopartite genome. To date, picornaviruses account for 94 species grouped into 40 genera (http://www.ictvonline.org/) (Table 5). Viruses belonging to this family can cause a wide variety of diseases in humans and other vertebrates, affecting the respiratory and gastrointestinal tract, central nervous system, heart, skeletal muscle and liver. The most studied species contain viruses that are pathogenic to mammals and birds and associated with human and livestock diseases (Lewis-Rogers 2008; Lewis-Rogers and Crandall 2010). Interestingly, this family contains viruses that can be found in almost all types of environment, in a great variety of different host species, which include humans, other mammals and birds, but also ectotherms such as amphibians (Reuter et al. 2015) and fish (Barbknecht et al. 2014; Mayo and Pringle 1998). Picornaviruses are mainly species-specific, but the constant increase in the number of picornavirus genera indicates that the diversity of *Picornaviridae* and their hosts is far from being fully delineated.

In humans, picornaviruses include causative agents of respiratory, cardiac, liver, neurological and systemic pathologies, which are among the most common cause of infections in developed countries (Rotbart 2002; Whitton et al. 2005; Chuchaona et al. 2017). Transmission mode is human-to-human, but the routes are diverse: picornaviruses can be transmitted to humans via the fecal-oral route, by indirect inhalation or even by direct contact between two infected individuals (Whitton et al. 2005). Several picornaviruses have been proposed to be zoonotic, but this has neither been proven nor widely accepted (e.g. Kriegshäuser et al. 2005; Wu et al. 2012; see below for LV).

The genome of picornaviruses is approximately 7000-9500 nucleotides long, enclosed in a capsid of about 30 nm diameter, with a general organization preserved in all picornavirus genera, with a few exceptions (Figure 4). The genome contains a single open reading frame (ORF) (Johansson et al. 2002), and the first 335-1199 bases (the length may vary according to the species) represent the 5'-untranslated region (UTR). Viral genomic RNA has a viral protein (VPg) covalently bound to the 5´-terminus, and an internal ribosome entry site (IRES), a genomic region used for initiation of internal translation. The 5'-UTR folds into a

more complex secondary structure (Doherty et al. 1999). The single ORF encodes a long polyprotein, which can be divided into three main segments: P1, encoding capsid proteins VP1-4 (and in some genera also for protein L, which precedes the structural proteins); and P2 and P3 regions, which encode non-structural (or replication) proteins 2A-C and 3A-D, respectively. The final section of the genome is 3'-UTR, consisting of 47 to 125 bases (depending on the species) followed by a short poly(A) tail at the 3' end.

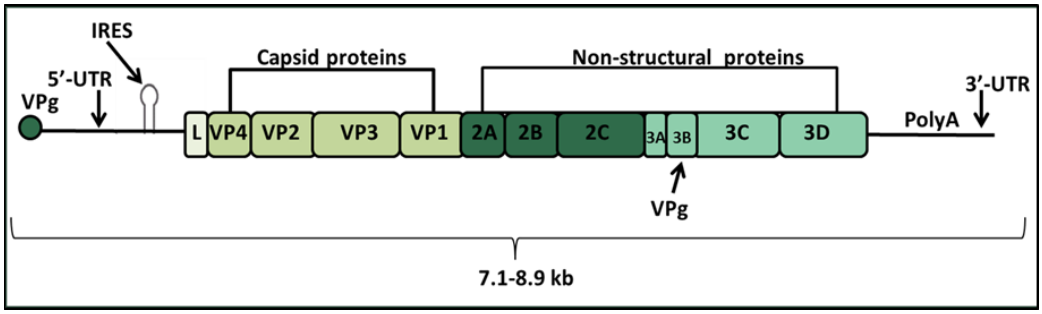

Figure 4. *Schematic representation of the picornavirus genome. The regions of the genome coding for the respective proteins are highlighted. VPg, viral protein genome-linked; UTR, untranslated region; IRES, internal ribosome entry site.*

Table 5. *Picornaviridae: genera and species approved to date, as of February 2018 (http://www.ictvonline.org/), disease(s) associations (if any), host species, and the year of taxonomy assignment.*

Genus	Species	Associated diseases	Host	Year of approval
Aalivirus	Aalivirus A	To be determined	Ducks	2018
Ampivirus	Ampivirus A	To be determined	Common newts	2017
Aphthovirus	Foot-and-mouth disease virus	Foot-and-mouth disease	Cloven-hooved animals	1978
	Bovine rhinitis A virus	Upper respiratory tract infections	Ruminants, pigs, horses	2012
	Bovine rhinitis B virus		Ruminants, pigs, horses	2009
	Equine rhinitis A virus	Upper respiratory tract infections Equine respiratory infections	Ruminants, pigs, horses	1976
Aquamavirus	Aquamavirus A	Upper respiratory tract disease	Seals	2012
Avihepatovirus	Avihepatovirus A	Goose hemorrhagic hepatitis	Ducks and gooses	2009
Avisivirus	Avisivirus A	Gastrointestinal disease	Turkey	2013
	Avisivirus B		Chickens	2016
	Avisivirus C		Chickens	2026
Bopivirus	Bopivirus A	To be determined	Cattle	2018
Cardiovirus	Cardiovirus A	Myocarditis	Pigs, rodents, human	1978
	Cardiovirus B	Gastroenteritis, influenza-like disease	Human	1971
	Cardiovirus C	To be determined	Rodents	2014
Cosavirus	Cosavirus A	Gastrointestinal disease	Human	2012
	Cosavirus B		Human	2016
	Cosavirus D		Human	2016
	Cosavirus E		Human	2016
	Cosavirus F		Human	2016
Crohivirus	Crohivirus A	To be determined	Shrew	2017
	Crohivirus B		Bats	2017
Dicipivirus	Cadicivirus A		Dogs	2012
Enterovirus	Enterovirus A	Paralysis (non-polio and polio-type), summer cold, meningitis, diarrhea; poliomyelitis, aseptic meningitis and encephalitis, herpangina, hand, foot and mouth disease, acute hemorrhagic conjunctivitis, pleurodynia, summer grippe; common cold	Human, monkyes, cows	1976
	Enterovirus B		Human, monkyes, dogs	1976
	Enterovirus C		Human, dogs	1971
	Enterovirus D		Human, monkyes	1976
	Enterovirus E		Cows, dogs, goats, deers, sheeps	1976
	Enterovirus F		Cows, gooses, horses, deers, sheeps, possums	2012

	Enterovirus G		Human, monkeys, sheeps, pigs	2008
	Enterovirus H		Human, monkeys	2004
	Enterovirus I		Dromedaries	2016
	Enterovirus J		Monkeys	2012
	Enterovirus K		Rodents	2017
	Enterovirus L		Monkeys	2017
	Rhinovirus A	Common cold	Human	1971
	Rhinovirus B		Human	1976
	Rhinovirus C		Human	2009
Erbovirus	Erbovirus A	Upper respiratory tract disease with viremia and fecal shedding	Horse	1999
Gallivirus	Gallivirus A	To be determined	Turkey, chicken	2013
Harkavirus	Harkavirus A	To be determined	Common kestrel	2016
Hepatovirus	Hepatovirus A	Mild hepatitis, rarely fulminate acute hepatitis, reduced hatching, tremors and/or ataxia	Human, monkeys	1991
	Hepatovirus B	To be determined	Seals	2016
	Hepatovirus C		Bats	2016
	Hepatovirus D		Rodents	2016
	Hepatovirus E		Rodents	2016
	Hepatovirus F		Rodents, woodchucks	2016
	Hepatovirus G		Bats	2016
	Hepatovirus H		Hedgehogs, bats and tree shrews	2016
	Hepatovirus I		Shrews	2016
Hunnivirus	Hunnivirus A	To be determined	Cattle	2013
Kobuvirus	Aichivirus A	Gastroenteritis	Human, cattle	1999
	Aichivirus B		Cattle, pigs	2004
	Aichivirus C		Pigs	2012
	Aichivirus D	To be determined	Cattle	2016
	Aichivirus E		Rabbits	2016
	Aichivirus F		Bats	2016
Kunsagivirus	Kunsagivirus A	To be determined	Birds	2014
	Kunsagivirus B		Birds	2017
	Kunsagivirus C		Monkyes	2017
Limnipivirus	Limnipivirus A	To be determined	Bluegill fish	2015
	Limnipivirus B		Common carp	2015
	Limnipivirus C		Fathead minnows, brassy minnows	2015
Megrivirus	Megrivirus A	Hepatitis	Turkey, ducks, gooses	2012

