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Sindbis virus as a human pathogen: Epidemiology, virology, genetic susceptibility and pathogenesis

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ACADEMIC DISSERTATION

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”When I learn something new, I may change my mind– how about you?”
-Lord John Maynard Keynes

Contents

1. List of original publications

This thesis is based on the following publications. Some unpublished data are also presented.

I Sane J, Guedes S, Kurkela S, Lyytikäinen O, Vapalahti O. Epidemiological analysis of mosquito-borne Pogosta disease in Finland, 2009. Eurosurveillance 2010; 15:19462.

II Sane J, Guedes S, Ollgren J, Kurkela S, Klemets P, Vapalahti O, Kela E, Lyytikäinen O, Nuorti JP. Epidemic Sindbis virus infection in Finland: a population-based case-control study of risk factors. The Journal of Infectious Diseases 2011; 204:459-66.

III Sane J, Kurkela S, Putkuri N, Huhtamo E, Vaheri A, Vapalahti O. Complete coding sequence and molecular epidemiological analysis of Sindbis virus isolates from mosquitoes and humans, Finland. The Journal of General Virology 2012; 93: 1984-1990.

IV Sane J, Kurkela S, Levanov L, Nikkari S, Vaheri A, Vapalahti O. Development and evaluation of a real-time RT-PCR assay for Sindbis virus detection. The Journal of Virological Methods 2012; 179:185-188.

V Sane J, Kurkela S, Desdouits M, Kalimo H, Mazalrey S, Lokki ML, Vaheri A, Helve T, Törnwall J, Huerre M, Butler-Browne G, Ceccaldi PE, Gessain A, Vapalahti O. Prolonged myalgia in Sindbis virus infection: case description and *in vitro* infection of myotubes and myoblasts. The Journal of Infectious Diseases 2012; 206:407-14.

VI Sane J, Kurkela S, Lokki ML, Miettinen A, Helve T, Vaheri A, Vapalahti O. Clinical Sindbis alphavirus infection is associated with HLA-DRB1*01 allele and production of autoantibodies. Clinical Infectious Diseases 2012; 55:358-63.

The publications are referred to in the text by their Roman numerals. The original articles are reprinted with the permission of their copyright holders.

2. Abbreviations

AMA	antimitochondrial antibody
ANA	antinuclear antibody
Arbovirus	arthropod-borne virus
BFV	Barmah Forest virus
CHIKV	Chikungunya virus
CI	confidence/credible interval
CPE	cytopathic effect
ct	cycle threshold
EEV	eastern equine encephalitis virus
EIA	enzyme immunoassay
FAM	6-carboxyfluorescein
FCS	fetal calf serum
HI	hemagglutination inhibition
HLA	human leukocyte antigen
IF	immunofluorescence
IFN	interferon
Ig	immunoglobuline
IL	interleukin
MAYV	Mayaro virus
MEM	minimum essential medium
MGB	minor groove binder
MHC	major histocompatibility complex
NIDR	National Infectious Disease Registry
ONNV	O´Nyong Nyong virus
mOR	matched odds ratio
ORF	open reading frame
p.i.	post infection
PAR	population attributable risk
PCR	polymerase chain reaction
PFU	plaque forming unit
RF	rheumatoid factor
RRV	Ross River virus
RT	reverse transcriptase
SINV	Sindbis virus
VEEV	Venezuelan equine encephalitis virus
WEEV	western equine encephalitis virus

3. Abstract

Sindbis virus (SINV), an arthropod-borne, enveloped single-stranded RNA virus belongs to genus *Alphavirus* in the family *Togaviridae*. SINV is found in Eurasia, Africa, and Australia but clinical SINV infections occur mostly in Northern Europe and particularly in Finland where the infection is known as Pogosta disease. SINV epidemics have a peculiar cyclic appearance in Finland and since 1974, major outbreaks involving hundreds or even thousands of cases have occurred approximately every seven years. This study aimed to characterise descriptive, analytical and molecular epidemiology, to develop a PCR-based detection method and to study the pathogenesis and genetic susceptibility to SINV infection.

The epidemiology of a SINV outbreak in 2009 was described and compared to the outbreak pattern of previous major epidemics. The anticipated seven-year cycle did not recur as a relatively mild epidemic was observed. The data indicated that changes in grouse population and weather factors may contribute to the human epidemiology of SINV infection as speculated earlier and supported by another recent study. We identified risk factors for SINV infection in a large population based case-control study including 337 cases and 934 controls and extended the data on clinical features and patient characteristics. In the final multivariable logistic regression analysis, mosquito bites and spending time in woods or marshland remained as independent risk factors with a significant dose-response effect. Exposure to other arthropods was not positively associated with SINV infection. Population attributable risk for mosquito bites was 87.2%. A new estimate of the median incubation period of SINV infection was 4 days. The SINV-infected patients were found to have previous or ongoing medical conditions affecting joint or connective tissues more frequently than controls.

SINV was isolated from mosquitoes in Finland for the first time. Sequence analyses on the full-length coding sequences of all Finnish SINV strains, significantly expanding the available data on SINV sequences, demonstrated that the novel SINV isolate is closely related to Finnish human SINV isolates and to strains isolated previously from mosquitoes in Sweden and Russia. The data

further established that SINV has had a local circulation in the endemic Northern Europe during the past decades. Sequence analyses also identified amino acid signatures, particularly in the nsP3 protein, shared by Northern European strains that may be associated with vector or host species adaptation.

We developed a specific and sensitive real-time RT-PCR assay for the detection of SINV RNA with a detection limit of 9 copies/reaction. The assay demonstrated low levels of viraemia ($<10^3$ copies/ml) in sera of SINV-infected patients and only 12% of the serum samples were positive in the assay. Thus, the assay was found to have limited value as a diagnostic technique using serum samples but could be used for screening SINV in e.g. wildlife. These findings on low viral load may be associated with the pathogenesis and epidemiology of SINV infection.

The pathogenesis of myalgia was studied by examining a unique muscle biopsy obtained from a SINV-infected patient with chronic myalgia and arthralgia and by performing SINV infections of primary human myoblasts and myotubes. Evidence of muscle regeneration due to previous necrosis likely caused by earlier SINV infection was found in the biopsy. We further showed that human primary myoblasts and myotubes were susceptible in vitro for SINV infection. Overall, the study provided new information on SINV tropism and the mechanisms behind myalgia in SINV infection.

The role of *HLA* and complement *C4* genes in the susceptibility and outcome of SINV infection were investigated. The frequency of *DRB1*01* allele was significantly higher in patients with SINV infection than in the reference population (OR=3.3, P=0.003). The frequency of *DRB1*01* allele was particularly prominent among patients who at three years post infection experienced joint manifestations. The data further suggested that the combination of *HLA-B*35- DRB1*01* alleles and *C4B* deficiency may be associated with a more prominent or persistent form of the disease. A set of autoantibodies was measured in SINV-infected patients at the acute phase and three years post infection to assess autoreactivity. The frequency of rheumatoid factor at three years post infection was 29.5% and showed significant increase (P=0.02) during the three-year follow-up. The antinuclear and anti-mitochondrial antibodies were also present in serum three

years post infection with frequencies of 15.9% and 6.8%, respectively. Thus, the data demonstrated that symptomatic SINV infections show association to the HLA system and that autoantibody titres are elevated in patients three years post infection. These findings indicate that SINV-induced arthritis shares features with autoimmune diseases.

4. Review of the literature

4.1 Arthropod-borne viruses – emerging cause of epidemics

Numerous medically important viruses are arboviruses (arthropod-borne viruses). Almost all arboviruses are RNA viruses that have a potential to rapidly adapt to new vectors and hosts due to high genetic plasticity and mutation rates, which are characteristic features of RNA viruses. The viruses replicate in their arthropod vectors such as mosquitoes or ticks before transmission to vertebrates. These viruses circulate in wild animals and are occasionally responsible for major epidemics in humans. The ability of these infectious agents to cause disease in humans is associated with epidemiological and ecological factors, host genetics, as well as changes in viral genetics. The possible effects of ongoing global warming on the epidemiology of arboviruses have been investigated and it has been strongly suggested that global warming is associated with increased vector distribution and that it enhances the transmission potential particularly in temperate climates [207]. However, it has also been argued that climate change is not the most significant factor behind the increased incidence of vector-borne disease although the contribution is evident. Factors such as deforestration, decreased hygiene and importantly, increased frequency of travel to and from vector-borne disease endemic areas have a major impact [120,153]. Examples of increasingly important arboviruses that have during recent years caused large outbreaks include dengue virus, a major arboviral pathogen responsible for severe and sometimes fatal hemorrhagic fever/shock syndrome, West Nile virus (WNV), which caused a widespread epidemic in North America in 1999, and Chikungunya virus (CHIKV) (reviewed below in detail) [207].

4.2 General introduction to alphaviruses

4.2.1 Taxonomy and phylogeny

The family *Togaviridae* is comprised of two genera: *Alphavirus and Rubivirus*. The *Rubivirus* genus includes the causative agent of rubella (German measles), namely rubella virus. The *Alphavirus* genus is divided into seven antigenic complexes (Table 1) based on serological cross-reactivity and contains 29 recognized species [147, 149].

Table 1. The recognized species of *Alphavirus* genus [43,147].

Virus species	Antigenic complex	Abbreviation	Origin
Salmon pancreatic disease		SPDV	United Kingdom
Southern elephant seal		SESV	Australia, 2001
Barmah Forest	BF	BFV	Australia, 1974
Middelburg	MID	MIDV	South Africa, 1957
Ndumu	NDU	NDUV	South Africa, 1959
Getah	SF	GETV	Malaysia, 1955
Ross River	SF	RRV	Australia, 1959
Bebaru	SF	BEBV	Malaysia, 1956
Semliki Forest	SF	SFV	Uganda, 1942
Mayaro	SF	MAYV	Trinidad, 1954
Una	SF	UNAV	Brazil, 1959
Chikungunya	SF	CHIKV	Tanzania, 1953
O'nyong nyong	SF	ONNV	Uganda, 1959
Venezuelan equine encephalitis	VEE	VEEV	Venezuela, 1938
Mosso das Pedras virus	VEE	-	Brazil, 1978
Everglades	VEE	EVEV	Florida, US,1963
Tonate	VEE	TONV	French Guiana, 1973
Mucambo	VEE	MUCV	Brazil, 1954
Pixuna	VEE	PIXV	Brazil, 1961
Cabassou	VEE	CABV	French Guiana,1968

Rio Negro (Ag80-663)	VEE	RNV	-
Eastern equine encephalitis	EEEV	EEEV	Maryland,US,1933
Aura	WEE	AURAV	Brazil, 1959
Fort Morgan	WEE	FMV	Colorado, US, 1973
Highlands J	WEE	HJV	Florida,US, 1960
Sindbis	WEE	SINV	Egypt, 1952
Trocara	WEE	TROV	Brazil, 1984
Western equine encephalitis	WEE	WEEV	California, US,1930
Whataroa	WEE	WHAV	New Zealand,1962

A phylogenetic tree based on full-length genomes of alphaviruses shows similar grouping as the serological classification (Figure 1). Studies on the evolutionary history of alphaviruses have proposed that the origin of the genus could be either in the Old or the New World and several transoceanic introductions must have occurred to explain the current distribution [103,149]. Recombination, rarely occurring among alphaviruses, of Sindbis virus (SINV) and eastern equine encephalitis virus (EEEV) resulted in western equine encephalitis virus (WEEV) in the New World approximately 1300-1900 years ago [209]. A recent phylogenetic study, however, suggested that alphaviruses originate from the Pacific Ocean and thereafter spread to terrestrial vertebrate hosts and mosquito vectors in the New World as well as the Old World [43]. The proposed model was based on the finding that alphaviruses found in the ocean are at root positions in the phylogenetic trees indicating that they represent ancestral viruses. Since the ancestral alphaviruses assumingly spread between the continents long before human travel, it is likely that the zoonotic hosts, most likely birds, are responsible for virus dissemination across the world [43]. For instance, the geographical distribution of SINV, a member of the WEEV complex, shows apparent correlation to major bird migration patterns [115].