	Megrivirus B	To be determined	Pigeons	2017
	Megrivirus C		Chickens	2017
	Megrivirus D		Ducks, gooses	2017
	Megrivirus E		Penguins	2017
Mischivirus	Mischivirus A	To be determined	Bats	2013
	Mischivirus B		Bats	2016
	Mischivirus C		Bats	2016
Mosavirus	Mosavirus A	To be determined	Canyon mouse	2013
Orivirus	Orivirus A	To be determined	Chicken	2017
Oscivirus	Oscivirus A	To be determined	Birds	2013
Parechovirus	Parechovirus A (human parechovirus)	Mild, gastrointestinal or respiratory illness, implicated in cases of myocarditis and encephalitis	Human, rodents	1997
	Parechovirus B (Ljungan virus)	To be determined; has been associated with myocarditis, type 1 diabetes and gestational diseases*	Rodents	2002
	Parechovirus C (Sebokele virus)	To be determined	Rodents	2016
	Parechovirus D (Ferret parechovirus)		Ferrets	2016
Pasivirus	Pasivirus A	To be determined	Pigs	2013
Passerivirus	Passerivirus A	To be determined	Birds	2013
Potamipivirus	Potamipivirus A	Hemorrhages at the head and the tail	Birds	2015
Rabovirus	Rabovirus A	To be determined	Norway rat	2016
Rosavirus	Rosavirus A	To be determined	Human, rodents	2013
Sakobuvirus	Sakobuvirus A	To be determined	Cats	2014
Salivirus	Salivirus A	Gastroenteritis	Human, chimpanzee	2012
Sapelovirus	Sapelovirus A,	Diseases of the reproductive tract	Pigs	1999
	Sapelovirus B	To be determined	Monkeys	2009
	Avian sapelovirus		Birds	2009
Senecavirus	Senecavirus A	Oncolytic virus	Pigs, cows	2009
Shanbavirus	Shanbavirus A	To be determined	Bats	2017
Sicinivirus	Sicinivirus A	Poultry disease	Birds	2014
Teschovirus	Teschovirus A	Porcine polioencephalomyelitis	Pigs	1999
Torchivirus	Torchivirus A	Diphtheroid-necrotizing stomatitis and pharyngitis, conjunctivitis, rhinitis, pneumonia, enteritis and ascites	Tortoises	2016
Tremovirus	Tremovirus A	Reduced hatching; tremors and/or ataxia	Chickens, pheasants, quails, turkeys	2009

* see below for further information.

1.2.1 Parechoviruses

Genus *Parechovirus* is one of the few picornavirus genera that contains viruses infecting humans. As listed in Table 5, this genus is currently represented by four species known in the literature as: *Human parechovirus* (HPeV), *Ljungan virus* (LV), *Sebokele virus* and *Ferret parechovirus*. However, for clarity, the taxonomy of these parechoviruses has recently been changed by the ICTV (http://www.ictvonline.org/) to *Parechovirus A*, *B*, *C*, and *D*, respectively.

The first two parechovirus types were originally called echovirus 22 and 23, and therefore assigned to the genus *Enterovirus*, but subsequent sequence analysis showed that they did not possess the characteristics of any known picornavirus thus far (Ghazi et al. 1998). Eventually, *Parechovirus* genus was proposed and accepted (King et al. 1999). Viruses in this genus form a separate lineage from all other picornaviruses (Johansson et al. 2002). In 1998, a new parecho-like virus was isolated in wild populations of bank voles in Sweden during the study of myocarditis patients. The suspected pathogen was named the 'Ljungan virus' after the river near the site of its discovery (Niklasson et al. 1998). A detailed comparison of LV genome with members of all genera of the *Picornaviridae* family confirmed the membership of LV to the *Parechovirus* genus (Johansson et al. 2002).

Only recently, two more species, named *Sebokele virus* and *Ferret parechovirus*, were included in the *Parechovirus* genus (Joffret et al. 2013; Smits et al. 2013). Sebokele virus 1 (SEBV1) was originally isolated in 1972 from African wood mice (*Hylomyscus simus*) samples collected in Central African Republic (Digoutte and Germain 1985). At that time, however, the attempts to identify the virus were unsuccessful, and therefore it was classified as an un-identified arbovirus. The full genome was characterized in 2013, and phylogenetic analysis confirmed that it is closely related to LV in the genus *Parechovirus*. In SEBV1, two structurally different 2A protein motifs were found to show the typical LV-like organization (Joffret et al. 2013). The genome of the *Ferret parechovirus* (MpPeV1) was characterized in 2013 from samples collected from ferrets in the Netherlands and Sweden. The genome showed the typical parechovirus genome organization, even if it shared less than 43% identity with the entire polyprotein of HPeVs and LVs (Smits et al. 2013).

However, other than the genome sequences, there is very limited information about the types within these two species and their genetic diversity.

1.2.2 *Human parechovirus* (*Parechovirus A*)

To date, the species *Human parechovirus* contains 19 different types based on the differences in the VP1 capsid protein sequence (Böttcher et al. 2014). Since the isolation of the first human parechovirus 1 (HPeV-1) isolate in the USA in 1956 (Wigand and Sabin 1961), parechoviruses have been recognized as a worldwide causative agent of gastrointestinal and mild respiratory infection in children (Tanaka et al. 2017). Seroprevalence of HPeV-1 antibodies is exceptionally high: HPeV antibodies can be detected in children under the age of two (72%, Ehrnst and Eriksson 1993; 95%, Joki-Korpela and Hyypiä 1998; 88%, Abed et al. 2007), and they exceed 95% prevalence in adult populations (Joki-Korpela and Hyypiä 2001; Harvala and Simmonds 2009; Westerhuis et al. 2013). HPeV-1 infections have been reported worldwide, for example, in the USA, Canada, Sweden, Finland, Israel and Japan (Stanway et al. 2000; and references therein). The clinical manifestations of HPeV-1 are mainly gastrointestinal and respiratory (Stanway et al. 2000), but Harvala and Simmonds (2009) also observed that HPeV infections can represent a significant cause of central nervous system (CNS) related diseases in infants, supporting the introduction of routine HPeV screening of cerebrospinal fluid (CSF) samples (Harvala et al. 2008). HPeV-3 is considered the most pathogenic strain, being associated with paralysis, neonatal sepsis-like disease and sudden infant death syndrome (SIDS) in infected children (Harvala and Simmonds 2009; Jääskeläinen et al. 2013; Kolehmainen et al. 2014). In addition, HPeV-4 has been detected in infants with sepsis-like disease (Jääskeläinen et al. 2013; Kolehmainen et al. 2014). More recently, HPeV-3 has been confirmed to cause severe symptoms in adults such as fever, myalgia, muscle weakness and sore throat (Tanaka et al. 2017). HPeV infections are routinely diagnosed by RT-qPCR on various body fluids (Harvala and Simmonds 2009; Jääskeläinen et al. 2013; Kolehmainen et al. 2014; de Crom et al. 2013).

1.2.3 *Ljungan virus (Parechovirus B)*

Interest in the Ljungan virus (LV) stems from the observations 20 years ago when significant temporal correlation between cyclical changes in the abundance of bank voles and the incidence of human disease was detected in northern Sweden (Niklasson et al. 1998, 2007). These authors noted that the number of new cases of Guillain-Barré syndrome, type 1 diabetes (T1D), and intrauterine fetal death (IUFD), as well as deaths caused by myocarditis correlated with rodent cycles in northern Sweden, although with different time lags between peak rodent density and peak incidence. These observations led them to hypothesize that one or more infectious agents, carried primarily by voles, could have caused these diseases in this area. LV was one of the candidate viruses isolated several years previously from *M. glareolus* trapped in Medelpad County, Sweden, as part of a survey aimed to determine the cause of an outbreak of myocarditis in a group of orienteering athletes (including 6 fatalities) between 1989 and 1992 (Niklasson et al. 1998; 1999). The first three isolates were named Ljungan 87-012; 174F and 145SL, respectively. The isolates were found to be related using a cross immunofluorescence test, but no cross-reaction was found with viruses belonging to other genera (Niklasson et al. 1999). In addition, molecular analysis showed that LV isolates were highly similar to other members of the *Picornaviridae* family, in particular with HPeV-3. The two viruses shared high sequence identity for structural proteins and for proteins responsible for the replication of the viral genome (Niklasson et al. 1999; Ekström et al. 2007). LV was assigned to species in 2002 by ICTV.