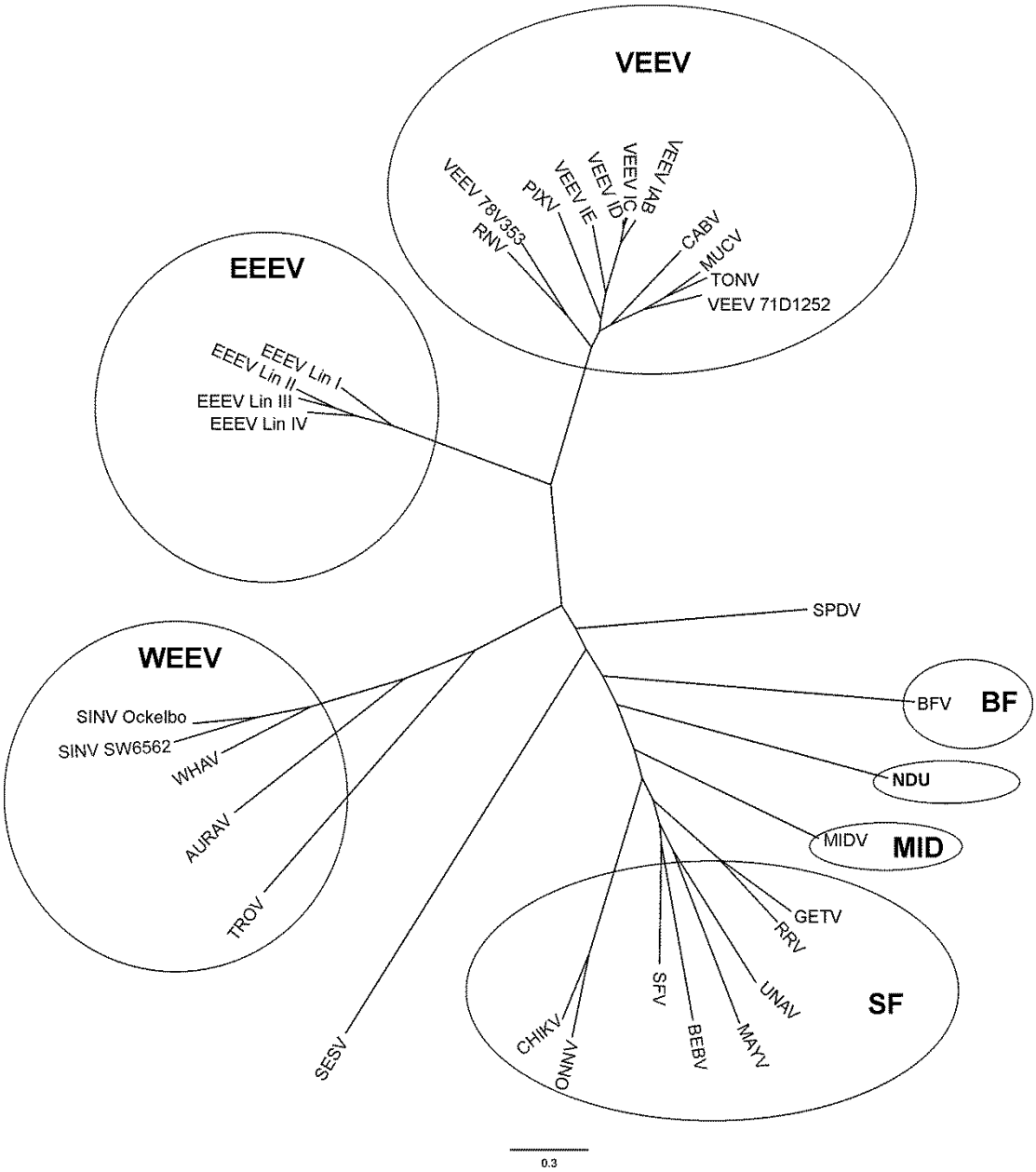

Figure 1. The radial layout of maximum likelihood phylogenetic tree based on full-genome alignment of the coding sequences of different species of alphaviruses and their subtypes. The antigenic complexes are indicated with circles and bolded text. To prevent bias, recombinant WEEV complex alphaviruses were excluded. The tree was constructed with maximum likelihood algorithm on RAxML program [181] using general time reversible model (GTR) with gamma distribution. The abbreviations of the viruses are clarified in Table 1.

4.2.2 Genome structure and replication strategy

Alphaviruses are enveloped, positive-sense single-stranded RNA viruses. The virions of alphaviruses are spherical and approximately 70 nm in diameter. The size of the genome is roughly 11.7 kb and includes 5´ teminal cap structure and 3`poly (A) tail [184]. It encodes two separate open reading frames that are separated by a short non-coding sequence. The 5´-region of the genomic RNA encodes four non-structural proteins (nsP1-nsP4) while the 3´-region, the subgenomic 26S RNA, is translated into structural proteins C, E3, E2, 6K and E1 [184,185] (Figure 2). The lipid bilayer, derived from the host cell, encloses heterodimeric protein spikes (n=80) composed of two envelope glycoproteins, E1 and E2, that are the immunodominant proteins. The interaction between E1 and E2 in a one-to-one relationship results in a firm structure across the membrane. Small quantities of the membrane-associated protein, 6K, are also found in virus particles and some alphaviruses contain a third envelope protein, E3. It has been suggested that E3 may have an enzymatic or functional role in virus assembly [142]. The nucleocapsid core consists of 240 copies of capsid proteins, which interact with the genomic RNA. Both nucleocapsid and envelope proteins are organized in a T=4 icosahedral symmetry [51,185,202].

The alphavirus life cycle starts with virus attachment to the receptors on the host cell surface (Figure 2). C-type lectins DC-(Dendritic Cell) and L-(Liver/Lymph Node) SIGN (Specific ICAM-3 Grabbing Non-Integrin), expressed in many early target cells of arbovirus infection including DCs and other cells of monocyte phagocyte system, have been implicated as attachment receptors for SINV [88]. In addition, heparan sulphate (HS) has been shown to act as a binding or capture receptor for several alphaviruses [217]. After the attachment, the virus enters the cell by receptor-mediated endocytosis in clathrin coated pits [33,67] where low pH triggers the destabilization of E1-E2 heterodimers [54,60]. Subsequently, the trimerisation of E1 occurs and its fusion peptides are exposed. As a consequence, viral and endosomal membranes fuse and the nucleocapsid core is disassembled and released into the cytoplasm of the host cell. The non-structural proteins are initially translated from the full-length genomic RNA. In most alphaviruses, the

translation results in two different polyproteins, P123 or larger P1234 that are proteolytically cleaved by viral proteases to yield individual proteins [185]. A study on SINV showed that the larger polyprotein occur as a result of translational read through of an opal termination codon. The read through takes place with only 10–20% efficiency and thus, P123 is the principal translational product [105]. However, some alphaviruses lack opal codon and only produce P1234 [188]. The non-structural proteins are responsible for the replication and formation of complementary minus-stranded RNA, which serves as a template for further synthesis of the positive-stranded RNA. The nsP1 protein, carrying RNA methyltransferase activity, is important in capping of viral RNA and initiation of minus-stranded RNA synthesis [8]. The nsP2 region encodes helicase and protease domains [161] whereas the nsP4 is the viral RNA polymerase [81]. The nsP3 is probably involved in the transcription process at an early stage of the infection but its role is still less well understood. A recent study found that the C-terminal proline-rich sequence motif of the nsP3 protein, common for many alphaviruses, serve as a target site for Src-homology 3 (SH3) domains of amphiphysin-1 and -2 and the SH3 domain-mediated binding of nsP3 with amphiphysin is important in promoting viral RNA replication [131].

Subgenomic mRNA is synthesized from the full-length intermediate minus-stranded RNA later in the infection and the translation of the mRNA produces the structural proteins. The capsid protein C is translated first and the assembly of the newly synthesized genomic RNA and capsid make the nucleocapsid [185]. The viral membrane glycoproteins E1 and pE2 (precursor to the E3 and E2) are assembled in the endoplasmic reticulum (ER) and cleaved by signalase [110]. pE2 is cleaved by furin in the Golgi and the E1-E2 heterodimers are then transferred to the plasma membrane. At the plasma membrane, nucleocapsid interacts with the cytoplasmic part of E2, becomes enveloped and the virion buds from the membrane [185].

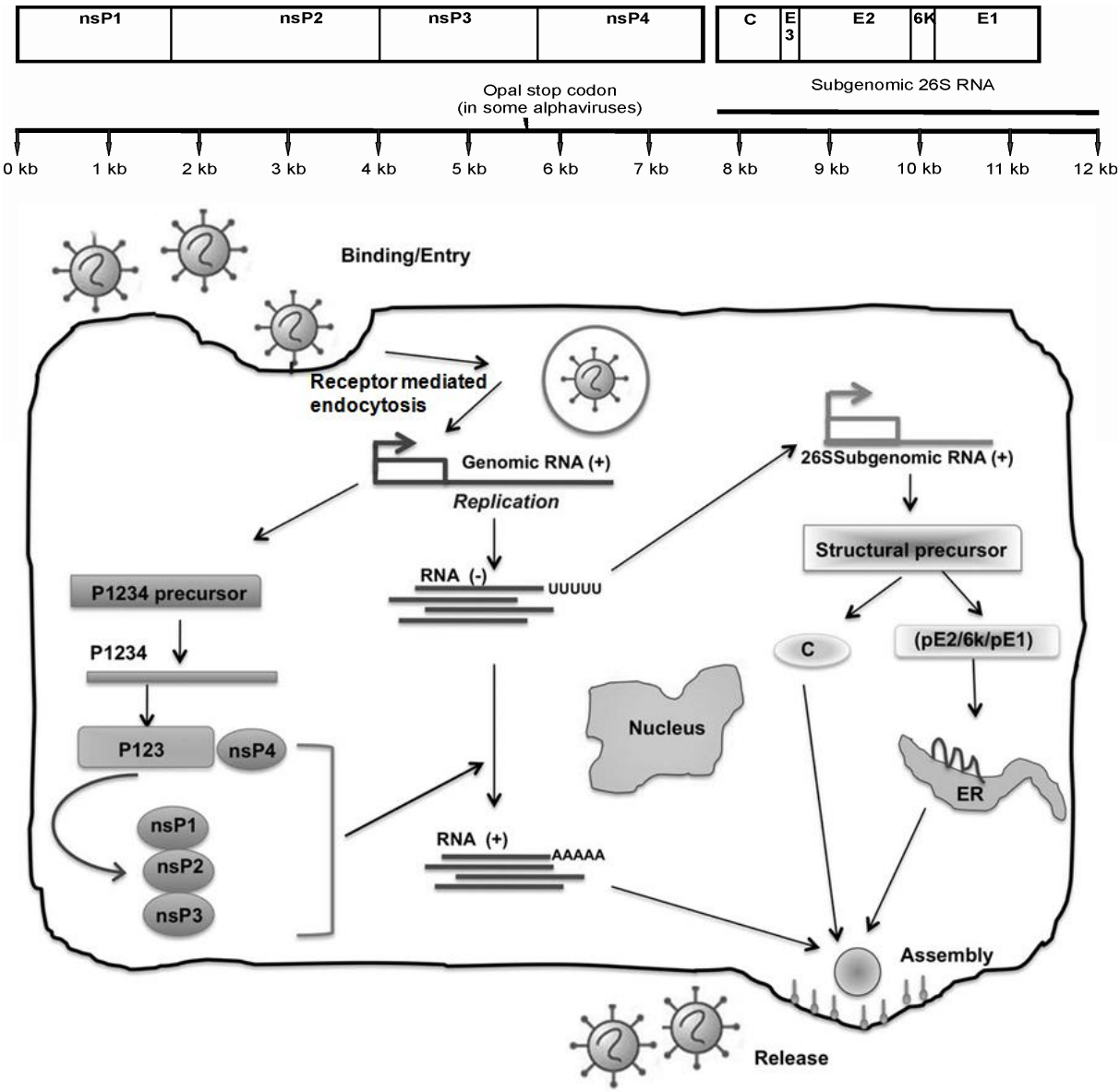

Figure 2. The lifecycle of alphaviruses. Modified from [193]. The insert above shows the organisation of the alphavirus genome.

4.3 Epidemiology of alphaviruses

Alphaviruses have been isolated throughout the world except in the Antarctica and are responsible for a wide range of diseases in humans and animals. The alphavirus genus includes two distinct subgroups, the Old World and the New World alphaviruses, based on the geographical distribution and the differences in

the associated clinical disease (Figure 3). The Old World alphaviruses including Ross River virus (RRV), O´Nyong Nyong virus, (ONNV), SINV, Barmah Forest virus (BFV) and CHIKV are arthritogenic viruses and characteristically cause a disease manifesting as febrile rash-arthritis with myalgia [186]. Semliki Forest virus (SFV), found in Africa, is classified as an Old World alphavirus but the association to arthritogenic disease is poorly described. Alphaviruses found in the New World including Venezuelan equine encephalitis virus (VEEV), EEV, and WEEV, are associated with severe neuropathogenic disease often resulting in encephalitis [149,216]. However, Mayaro virus (MAYV) cause arthritogenic disease and is sometimes grouped as an Old World alphavirus in the literature [149] due to the similarities in the clinical features but the virus is only found in the New World [103]. The neuropathogenic alphaviruses have been classified as possible biological weapons [35].