The viral RNA genome of LV is about 7.6 kb long, the virion is 27 nm in diameter, and the protein sequences encoded by the RNA are known (Johansson et al. 2002). Structural analysis showed that the capsid proteins share the same three-dimensional structure of other picornaviruses (Samsioe et al. 2009). The 5'-UTR region shows significant structural differences from that of HPeV, although it shares the general organization with the rest of the genome. The main difference between the LV genome and the majority of the other picornaviruses genomes is the presence of two consecutive, but different, nonstructural 2A proteins. The first one of these proteins is Aphthovirus-like 2A, mediating translation, and the second is Parechovirus-like 2A, involved in virus replication (Johansson et al. 2002; Yang et al. 2017). Up to now, eight strains of LV isolated from Swedish and American voles

have been sequenced and made available in GenBank. Based on the genome sequence analyses and phylogenetic relationships, the sequences group into four genotypes: LV-87-012 and LV-174F belong to genotype 1, since they have 93% sequence identity; LV-145SL, LV342 and LV340 belong to genotype 2, having >92% sequence identity; M1146 represents genotype 3; and LV-7855 represents genotype 4 (Lindberg and Johansson 2002; Johansson et al. 2002; Johansson et al. 2003; Tolf et al. 2009; Pounder et al. 2015). The complete genome characterization of a new parechovirus related to LV was recently reported from wild birds in Hungary; the viral RNA was detected from fecal samples of common kestrels (*Falco tinnunculus*) and red-footed falcons (*Falco vespertinus*) (Pankovics et al. 2017).

The most common methods for diagnosing LV infection include detection of the antibodies against the virus, detection of viral RNA, and virus isolation. The main tool utilized to detect LV-RNA is a sensitive RT-qPCR assay. Specific LV primers, targeting the 5'-UTR region of the LV genome, were designed by Donoso Mantke et al. (2007). The assay was verified using viral RNA isolated from the six LV strains known at the time, and the specificity was confirmed for different tissue types (Samsioe et al. 2008; Hauffe et al. 2010; Kallies 2012 and references therein; Niklasson et al. 2009; Salisbury et al. 2014; Romeo et al. 2014). High copy number of LV-RNA was noted in all tissues analyzed (Donoso Mantke et al. 2007). Positive samples are identified as a band of 185 bp on agarose gel; however, most publications using this protocol have confirmed the result by sequencing the fragment and comparing the outcome to sequences in public databases. Nix and colleagues developed a novel PCR-based assay targeting the conserved 5'-UTR region of the *Parechovirus* genus (Johansson et al. 2002; Nix et al. 2008) that worked more efficiently on a broad range of specimens, including CSF, stool, rectal and nasopharyngeal swabs, lung and spleen tissues (Nix et al. 2008). The differentiation of HPeVs from LV is achieved by sequencing the PCR amplicon. An IFA assay has also been developed for the detection of LV in humans and other animals, which has proved to be sensitive. In addition, the specificity of the test to LV has also been confirmed (Jääskeläinen et al. 2013; Forbes et al. 2014; Jääskeläinen et al. 2016).

Isolation of the virus directly from human tissues and stool samples, or from tissues of wild fauna has been unsuccessful thus far (Niklasson et al. 2007; Tapia et al. 2010), although Niklasson and colleagues succeeded in obtaining LV isolate from 1-day-old suckling mice after intra-cerebral inoculation. However, they observed only a low cytopathogenic effect

(CPE). From here, Johansson and colleagues managed to achieve an efficient system for propagating LV in cell culture, allowing *in vitro* radiolabelling and purification of LV strain 87-012 (Niklasson et al. 1999; Johansson et al. 2004).

Of particular interest (e.g. Greene Mcdonald 2009) is the idea that LV may be associated with metabolic disease (Niklasson et al. 2006; 2007; Samsioe et al. 2006). The role of intrauterine exposure to LV on onset of T1D was tested in outbred CD-1 mice, demonstrating that LV intrauterine infection leads to glucose intolerance in the offspring or malformation of the fetal pups causing death in the neonatal period. However, LV infection by itself was not enough to cause disease, a stress component was also needed (Niklasson et al. 2006; Samsioe et al. 2006; Holmberg et al. 2009). As for the association between T1D and LV in humans, four studies have tested blood serum from T1D children and controls for the presence of LV antibodies, all concluding that T1D patients had significantly higher levels of LV antibodies compared to controls (Niklasson et al. 2003; Nilsson et al. 2009; 2013; 2015). On the other hand, two other studies using stool samples to examine LV prevalence in Norwegian infants with a genetic risk for developing T1D found no evidence of LV (Tapia et al. 2008; 2010). However, these two sets of results are not necessarily in contrast with one another. If LV is associated with T1D, it is probably responsible for triggering the onset of diabetes and not the development of diabetes itself, in a similar manner to what has been hypothesized for enteroviruses. That is, viral infection peaks in the months preceding onset of T1D, initiating an autoimmune response (Laitinen et al. 2014; Hyöty 2016; Honkanen et al. 2017). This would explain why LV antibodies are found in the blood serum of T1D children (as a result of being exposed to the virus), but LV nucleic acids are not found in the stools of T1D patients (because viral shedding would presumably have occurred during infection, in the months preceding T1D onset; Rodriguez-calvo 2018).

The early findings that LV was correlated with gestational disease in laboratory mice (Niklasson et al. 2006; 2007; Samsioe et al. 2006; 2008; 2009), also initiated studies on the possible role of LV in the same diseases in humans. As mentioned above, a correlation between the incidence of IUFD cases and cyclic variation of wild rodents was observed in northern Sweden; however, no signs of diseases were detected in histopathologic analysis of the placenta and brain tissues of fetuses (Niklasson et al. 2007). Later studies also

proposed a correlation between the incidence of SIDS and fluctuations in rodent densities (Niklasson et al. 2009), but the LV screening was not repeated on larger cohorts of sample and findings were not confirmed using any other virological methods; hence, these results have been challenged (Krous and Langlois 2009; 2010). In further experiments the presence of LV-specific antigens and LV-RNA were detected in tissues from fetuses with anencephaly and hydrocephaly (Niklasson et al. 2009), and infection of pregnant laboratory mice with LV-145SL led to clinical signs of encephalitis in all infected but not uninfected offspring. Although sample size was also limited in these studies, the authors suggested that LV should be included in the list of viruses associated with CNS malformations (Niklasson et al. 2009).

In addition to human studies, investigation of LV distribution and prevalence in non-human animals has continued since the discovery of the virus, but these investigations have been mainly limited to a single country and/or few host species. Therefore, information about LV host range and geographical distribution are still scarce (e.g. Tolf et al. 2009; Hauffe et al. 2010; Romeo et al. 2014; Kallies et al. 2012; Salisbury et al. 2014; Jääskeläinen et al. 2013; Forbes et al. 2014). It has been hypothesized that rodents could act as reservoirs and/or vectors of LV, possibly transmitting the infection through the oral-fecal route, or by inhalation of aerosolized excreta, as is the case for other viruses in the same family for which rodents are the primary hosts, such as HPeV (Krous and Langlois 2010; McDonald 2009). A fecal-oral route is supported by the finding that LV was isolated from fecal pellets in voles (Niklasson et al. 1999), although not from humans, as mentioned above. Human picornaviruses are typically transmitted via the fecal-oral route and can be detected in stool for a long period of time (Wu et al. 2017); thus, LV may be transmitted in a similar manner.

Despite the interest in LV as a potential human pathogen, this virus has not yet been conclusively identified as an etiological agent of any disease (Niklasson et al. 2007; Krous and Langlois 2010). In particular, symptoms of LV and its role in CNS diseases are virtually unknown. In addition, LV distribution and prevalence in potential rodent host species across Europe is an important gap in the understanding of the zoonotic potential of this virus. The aim of this thesis was to fill these knowledge gaps.

2 AIMS OF THE STUDY

The primary aims of the present study were:

i) to investigate the association of Ljungan virus with human infections with unknown aetiology and CNS disease

ii) to determine the prevalence and distribution of LV in human and other animal populations in Europe

The specific objectives of the thesis were:

i) to establish if the co-existence of LV with other rodent-borne viruses leads to increased severity of nephropathia epidemica in humans

ii) to investigate whether LV causes neurological symptoms in humans

iii) to determine the distribution of LV in small mammals throughout Europe

iv) to identify the individual and environmental factors affecting LV prevalence in animal hosts.

3 MATERIALS AND METHODS

3.1 Patient samples (I and III)

Serum and CSF samples from 116 Finnish patients (I) were screened for the presence of LCMV, LV and CPXV reactive IgG antibodies and seroconversions. Seroconversion was considered to occur when the first sample taken at hospitalization was anti-LV/LCMV/CPXV IgG negative, followed by a second sample taken during or after hospitalization, which was anti-LV/LCMV/CPXV IgG positive. Clinical outcomes were compared between patients who were only positive to PUUV-IgG, and those who were positive to PUUV-IgG and also presented antibodies to LCMV, CPXV and/or LV. Serum samples were collected from Tampere University Hospital (Finland) in 2000 - 2009 from patients hospitalized due to NE, and analyzed at the University of Helsinki. For each patient, the first sample was collected on the day of admittance to the hospital, and subsequent samples were taken during the convalescent phase. PUUV infections were confirmed for all the patients at the Pirkanmaa Hospital District Laboratory Centre (Finland). All patient samples were treated anonymously (research permits Tampere University Hospital, Tampere, Finland, N. 99256 and R04180).

Sera and CSF samples were collected from 400 patients with suspected neurological infections from December 2013 to December 2014 (Table 1 in III). The cohort included patients from 5 to 50 years of age. All samples were routinely screened at the Hospital District of Helsinki and Uusimaa, Finland (HUSLAB) for several causative agents of suspected neurological infections, such as herpes simplex viruses (HSV) type 1 and type 2, varicella zoster viruses (VZV), human herpesvirus 6 (HHV6), and *Mycoplasma pneumoniae*. CSF, and serum samples from 380 patients were also tested for both LCMV and LV nucleic acids. All patient samples were treated anonymously (research permits TYH2016258 and TYH2017257, HUS permit code: DKMIKR, Helsinki, Finland).

3.2 Small mammal samples (II and IV)

The distribution of LV in rodent and insectivore hosts across ten European countries was analyzed using liver samples collected during the EU FP7 project EDENext (2011-2014, Emerging diseases in a changing European environment; http://www.edenext.eu/). During this thesis, the author was directly involved in the live-trapping of rodents across northern Italy, and sampling bank voles from biobanks in Sweden. In the Baltic States, Croatia, France, Germany, Italy, Slovakia, Slovenia and The Netherlands, animals were live-trapped and euthanized the same day; animals were dissected and liver samples were stored at -20 °C. In Finland and Sweden, animals were snap trapped using Finnish traps (Etutuote Ky, Vaasa, Finland), frozen at -20 °C and dissected at a later date. All samples were shipped on dry ice to the Fondazione Edmund Mach (Italy), where molecular screening was performed (Table 1 in II and Table 1 in IV). In addition, bank voles were trapped around Umeå (Sweden) in spring and fall from 2009–2012 as part of a long-term study. For these samples, the seasonal variation in LV prevalence was investigated. Finally, at the long term trapping site in Kilpisjärvi (Finland), Norwegian lemmings were trapped before, during and after a particularly strong oscillation in density, which occurred in 2011, in order to investigate the role of LV in lemming population cycles.

For each individual sampled, the following metadata were collected: sex, age and body mass, trapping season, trapping site, trapping year, land use, elevation, and mean temperature and rainfall of the previous six months. This time period was considered to be sufficient in order to establish if these two factors had an influence on viruses prevalence in rodents, consistently with previous literature (Karki et al. 2016; Díaz et al. 2010; Haredasht et al. 2013).

All procedures were carried out with permission from the ethical committees in the respective countries according to their national laws. The sampling carried out during this thesis in Sweden was approved by the Animal Ethics Committees of 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). Similarly, in Italy, animal trapping and handling procedures were authorized by the “Comitato Faunistico Provinciale della Provincia di Trento”, protocol n° 595, issued on 04 May 2011. In Finland, snap trapping does not require ethical permits under the

Finnish Act on Animal Experimentation (62/2006) and by the decision of Finnish Animal Experiment Board (16 May 2007).

3.3 Serological methods

3.3.1 Immunofluorescence assay (IFA)

Human serum samples were analyzed for the presence of LCMV-, LV- and CPXV- reactive IgG antibodies (I), or tested for LCMV- and LV-reactive IgG and IgM antibodies (III) by immunofluoroassay (IFA). Briefly, cultured virus strains were inoculated onto Vero cells and cells were collected when the CPE was visible, after approximately 2-3 days. At this point, the liquid was poured away, and the cells were detached from the cell culture flasks with a trypsin-EDTA treatment. The cells were washed five times with phosphate-buffered saline (PBS), before mixing with non-infected cells. The mix was finally diluted with PBS and added to the wells of diagnostic slides (Paul Marienfeld GmbH and Co. KG, Lauda-Königshofen, Germany). All the slides were allowed to dry overnight before fixing them with ice-cold acetone and stored dry at -70 °C ready for use.

For detection of the presence of IgG and IgM antibodies in human serum samples, they were diluted 1:20 in PBS and 20 µL aliquots were added to each well on the previously prepared slides. To detect the presence of IgG antibodies, the slides were incubated for 60 minutes at 37 °C in the case of LV, while the incubation time was 30 minutes at 37 °C for LCMV and CPXV. Slides were washed three times in PBS, rinsed with MilliQ water for five minutes and air dried. At this point, fluorescein (FITC)-AffiniPure F(ab')2 fragment goat anti-human IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies, diluted 1:100 in PBS, were then added to each well of the slides, which were then incubated again for 30 minutes at 37 °C for all viruses. The same washing and drying steps were then repeated. For IgM antibody detection, the protocol was similar, but the first incubation phase at 37°C

lasted 2 hours for all three viruses. The secondary antibody used in this case was the FITC labelled AffiniPure goat anti-human IgM conjugate (Jackson ImmunoResearch Europe Ltd, UK), diluted 1:50 in PBS. Following slide preparation, cover slips were mounted and slides were inspected under a fluorescence microscope (Leica Microsystems, Espoo, Finland) with a FITC filter (Figure 5A, 5B). The methods used for other microbial agents are listed in Tables 1 and 2 (III).

3.4 Molecular methods

3.4.1 RNA extraction and PCR amplification from human serum and CSF samples

RNA was extracted from human serum and CSF samples (I) using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). In order to screen for arena- and parechoviruses, extracted RNA was amplified using RT-PCR with the Invitrogen SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA). For arenaviruses, two forward RiViGene primers were used: LVL3359D Yplus (5'-agaatcagtgaaagggaaagcaay) and LVL3359G Yplus (5'-agaattagtgaaagggagagtaat); and two reverse primers: LVL3754D Rminus (5'-cacatcattggtccccatttactgtgr) and LVL3754A Rminus (5'-cacatcattggtccccatttactatgr). For LV, MetaBion primers were used: reverse primer pan-parechovirus (PEV) RV (5'-gtaacaswwgcctctggg) and forward primer PEV FW (5'-gtacctycwggccatccttg). Protocols are detailed in I. PCR-products were sequenced at the Institute for Molecular Medicine (FIMM, Finland) or DNA Sequencing and Genomics Laboratory (Institute of Biotechnology, University of Helsinki, Finland).

3.4.2 RNA extraction and PCR amplification from small mammal liver samples

For small mammals, LV-RNA extraction from liver was performed using the RNeasy Lipid Tissue MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. LV-specific RT-PCR was performed according to Donoso Mantke et al. (2007) with the exception that Qiagen OneStep RT-PCR kit (Qiagen) was used in the protocol (II). The 185 bp PCR products were purified from agarose gel and Sanger-sequenced in both directions. Sequences were assembled using Sequencher DNA sequence analysis software (version 4.7, Gene Codes, Ann Arbor, Michigan, USA) and aligned against GenBank sequences using BLASTn (Basic Alignment Search Tool; National Center for Biotechnology Information Bethesda MD, USA, 2017) to confirm that the amplified fragment originated from LV.

3.5 Phylogenetic analysis

Phylogenetic analysis of the 185 bp fragments was performed in order to investigate the genetic variation between LV isolates. Since the fragments were already short, only 127 sequences which were 100% complete were used; this conservative choice excluded the use of the five LV genomes available online. Unique sequences were identified using DNAcollapser (http://users-birc.au.dk/biopv/php/fabox/dnacollapser) and aligned using ClustalX (Larkin et al. 2007) with default settings. JModeltest2 available online on the CIPRES Science Gateway portal (Miller et al. 2010) was used to determine the most suitable model of DNA substitution for the dataset. The Bayesian MCMC approach and a bootstrapped distance-based method were used to generate the phylogenies. The transitional model with invariant sites and gamma distributed rates were applied using MCMC in MrBayes (Huelsenbeck and Ronquist 2001) (Huelsenbeck and Ronquist, 2001). The run was performed with 10 million generations. A consensus tree was built from the last 50,000 trees (Figure 6). The statistical support for the nodes was calculated using the

approximate log likelihood ratio test (aLRT) and a Bayesian posterior probability (BPP) algorithm. A distance method was implemented with the PhyML package (Guindon et al. 2010) using the same model and 1000 bootstraps were generated. The proportion of conserved sites was calculated with MEGA7 (Molecular Evolutionary Genetics Analysis version 7.0, Kumar, Stecher, and Tamura 2016).

3.6 Statistical analysis

In order to evaluate the effects of individual characteristics (i.e. sex, age) and sequential or co-infections on IgG seropositivity to LV, CPXV and LCMV in humans, generalized linear mixed models (GLMMs) were constructed in SAS version 9.3 (SAS Institute, Inc., Cary, North Carolina), with a binary distribution and logit link function (I).