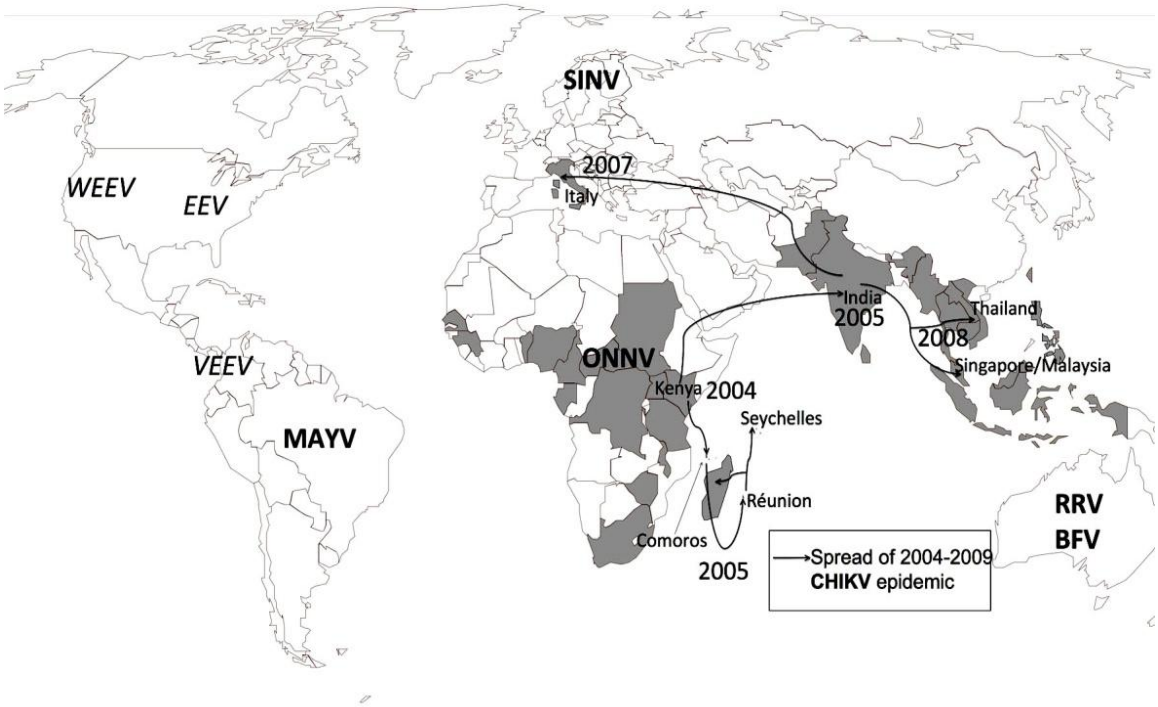

Figure 3. Geographical distribution of medically important alphaviruses. The areas where most of the human infections of each virus have been reported are indicated with the virus name. The arthritogenic alphaviruses are bolded and the neuropathogenic viruses marked with italics. Countries where people have become infected with CHIKV are highlighted with grey and the arrows show the spread of the CHIKV epidemic in 2004–2009. Data obtained from [148,182,206,207].

4.3.1 New World alphaviruses

WEEV is found in the western U.S. and South America, and statistics from the Centers for Disease Control and Prevention [6] show that 640 human cases have been reported in the U.S. from 1964 to 2010. However, no cases have been reported during the last decade. The mortality in humans is 3-7% [216]. EEV infections are reported in the eastern and northern U.S. The human mortality is high, up to 75%, and in 1964–2010, 270 human cases were reported in the U.S. (10 cases were reported in 2010) [6]. The distribution of VEEV includes Central America, and the northern and eastern parts of South America [208]. Severe encephalitis in humans infected with VEEV is rare compared to EEV and WEEV and the human mortality is approximately 1% [208]. Horses in particular are susceptible to neurological disorders caused by VEEV. However, VEEV was responsible for a major outbreak in 1995 in Venezuela and Colombia where up to 100,000 human cases were reported [210]. MAYV has been associated with several small sporadic epidemics of rash-arthritis in South America, particularly in Brazil and Bolivia [192].

4.3.2 SINV epidemiology in Finland

SINV and antibodies to SINV are widely found in wildlife and in humans across Eurasia, Africa and Oceania [74,206]. However, clinical SINV infections have been reported mostly in Northern Europe and particularly in Finland where the infection is known as Pogosta disease [96]. The underlying factors explaining the high incidence in Finland are unclear. SINV epidemics in Finland have emerged in approximately seven-year cycles with hundreds or even thousands of reported annual cases (Figure 4) [18,96]. The first epidemic took place in 1974, and the largest epidemic occurred in 1995 when 1,310 serologically confirmed cases were reported. Since 1995, all laboratory-confirmed cases of SINV infection have been reported to the National Infectious Disease Registry (NIDR) at the National Institute for Health and Welfare (THL). Two accredited laboratories in Finland (in Helsinki and Turku) perform serological testing for SINV, of which approximately

70-80% is performed in Helsinki. SINV infections occur mostly in August and September. It has been estimated that the age-standardised seroprevalence of SINV infection in Finland is 5.2% and that the prevalence as well as the average annualised incidence have been highest in North Karelia in eastern Finland [94]. The incidence has been highest among females aged 50–59 years and the seroprevalence increases with age peaking at the age group 60-69 [94]. It has been estimated that there are 17 times more subclinical than symptomatic SINV infections [18]. A recent study indicated that certain climatic conditions such as temperature and precipitation as well as the density of hatch-year black grouse are significant determinants of occurrence and incidence of human SINV infections in Finland [75].