A generalized linear model (GLM) with a binomial error distribution was built to estimate which parameters (sex, age and body mass, trapping season, trapping site, trapping year, land use, elevation, mean temperature and mean rainfall of the previous six months) were correlated with LV prevalence in bank voles trapped at the Umeå sampling site, as well as in bank voles trapped only in autumn, in all the sampling sites (II). In order to assess the influence of these same individual and environmental factors on LV occurrence in *M. glareolus* in other European countries sampled, a GLMM with a binomial error distribution was constructed (IV). In the GLM analyses in II and IV, the binary response variable was the presence or absence of LV nucleic acids. Starting from the full models, which included all non-collinear explanatory variables, a stepwise model selection based on Akaike Information Criterion (AIC; Akaike 1998) was carried out; the models with the lowest AIC were then selected as best model. All statistical analyses were performed using the R package 'stats' (R Development Core Team 2016).

4 RESULTS AND DISCUSSION

Since its discovery, a number of publications have implicated LV in human disease, including those related to the CNS. Early studies showed that LV infected rodents and inoculated laboratory animals displayed human disease-like symptoms; hence, it was suggested that LV had zoonotic potential. Up to now, knowledge of geographical and host distribution of LV has been patchy, and LV has not been proven to be etiological agent for any (human) disease.

Because the etiology of possible LV infection in humans is unknown, large sera sample sets and their metadata of cohorts of patients with NE infected with rodent-borne viruses (PUUV, LCMV and/or CPXV), already accessible at the University of Helsinki were used to attempt to detect LV seroconversions and associated symptoms. Other cohorts, presenting CNS disease, were used to attempt to detect LV nucleic acids in sera and associate this detection with CNS symptoms.

4.1 Association of LV with LCMV and CPXV in PUUV infection outcomes

Screening patients hospitalized for NE, and determining which were positive to PUUV-IgG antibodies and also to LV-antibodies, gave the opportunity to deepen our knowledge about possible symptoms linked to LV exposure, and to verify whether LV could be responsible for a more severe outcome of the disease caused by the rodent-borne PUUV. PUUV-positive patients were chosen because PUUV and LV share the same rodent host, thus it was assumed that a high proportion PUUV-positive samples could also be LV seropositive (if the hypothesis that LV is rodent-borne is correct). Therefore, in order to compare the outcomes of PUUV-positive and PUUV + LV-positive patients, a detailed list of symptoms was recorded for each patient. The seropositivity for two other rodent-borne viruses, LCMV

and CPXV, was also investigated. It is known that virus-virus interactions within the same host can alter the outcome of an infection (DaPalma et al. 2010). However, the presence of viral co-infection is not necessarily associated with a more severe disease course. Both an increase in disease severity in relation to multiple infections and the absence of this association have been reported (Griffiths et al. 2011; Cebey López et al. 2016).

534 serum samples were acquired from 116 patients hospitalized at Tampere University Hospital in Finland (I). High levels of LV antibodies were reported in this patient cohort (47.8%) in line with previous results obtained from the screening of other Finnish patients (38%, 36%, Jääskeläinen et al. 2013; 2016). Seroprevalence to CPXV was 32.4%, while that of LCMV was relatively low (8.5%). NE symptoms were observed in all patients co-infected with PUUV and either LV, LCMV or CPXV, but the outcome for co-infected patients was not different from typical NE (typical symptoms related to PUUV infection are listed in section 1.1.2). Specifically, there were no statistical differences in clinical severity or laboratory findings between PUUV cases and PUUV-LV cases. However, due to the low number of samples with seroconversion, the results are inconclusive. NE patients with LCMV seroconversions had lower plasma creatinine concentrations and lower platelet counts than patients with no LCMV seroconversion. However, co-infections could not be confirmed by PCR, and due to low number of patients, it can only be speculated as to whether this LCMV seroconversion had an impact on the severity of the acute symptoms of PUUV infection and kidney functions. LCMV seroprevalence in Finland is similar to levels found in other European countries like Spain (1.7%; Lledó et al. 2003) and the Netherlands (2.6%; Elbers et al. 1999). Although there were fifteen LV, five LCMV and one CPXV seroconversions (Figure 5A and 5B) detected among these patients during sample collection, all patients were PCR negative to LV, LCMV and CPXV nucleic acids, so it was not possible to confirm the LV isolate(s) infecting humans.

These results showed that co-infection between PUUV and LV, LCMV and/or CPXV had little or no impact on the severity of the outcome in the patient cohort analyzed. No other studies of LV seroconversion or co-infection with rodent-borne zoonotic viruses have been reported thus far. However, these negative results do not allow conclusions to be made about the role of LV in human disease, even if we compare them to other virus-virus co-infection studies, since these show that co-infections of distantly related pathogens with

known etiology may have contrasting outcomes (i.e., they either increase or decrease disease severity). For example, Benhammou and colleagues (2018) compared hepatitis B virus/human immunodeficiency virus (HIV) and hepatitis C virus/HIV co-infected groups to HIV mono-infected group of pregnant women for pregnancy outcomes, showing that co-infections were not associated with a higher risk of any adverse pregnancy or neonatal outcomes. In studies conducted on children hospitalized with acute diarrhea, co-infection cases of HPeV with other common viral gastroenteritis agents (human rotaviruses, human caliciviruses, astroviruses, and adenoviruses) were detected using RT-qPCR. No significant differences in fever and vomiting rate, or mean duration and frequency of diarrhea and vomiting, were found between the positive and negative case groups of HPeV co-infections (Zhang et al. 2011). On the other hand, studies reporting patients with respiratory diseases reported poor host health and enhanced pathogen abundance in cases of co-infection with multiple respiratory viruses compared with single infections. Co-infection of viruses such as influenza virus, rhinovirus, enterovirus, parainfluenza virus and respiratory syncytial virus were associated with more severe outcome and a higher risk of admission to the intensive care unit or death compared to patients with single infections (Goka et al. 2013; Crotty et al. 2015).

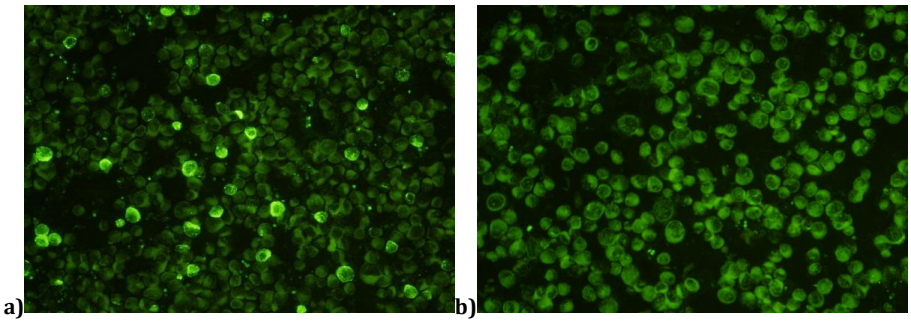

***Figure 5**: Comparison of different patterns in the LV IFA test examined under a fluorescence microscope.* ***a)** LV-positive sample in IgG IFA.* ***b)** LV-negative sample in IgG IFA.*

4.2 Association of LV (and LCMV) with suspected neurological infection

The diagnosis of many patients with neurological symptoms is often inconclusive, although, causes of CNS symptoms may be intrinsic (e.g. vasculitis) or extrinsic, including viral infections. Previous studies of human fetuses with CNS malformations revealed the presence of LV-antigens and RNA in brain tissue (Niklasson et al. 2009; Samsioe et al. 2009). Therefore, LV was purported to play an important role in CNS diseases in humans, but confirmation of this hypothesis is still lacking.

Here the role of LV as a possible causative agent of suspected neurological infections was examined for the first time in 400 Finnish patients, ranging in age from 5 to 50 years old, hospitalized with CNS symptoms. CSF and serum samples were tested for LV sero- and RNA-prevalence, together with other pathogens listed among the most common causes of CNS infections: HSV1 and HSV2, VZV, HHV6, *M. pneumoniae* and *Borrelia* antibodies (as an indicator for neuroborreliosis) (Granerod et al. 2010; De Ory et al. 2012; Kleines et al. 2014). The samples were also screened for LCMV because this virus has been proven to lead to the development of fever, malaise, headaches, seizure and in some cases fatal meningitis in adults (Kang and McGavern 2008; Wilson and Peters 2014).

A causative microbial agent was confirmed in only 15.5% of the 400 CNS samples. *M. pneumoniae* (26/400; 6.5%) and *Borrelia* spp. (18/400; 4.5%) infections were the most common causes of neurological symptoms, and mostly in young patients. These data supported previous results showing that *M. pneumoniae* infection and neuroborreliosis have higher incidence in children (Bitnun et al. 2003; Brown et al. 2016; Esposito et al. 2013). Enteroviruses and herpes simplex viruses were the most prevalent viral agents confirmed by PCR from CSF. Although IgG seroprevalences for LV and LCMV were 33.8% and 5.0% respectively, none of the sera was positive for anti-LV IgM antibodies, while only one serum sample was positive for anti-LCMV IgM. The serum sample found to be positive to both anti-LCMV IgM and IgG was from a patient diagnosed for acute neuroborreliosis. This suggests that the IgM positivity to LCMV may be nonspecific. No LV or LCMV infections were detected by PCR in CSF, and no correlation between LV seropositivity and CNS

symptoms was found in patients screened here, in line with results in 4.1. As for the 85% undiagnosed patients, their neurological symptoms may have had another etiology (e.g. autoimmunological origin, Salvarani et al. 2007), or been caused by pathogens, which are involved in CNS disease, but have not been reported or tested here, e.g. Epstein–Barr virus, cytomegaloviruses or bacteria other than *Borrelia* (Kleines et al. 2014; Wilson and Peters 2014).

The prevalence of LV antibodies in this cohort (33.8%) was similar to those used in (I) and in previous studies (see section below for details). This could be considered a minimum prevalence in Finland since the serological test used was based on antibodies against the Swedish serotypes LV87-012 and 145SLG (Jääskeläinen et al. 2013). This is because no Finnish LV serotypes have been isolated, and there is no information about the prevalence of any LV strain in this country. Since the antigenic properties of Finnish isolates may differ from those of the Swedish ones, the currently available assay may underestimate LV seroprevalence in Finland.

Overall, results presented in 4.1 and 4.2 strongly suggest that there is no association of LV or antigenically related virus with any symptoms of acute infection, during seroconversion or in cases of suspected neurological infection. This evidence corroborates previous reports, which did not show unequivocally that LV causes human disease (see section 1.2.3 for more detailed discussion). In addition, there are only serological data regarding the exposure of humans to LV (Nilsson et al. 2009; 2015; Jääskeläinen et al. 2013; 2016; Van Cuong et al. 2015), and no study thus far, including this one, has been able to identify LV nucleic acids in blood or excreta, precluding the identification of human isolates or potential mode of transmission.

Interestingly, LV seroprevalence in the present investigation (I: 47.8%; III: 33.8%) was similar to that in previous studies of other patient cohorts in Finland (38%, Jääskeläinen et al. 2013; 36%; Jääskeläinen et al. 2016). In addition, in human studies including the present one (III), patients with higher level of LV antibodies were found to be younger compared to the rest of the cohort (Van Cuong et al. 2015; Jääskeläinen et al. 2016). The unexpectedly high seroprevalence in children and adolescents and subsequent decline of LV antibodies in older age groups raise the possibility that LV or an LV-related virus may be transmitted

from human-to-human since this is a typical pattern for such virus transmission (Van Cuong et al. 2015; Jääskeläinen et al. 2016). In addition, LV was found to be more prevalent in urbanized compared to rural parts of Finland (III; Jääskeläinen et al. 2016), similar to findings for HPeVs (Harvala and Simmonds 2009), but in contrast to zoonotic viruses transmitted by rodents (LCMV: Wilson and Peters 2014; PUUV: Bergstedt Oscarsson et al. 2016; but see Tagliapietra et al. 2018). In general, the LV-seroprevalence in the cohorts studied may be biased because of the serological method used here, based on Swedish LV isolates, which may differ from the Finnish ones. However, it should be underlined again that, as pointed out by Jääskeläinen et al. (2016), high seropositivity is suggestive of human contact with virus, but proof of a causal role of LV in any disease is still lacking.

4.3 Geographical distribution and prevalence of LV in rodent hosts in Europe

Detailed knowledge of host and geographical distribution of LV is important background information to assess the role of this virus as a zoonotic rodent-borne pathogen. As mentioned above, LV was first discovered in bank voles trapped in Sweden in 1998. In addition to bank voles, LV and/or LV antibodies have been reported later from other rodent species in Sweden including the grey red-backed vole *Myodes rufocanus*, field vole *Microtus agrestis*, Norway lemming *Lemmus lemmus* and wood lemming *Myopus schisticolor*; in Denmark (bank vole); in USA (montane vole *Microtus montanus*, southern red-backed vole *Myodes gapperi)*; in Italy (bank vole, yellow-necked mouse, red squirrel *Sciurus vulgaris)*; in Germany (bank vole, field vole, common vole, yellow-necked mouse, striped field mouse *Apodemus agrarius*, wood mouse *Apodemus sylvaticus*, harvest mouse *Micromys minutus*, house mouse *Mus musculus*, and Norway rat); in the UK (bank vole, field vole, wood mouse and house mouse) and in Finland (bank vole, field vole) (Niklasson et al. 2003, 2006; Johansson et al. 2003; Tolf et al. 2009; Hauffe et al. 2010; Romeo et al. 2014; Kallies et al. 2012; Salisbury et al. 2014; Jääskeläinen et al. 2013; Forbes et al. 2014). In 2015, LV was

isolated from wild gull stool samples in Japan, providing the first evidence of a possible LV infection in birds, and suggesting the presence of a new LV genotype (Mitake et al. 2016).

Despite increasing interest in LV in wildlife, most scientific articles mentioned above include samples from a few individuals and/or single host population. Here, taking advantage of available biobanks and long term trapping programs across Europe, an initial intensive screening of bank voles (the host from which LV was first isolated) throughout Fennoscandia (mainly Sweden where LV was first discovered) was carried out (II). The search for LV was then extended (within the EU FP7 project EDENext) to eight other European countries, and 21 small mammal species, in one of the most intensive and systematic prevalence studies of LV conducted thus far (IV).

Overall, 1685 animals were screened for LV by RT-PCR (Table 1 in IV). LV nucleic acids were detected in nine out of ten EU countries and in those wild species tested previously: bank vole, field vole, yellow-necked mouse, grey red-backed vole, Norway lemming, wood lemming and house mouse. LV was detected for the first time here in two additional rodent species: northern red-backed vole (*M. rutilus*) and tundra vole (*M. oeconomus*), and for the first time in insectivores; in bicolored white-toothed shrew (*C. leucodon*) and Valais shrew (*S. antinorii*). This brings the number of LV hosts to 22 (including the Arctic fox *Alopex lagopus*, Niklasson 2008; and two species of birds, Pankovics et al. 2017). LV was not present in the common vole, black rat (*Rattus rattus*), grey squirrel (*Sciurus carolinensis*), striped field mouse and wood mouse. The fact that common vole, striped field mouse and wood mouse were not found to be LV PCR-positive could be due to small sample size. From a potential zoonotic point of view, it is interesting that the commensal black rat was LV PCR-negative, while the house mouse was positive. Also, interestingly, the autochthonous squirrel species (*S. vulgaris*) was found to be LV PCR-positive, but the grey squirrel, which was introduced in the EU several decades ago, was LV negative, even though these two species are known to exchange other viruses, e.g. *Squirrel poxvirus* (Rushton et al. 2006).

LV PCR-prevalence among the 885 screened bank voles (452 from Fennoscandia) was 15.2% (135/885; Figure 1 and Table 1 in II), ranging from a minimum of 3.7% (3/80, in Slovakia) to 25.9% (33/127, in Italy). LV-positive *M. glareolus* were found in every country where they were trapped and LV prevalence in this species was also the highest of all

rodent species tested: 13.3% *M. agrestis*, 11.5% *M. schisticolor*; 6.2% *M. oeconomus*, 5.9% *M. rutilus*; 4.2% *M. rufocanus*; 4.0% *M. musculus*; 3.7% *A. flavicollis*; 2.3% *L. lemmus*. Comparing the species for which we had a large sample size taken from several populations (therefore more accurate measure of prevalence: *M. glareolus*, *A. flavicollis, L. lemmus, M. musculus*), these results suggest that bank voles are the main host for LV as proposed by Niklasson et al. (2006). LV PCR-prevalence in bank voles was within the range of previous LV screening of bank vole in previous publications (Italy: 50.0%, Hauffe et al. 2010; UK: 27.0%; Salisbury et al. 2014; Germany: 8.4%, Kallies 2012). Bank voles act as reservoir for several rodent-borne viruses (Davis et al. 2005; Meerburg et al. 2009). In particular, *M. glareolus* is recognized as PUUV main rodent host (Voutilainen et al. 2012; Reil et al. 2017). PUUV exposures have been documented, both with serological and molecular screenings, in several populations of *M. glareolus*. Essbauer (2006) noted a high PUUV PCR-prevalence in bank voles trapped in Germany (34.5%), and those trapped in northern Sweden and Finland had levels of PUUV seroprevalence similar to LV PCR-prevalence showed here (17.6%: Olsson et al. 2005; 20%: Reil et al. 2017).