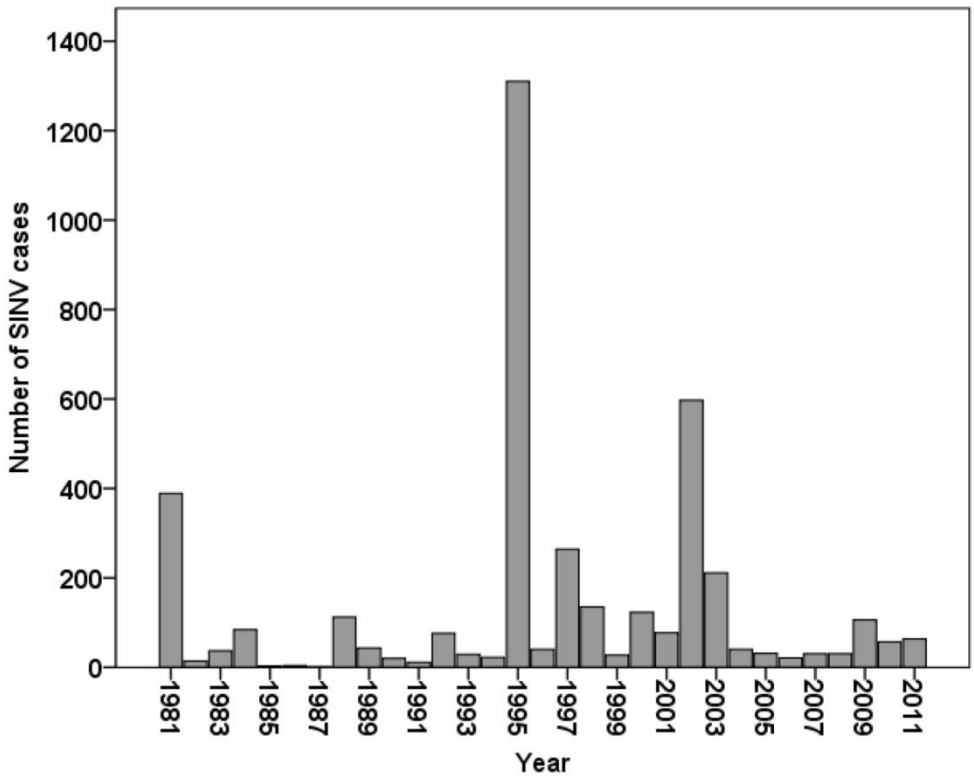

Figure 4. The number of serologically confirmed SINV infections in Finland annually, 1981-2011. Data from National Infectious Disease Registry (1995-2011) and [18] (1981–1994, diagnoses made at the Department of Virology, University of Helsinki, which accounted for 70–80% of all laboratory confirmed SINV infections in Finland).

4.3.3 Other Old World alphaviruses

RRV is responsible for approximately 5,000 and BFV for 1,000 human infections annually in Australia [7]. ONNV is found in Africa and during 1959–1962, the virus caused a major outbreak that began in Uganda and roughly 2 million cases were reported in Kenya, Tanzania and Uganda [214]. After years of silence with only a few sporadic cases reported, ONNV re-emerged again in Uganda in 1996 [162]. Outbreaks of CHIKV have occurred sporadically in Africa, Southeast Asia and India during the past 50 years [148,182]. The outbreaks have mostly been relatively mild but in 2004, a massive re-emergence of CHIKV started from the coast of Kenya [148,169].

4.3.4 Re-emergence of Chikungunya in 2004–2009 – more to come?

After the appearance of CHIKV in Kenya in 2004, the epidemic spread to the Island of Comoros in 2005 where 6,000 cases were reported (Figure 3). The massive outbreak spread further to other islands in the Indian Ocean, including Madagascar, Mayotte, Mauritius and Réunion [148]. The burden of CHIKV infection was particularly high on the island of Réunion where approximately 300,000 cases were reported, representing more than one third of the population of the island [169]. At the end of 2005, a separate epidemic caused by the same viral genotype spread from East Africa to India where millions of people became infected [207]. During 2006–2009 CHIKV spread further to Southeast Asia [62] and also to Europe (Emilia-Romagna, Italy) where local transmission of CHIKV had never occurred before [203]. More than 200 cases, including one fatal case, were reported in this north-eastern part of Italy in 2007. Further expansion of the outbreak was at least partly limited due to the relatively rapid implementation of an active surveillance system [203]. Overall, the case-fatality rate during the explosive outbreak of CHIKV was estimated to be between 0.1– 4.9% [156].

Lack of herd immunity in humans and in unknown hosts in the new areas where CHIKV emerged may partly explain the magnitude of CHIKV re-emergence. Probably the most important factor, however, was a single point

mutation that resulted in a change of amino acid (alanine to valine) at the position 226 of the E1 protein [169]. This change facilitated the adaption of the virus to a new vector, *Aedes albopictus*, and enhanced CHIKV transmission in areas where the distribution of the classical CHIKV vector, *Aedes aegypti*, was limited. Currently there is no evidence that the A226V mutation affects the viral virulence in humans. Due to the increased distribution of *Aedes albopictus*, assisted by tyre shipments [154], it is considered highly likely that CHIKV could spread further on and possibly even become endemic in Europe or in tropical America. This mosquito species was for example only recently found in Germany for the first time ever [211]. The spread of CHIKV, and other arboviruses, is further aided by the increased global travel, deforestation and widespread tropical urbanization [55,207].

4.3.5 Vectors, reservoir hosts and transmission

The geographical distribution of individual alphaviruses is constrained by certain ecological factors including reservoir host and vector restrictions. Genetic changes in viruses may enable vector adaptation and thus lead to emergence of alphaviruses to new geographical areas as was shown for CHIKV. Alphaviruses are mainly transmitted by various mosquito species, and numerous vertebrate species are known to be associated with the transmission cycle. However, trout and salmon infecting salmon pancreatic disease virus (SPDV) and its subtype sleeping disease virus (SDV) as well as Southern elephant seal virus (SESV) do not have established arthropod vectors although it has been speculated that sea louse, *Lepeophtheirus salmonis*, may be involved in the transmission of SPD*V* [43,149].

Ornitophilic mosquito species, *Culex* and *Culiseta,* have been suspected to be the primary vectors of SINV [45,135]. However, also *Aedes* species are involved since SINV has been isolated from *Aedes* mosquitoes [45,117]. SINV has also been isolated from ticks in Italy [57] but it has been unclear whether other arthropods could transmit the virus. SINV antibodies have been detected in grouse [18,94] and studies have hypothesized that grouse, as probable amplifying

hosts, may play a role in the occurrence of SINV outbreaks because SINV outbreaks have previously coincided with a decrease in grouse populations [111]. Migratory birds have also been found positive for SINV antibodies, thus suggesting that migratory birds may also play a role in the distribution of the virus [20, 94].

For RRV the principal vectors are the mosquitoes *Culex annulirostris* and *Aedes vigilax* and the vertebrate hosts include native marsupials [64,136]. BFV is transmitted by *Aedes* and *Culex species* [15,204] and ONNV by *Anopheles* [27,215] but the reservoir host is currently unknown for both viruses. The vectors of CHIKV in the urban transmission cycle in Asia, where humans act as major hosts, are *Aedes aegypti* and *Aedes albopictus* [126,127,150]. *Aedes albopictus*, as described above, was particularly involved in the recent CHIKV outbreak. Although similar urban transmission does also occur in Africa, African CHIKV circulates predominantly in an enzootic cycle, where non-human primates are the likely hosts and primatophilic *Aedes* mosquitoes act as principal vectors [34].

MAYV is transmitted by *Haemagogus* species and monkeys are the main amplifying hosts of the virus [72]. *Culex tarsalis* is the primary vector for WEEV and rodents as well as birds, such as house finches, have been indicated as reservoir hosts [63]. Birds and rodents have also been suggested as hosts for VEEV, which is transmitted by various mosquito species including *Aedes, Culex* and *Psorophora* species [208]. VEEV has a distinct enzootic and epidemic/epizootic cycle in which the epizootic strain arises by mutation from the enzootic virus [202] (Figure 5). EEV is primarily transmitted by *Culex melanura* in North America, where passerine birds are considered main hosts, and probably by *Culex melanoconion* in South America, where rodents possibly function as vertebrate hosts [171].