A preliminary phylogenetic analysis was conducted using the 185 bp LV-specific fragments representing 80 isolates (unpublished data). 53.5% of the sites were conserved, especially in the termini of the sequences. Figure 6 shows the phylogenetic tree: each branch represents one isolate, and each main cluster is represented by a different color. There are no obvious geographical or species-specific patterns, and there are no lineage relationships. Several species carried the same isolate; for example, *M. glareolus*, *L. lemmus* and *S. vulgaris* shared isolate 4, whereas, *M. glareolus*, *M. musculus* and *A. flavicollis* shared isolate 21. Several isolates were identified across different countries; for example, isolate 4, 5, 46 (Finland and Italy), isolate 21 (Italy and Sweden), isolate 49 (Finland, France, Germany, Italy and Slovakia). Most isolates (73/80) were found in bank voles but this is probably due to much higher number of LV PCR-positive samples from this species used in the study. A similar phylogenetic pattern (lacking geographical and host specificity of isolates) was found for CPXV based alignment of the complete coding regions from different mammalian hosts, including humans (Carroll et al. 2011). However, other viruses carried by rodents have been found to exhibit the opposite pattern. For example, phylogenetic analysis of the partial S segments of PUUV, obtained from bank voles, yellow-necked mice, wood mice and house mice, demonstrated geographically distinct clades (Essbauer et al.

2006). Similarly, S segment coding sequences of the Dobrava-Belgrade orthohantavirus (DOBV) strains from European yellow-necked and striped field mice, clearly showed that DOBV forms distinct evolutionary lineages which are host specific (Klempa et al. 2013). Since our results may be biased by the low number of bp analyzed, and only five genomes of laboratory cultured LV isolates are available in public database thus far, genetic analysis is ongoing to sequence longer and more evolutionary informative fragments of LV.

In conclusion, the presence of LV in 10 rodent and two insectivore species and in nine European countries confirms the wide geographical and host range of LV, as first hypothesized by Johansson et al (2003). In addition, the lack of host specific isolates or geographic pattern in isolate distribution suggests widespread transmission of LV throughout the EU and between small mammal communities.

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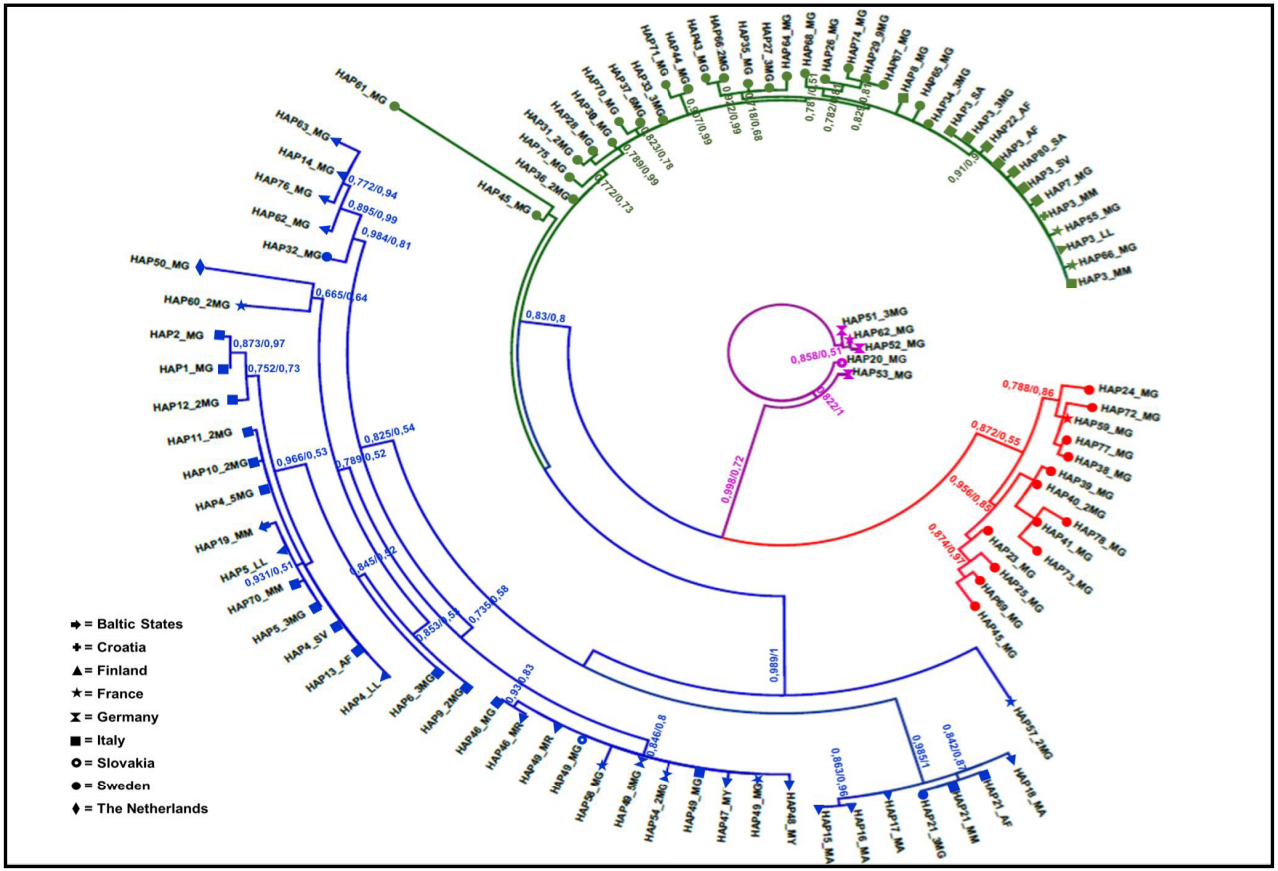

Figure 6: *Phylogenetic tree of 5'-UTR isolates of LV obtained using MrBayes and phyML. For each node, aLRT values obtained with phyML, and BPP values obtained from MrBayes are shown in the format aLRT/BPP. The tree is unrooted. The four main clusters are shown in different colors. Each branch represents an isolate.*
HAP= haplotype; MG= M. glareolus; MM= M. musculus; LL= L. lemmus; SV= S. vulgaris; AF= A. flavicollis; MR= M. rutilus; MY= M. schistocolor; MA= M. agrestis; SA= S. antinorii. The numbers before the name of the species indicate how many animals belong to that isolate.

4.4 Effect of individual and environmental factors on LV prevalence in bank voles

As a part of investigation into the zoonotic potential of LV, in order to estimate which individual and environmental factors were correlated with LV prevalence in bank voles, a generalized linear model (GLM) was built using LV PCR-screening results and metadata for 452 bank voles from Fennoscandia (Table 3 in II), and from Italy and Sweden (2010-2012) (Table 3 in IV). Then a GLMM was generated for 885 bank voles from five EU countries (Table 2 in IV). Results of the GLMs indicated that LV PCR-prevalence in *M. glareolus* was significantly higher in males compared to females ($P<0.05$, Table 2 in II), and that positive animals were mainly subadults, while the youngest and the oldest animals were less infected ($P<0.01$, Tables 2 and 3 in II; Tables 2 and 3 in IV). Virus prevalence was also significantly higher in autumn than in spring ($P<0.001$, Table 2 in II), both overall and in adults only. The generalized linear mixed model (GLMM) did not find any differences in sex, but indicated the same result for age group (subadults had the highest prevalence) and for season (highest prevalence was found in autumn). The fact that subadult bank voles have a higher LV prevalence than other age groups suggests that LV infection may not be chronic in this species, i.e. they clear the infection, unlike what happens in the same host for rodent-borne PUUV (Vapalahti et al. 2003; Kallio et al. 2007). Similarly to PUUV, juveniles may have limited exposure to the virus, having few contacts with other individuals while staying within the mother's home range (Verhagen et al. 1986); and they may still be covered by maternal antibodies. Age is often associated with viral exposure, for instance, in bank voles PUUV infection is associated with higher mobility in subadults (Escutenaire et al. 2002). Seasonal variation in LV prevalence may be explained by the bank vole reproductive cycle. Summer represents the breeding season in this species (Bujalska 1996; Tadin et al. 2014), during which there are more chances for the animals to come into contact with each other and transmit the virus, resulting in a higher prevalence of the virus in the following autumn months. The effect of seasons on population dynamics is known from several rodent species, e.g. common vole, field vole, Norwegian lemming (Korpimäki et al. 2004; Ulrich et al. 2008). In *A. flavicollis*, the number of LCMV infected mice increases in summer, during the breeding season, with a clear seasonal variation (Tagliapietra et al. 2009). Both the

clearing of the virus at the subadult stage, and an autumn rather than spring infection peak could also limit transmission of LV to humans since a low number of individuals are infected at any one time, shedding is limited, and humans are less likely to come into contact with rodents during the colder seasons, all characteristics in contrast to rodent-borne PUUV, which causes numerous human disease cases.