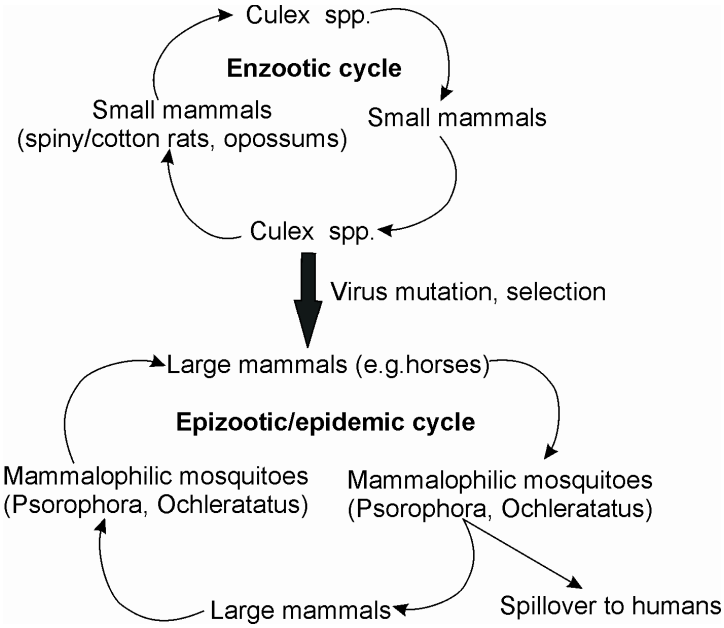

Figure 5. Transmission cycle of VEEV illustrating an example of an alphavirus for which epizootic/epidemic strains are generated by mutations from the enzootic viral strains resulting also in human infections. Modified from [206].

4.4 Clinical features of arthritogenic alphaviruses

4.4.1 Acute symptoms

Arthritogenic alphaviruses SINV, CHIKV, RRV, MAYV, ONNV and BFV cause disease typically presenting with fever, (poly)arthralgia or arthritis, myalgia, maculopapular rash, fatigue and headache [85,95,144,145,148,180,186,190,192,197]. In general, infections caused by CHIKV are considered most severe. Table 2 shows a comparison of the clinical features of acute CHIKV and SINV infections. The typical incubation period for arthritogenic alphaviruses ranges from 1 to 12 days [49,182,186]. For example in SINV infection, the incubation period was estimated to be 8–9 days based on one patient case [95]. The joints most commonly affected include knees, ankles, feet, fingers, and wrists [95,180,186,190]. Lymphadenopathy has been reported in CHIKV [187], ONNV [85], MAYV [192] and RRV infections [186] and a single case

of glomerulonephritis has been described both in RRV and BFV infections [47,83]. Two cases of foethal deaths in women who at the time had serologically confirmed acute SINV infection have been reported but the causality between SINV infection and the delivery of stillborn children is unclear [18]. Regarding gender distribution of arthritogenic alphavirus infections, no significant differences have been reported in general, but studies suggest that clinical SINV infection is more common among females than males [95,197].

During the recent CHIKV epidemic, atypical symptoms of CHIKV infection such as meningoencephalitis, myocarditis, pericarditis, nephritis and retinitis were for the first time associated with CHIKV and primarily observed in children, in the elderly and in patients with comorbidities [36,152]. Furthermore, more than 200 deaths were reported during the epidemic on the islands in the Indian Ocean [12].

Table 2. Clinical features of acute SINV and CHIKV infection: +, typical symptom; +/-, occur occasionally. Data from [95,180,182,190,197]

	CHIKV	SINV
Fever	+	+
Joint symptoms	+	+
Myalgia	+	+
Headache	+	+
Gastrointestinal symptoms	+	-
Rash	+	+
Fatigue	+	+
Petechiae and other mild haemorrhages	+/-	-
Severe complications	Neurological complications, hepatitis, myocarditis, pericarditis, acute renal failure, ocular manifestations	-
Leukopenia	+/-	-
Thrombocytopenia	+/-	-
Elevated liver enzymes	+/-	-
Subclinical infection	3-25 %	Occurs frequently

4.4.2 Persistent sequelae

The symptoms in the arthritogenic alphavirus infections are generally self-limiting but the viruses can cause prolonged joint manifestations that are particularly important from a public health perspective. A recent study on SINV indicated that one year after an acute SINV infection, 50% of the patients had joint symptoms and three years post infection (p.i.), 25% of the patients still suffered from joint pain attributable to previous infection and 4% had arthritis [93]. Persistent joint symptoms in SINV infection have also been observed in earlier studies [99,133,134,197]. Persistent, debilitating polyarticular arthralgia [13,16,151] as well as arthritis [44] have been reported in CHIKV infection. Prolonged joint manifestations also occur in RRV [25,186], BFV [41] and MAYV infections [191]. Long-term sequelae of ONNV infection are currently poorly known.

4.4.3 Diagnostics

Laboratory tests for arthritogenic alphaviruses are available in several countries and primarily based on serological assays that detect IgM and IgG antibodies including enzyme immunoassays (EIA), haemagglutination inhibition (HI) test, neutralization tests, indirect immunofluorescence (IFA) or immunoblot assays [76,100,118,159,182,186,200]. In the laboratories in Finland, EIA is mainly used in diagnostics. For the laboratory-confirmed diagnosis, seroconversion in paired serum samples and/or positive IgM result in EIA in a single serum sample is usually required [100,118,186]. For example in SINV infection, IgM and IgG antibodies become detectable within the first 8 and 11 days of illness, respectively [95]. Several methods based on viral RNA detection with PCR have also been developed [73,100,102,141,172] but mainly for CHIKV, since CHIKV infections, unlike SINV or RRV infections, cause high levels of viraemia that can last for days [100,141]. Figure 6 illustrates the diagnostic findings in CHIKV infection during the first days of symptoms [141].