Some authors have suggested that LV infection could impair the survival rate of rodents in the wild, especially in situation of high stress, such as high density population peaks (Niklasson et al. 2006; Niklasson et al. 2006; Samsioe et al. 2006). Previously, it has been shown that cowpox virus infection influences the dynamics of bank voles and wood mice by impairing the reproductive potential in the animals kept in captivity, without affecting either the morbidity or the mortality of the hosts (Feore et al. 1997). However, as the authors pointed out, observations made on laboratory mice may have different implications in wild rodent dynamics, since the latter can be more susceptible to the effects of an infection in their natural environment where conditions are not optimal. Cyclic lemming populations in Northern Finland were studied to determine the role of LV in the decline of populations at the end of the population cycle (unpublished data), but only a very small number of these animals resulted to be LV PCR-positive, trapped in different seasons, indicating that LV was not responsible for the mortality of lemmings at high density (Hauffe et al. 2015).

The GLMM also showed that rainfall has a significant effect on LV prevalence in bank voles in Europe ($P<0.05$, Table 3 in IV), so that precipitation in the six months before the trapping date is correlated with a lower LV occurrence. This could be due to rain and snow eliminating LV from topsoil and lowering transmission. However, precipitation could also be indirectly related to lower transmission because voles tend to be less active during rainy weather (Wróbel and Bogdziewicz 2015). On the contrary, other studies have revealed that prevalence of PUUV infection is higher among bank voles in wet or very humid habitats compared to dryer habitats (Verhagen et al. 1986; Olsson et al. 2010). It is known that LV is sensitive to heat (Ekström et al. 2007), but only in laboratory conditions, so the persistence of LV in the environment needs further investigation, as part of transmission studies.

5 CONCLUDING REMARKS

This thesis aimed at demonstrating whether there are signs of LV infection in humans and whether LV can be associated with any CNS symptoms. In addition, the distribution and prevalence of LV in European small mammals was estimated to assess which hosts could potentially act as vectors. Both serological and molecular methods were used to study LV.

The results of the role of LV as a potential human pathogen imply that it is unlikely that exposure to LV affects the severity of NE disease. Results presented here also suggest that there is no association of LV exposure or antigenically related viruses with any symptoms of suspected neurological infection. These results add to the growing number of studies (e.g. gestational disease: see references in section 1.2.3; NE and CNS disease: this study sections 4.1 and 4.2) concluding that there is no causal relationship between LV with human disease. In addition, LV has not been found in stools samples as would be expected if the virus was replicative in human host (Niklasson et al. 2007; Tapia et al. 2008; Tapia et al. 2010; Moore et al. 2015; Zhao et al. 2017). Finally, no epidemic findings (other than the original purported fatalities among athletes, which were assigned to LV without convincing evidence) have been recorded thus far. However, although no specific symptoms have been assigned to LV infection, LV seroprevalence was high in all patient cohorts analyzed here and previously, indicating that LV or an LV-like virus is circulating in the Finnish human population, apparently with null or minor disease associations.

It was shown that LV is widespread with a relatively high PCR-prevalence among many small mammals across Europe, even in some commensal species that come into close contact with humans. However, thus far, no LV isolates or nucleic acids have been recovered or detected from thousands of human sera or feces, despite picornaviruses being readily recoverable from these excreta (Stanway et al. 2000; Kapoor et al. 2008; Victoria et al. 2009; Tapparel et al. 2013). Therefore, it has not been possible to confirm whether LV isolates carried by wildlife are also transmitted to humans. In any case, the fact that LV seroprevalence is very high in younger patients, and wanes in later life suggests that LV transmission is unlikely to be rodent-borne and needs further investigation.

Future studies of LV should include sequences of LV genomes from wild and human hosts to understand isolate origin and transmission. In my opinion the only open question remaining about the association between the LV and human disease is that surrounding T1D, since, as mentioned in the Literature review, studies thus far have been inconclusive. A definitive resolution of this conundrum can be made by screening for LV in the months up to T1D onset, a task only possible by using samples from children followed over many years (before, during and after onset, as well as a control group). Studies sampling T1D patients in this manner are ongoing (e.g. https://teddy.epi.usf.edu/; Hagopian et al. 2011; Lönnrot et al. 2018), but do not at present include screening for LV.

ACKNOWLEDGEMENTS

Writing this note of thanks is the finishing touch on my dissertation. It has been a period of intensive learning for me, not only in the scientific arena, but also on a personal level. Writing this dissertation has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

Half of this study was carried out in the Department of Virology, at the University of Helsinki, and I would like to thank the head of the department, Professor Kalle Saksela, for providing appropriate working facilities. I thank the members of my thesis committee, Dr. Juha Laakkonen and Dr. Sisko Tauriainen, for their helpful comments and suggestions in general. I would like to express my gratitude to Dr. Laura Kakkola and Dr. Petri Susi for pre-examining this thesis and providing very helpful comments, which helped to improve this dissertation very much.

All co-authors of my thesis articles are thanked for their expertise and the help they provided. They are too many to be listed here, but I consider myself lucky for having been in touch with each one of them.

Many thanks to Dr. Olli Vapalahti for the financial support provided in many occasions.

Even though my time at the Meilahti campus was short, my stay there has been so enjoyable thanks to the whole "viral zoonosis group" who welcomed me into its laboratory and coffee room. Thanks for the brainstorming in the corridors, during lab work, and for the Friday meetings. And of course, thanks for always providing a reason for skumppa on Friday afternoons.
Mira Utriainen, Sanna Mäki, Irina Suomalainen, Johanna Martikainen and Kirsi Aaltonen are thanked for the technical assistance.

The other half of my doctoral project was carried out at the Fondazione Edmund Mach (FEM). I own many thanks to the lab staff there, Chiara Rossi and Matteo Girardi first, for invaluable help during the endless weeks spent in the lab together. The Fondazione has

become my second family during the past four years; I felt home among all the friendly faces I used to meet every day at work. Thanks to all the colleagues and friends for having made my time in FEM so pleasant.
Thanks to all my fellow “travelers” during this journey called Ph.D., with whom I have shared the best times, and the worst, in FEM but especially outside. At first we were just strangers arrived from the four corners of the earth, later we became family. We have all been there for one another, I have learned so much from every Ph.D. candidate, Masters student, postdoc, technician, technologist, and researcher met working at FEM (and I do not mean only professionally). I cannot express how privileged I consider myself for having crossed them on my path.

I also thank my best friends Elina and Francesca for providing all the encouragement and friendship that I needed. Thanks for listening to all my rants, especially in the last year of my PhD. And of course thanks to my mum and dad and my sister for the support and unconditional love.

However, the biggest thank of all goes to my Ph.D. advisors Heidi Hauffe and Anne Jääskeläinen, the two persons who have helped me the most during all this time. These two strong, amazing women are someone you will instantly love and never forget once you meet them. They are the funniest and sweetest advisors and among the smartest people I have ever met. They have been there for me throughout my entire Ph.D., helping and giving me inspiration even during tough times in the Ph.D. pursuit. The joy and enthusiasm they have for their research were contagious and motivational for me, and I hope that I could be as lively, enthusiastic, and energetic as Heidi and Anne during my whole career! They continuously provided insightful discussions about the research, and taught me, both consciously and unconsciously, how good research is done. It has been an honor to be their Ph.D. student.
Professor Emeritus Antti Vaheri also deserves thanks for his inspiring advises and guidance during these years. I appreciate all their contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating.

Heidi, I will never forget you words during one of our first weekly meeting during the first year of my Ph.D: "*it's not what you wish, but what you do that makes things happen*". I have made this my motto during these years. Thank you!

This research was funded by a FIRST PhD scholarship from the Fondazione E. Mach (FEM, Italy), EU grant FP7-261504 EDENext: Biology and control of vector-borne infections in Europe (to H Henttonen, Natural Resources Institute Finland (LUKE), Vantaa, Finland), the Center for International Mobility (CIMO, Finland), grant from Helsinki University Funding (Helsinki, Finland), Helsinki University Hospital (HUSLAB, Finland), Sigrid Jusélius Foundation (Helsinki, Finland), and Foundation for Research on Viral Diseases (VITUS, Finland), and additional sources to co-authors as listed in publications.

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