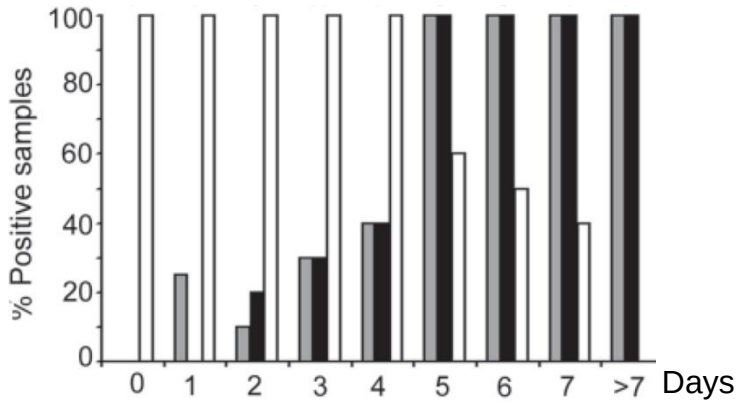

Figure 6. The percentage of IgM (grey bar), IgG (black) and viral RNA (white) positive samples during the acute phase of CHIKV infection. Reprinted from [141] with permission.

4.4.4 Treatment and prevention

Alphaviral arthritides are treated symptomatically as no specific antivirals are available. Nonsteroidal anti-inflammatory drugs (NSAID) can be used. However, a study in mice found that certain steroids are effective and selective inhibitors of alphaviruses [107], and a recent in vitro screening study identified compounds that are able to inhibit entry and replication of CHIKV replicons and infectious SFV [146]. Furthermore, a combination of ribavirin and recombinant interferon alpha (IFN-α) was found to have an anti-CHIKV and anti-SFV effect *in vitro* [17] .

The most efficient way to prevent alphavirus infections is to avoid mosquitoes using repellents, nets and suitable clothing [64]. Human vaccines against arthritogenic alphaviruses are currently not available although following the recent CHIKV outbreak, vaccine development against CHIKV has advanced. A study by the US army showed earlier that a live attenuated CHIKV vaccine was effective in safety and immunogenicity studies [37] and recently, a vaccine based on virus-like particles was shown to protect nonhuman primates against CHIKV [9]. The current understanding is that alphavirus infection confers lifelong immunity against repeat infections [206].

4.5 Pathogenesis of arthritogenic alphaviruses

Studies on the pathogenesis of arthritogenic alphaviruses have focused on arthralgia/arthritis, but also the mechanisms behind myalgia have recently been investigated. It has previously been implied that virus replication and subsequent cytopathic effect (CPE) in target tissues would be the major cause of pathogenic events, but several studies have recently highlighted the significant role of indirect mechanisms involving the immune-mediated pathogenesis, particularly in the persistence of arthralgia and myalgia [108,160,170,186]. The vast majority of the studies addressing the pathogenesis of arthritogenic alphaviruses have been performed on RRV and CHIKV.

4.5.1 Cell and tissue tropism

SINV is considered the prototype member of alphaviruses and has been widely used in studies of neural pathogenesis of alphaviruses in mice [58,89,175,195]. These studies focusing on central nervous system (CNS) manifestations have showed that SINV replicates in neural tissue causing encephalitis [58]. However, SINV is not associated with CNS symptoms in humans, and the studies on neural pathogenesis of SINV in mice are not further reviewed in this thesis summary. Yet, mouse models of SINV infection have also shown that in mice infected subcutaneously, virus replication is detected in skeletal muscle, showing onset of myositis, skin and connective tissue adjacent to articular joints [66,89,195]. A recent study found that SINV is able to replicate in human macrophages [10]. RRV has been shown to target bone, joint and skeletal muscle tissue in a mouse model [125], and in humans, RRV RNA has been detected in synovial fluid samples [178]. Moreover, macrophages have been found susceptible for RRV infection [79,112,205]. RRV infection in the skeletal muscle tissue of humans has not been directly demonstrated.

A study characterizing the tropism of clinical CHIKV strains from the recent epidemic found that CHIKV replicates in several human cells including epithelial and endothelial primary cells, fibroblasts as well as monocyte-derived

macrophages [179]. This *in vitro* study also found that peripheral blood mononuclear cells including B cells, T cells and monocytes were not susceptible to CHIKV infection [179]. However, another study concluded that blood monocytes are the main targets of CHIKV infection in the acute phase of illness as the cells were found susceptible *in vitro* and CHIKV-antigen positive monocytes were isolated from a patient with an acute CHIKV infection [68]. CHIKV has also been found in perivascular macrophages in a patient with chronic symptoms 18 months p.i. [71]. Replication of CHIKV has been evident *in vitro* in human muscle satellite cells, but not in differentiated myotubes [139]. Furthermore, CHIKV antigens were detected in the satellite cells of muscle biopsies from two patients with myositis [139]. In a non-human primate model, CHIKV was found to infect joints, muscles, lymphoid tissue and liver, and persistent infection was observed in macrophages [98].

4.5.2 Role of innate immunity

Antiviral cytokines, type I interferons IFN-α and –β, are abundantly produced, mainly by leukocytes (IFN-α) and fibroblasts (IFN-β), in various alphavirus infections [206]. *In vitro* studies have shown that fibroblasts [168] and monocytes [68] infected by CHIKV produce high levels of type I IFNs that are able to control the infection by binding to the IFN- α/β receptor (IFNAR) which then initiates an antiviral effector programme in infected and neighbouring cells. In patients with chronic CHIKV infection, high levels of IFN-α mRNA have been observed in blood mononuclear cells [71]. Inefficient type I IFN signalling was recently shown to cause severe CHIKV-associated disease in mice [28], highlighting an important role of IFN system in antiviral defence. RRV was found to induce type I IFN production in myeloid dendritic cells [174] and the induction of IFN- α/ β has also been observed in SINV mouse models [194]. NK cells, which have an important role in innate immunity against viruses, have been detected in inflammatory infiltrates around joint and skeletal muscle both in CHIKV and RRV mouse models [52,125]. NK cells have also been observed in patients with CHIKV [71] and RRV infection [48]. Furthermore, a study showed that the activation of complement

system, also a fundamental part of the innate immune response, contributed to inflammatory tissue destruction in RRV infection [124]. The role of complement factor C3 and its receptor was highlighted as a subsequent study demonstrated that mice deficient in complement receptor 3 (CR3) developed milder disease and reduced tissue destruction compared to RRV-infected wild-type mice. CR3 deficiency had no effect on viral replication [123].

Recent studies have highlighted the crucial role of macrophages and proinflammatory cytokines/chemokines in the pathogenesis of arthritogenic alphaviruses supporting the view that alphavirus-induced arthritis/arthralgia and myalgia is an inflammatory, immune-mediated disease. The major constituent of the inflammatory infiltrates in the skeletal muscles of RRV-infected mice was found to be macrophages [122,125] and by using compounds toxic to macrophages, muscle inflammation was prevented and the clearance of macrophages was found to correlate with the recovery [109]. Mouse and non-human primate models of CHIKV infection have also showed a high degree of macrophage and monocyte infiltration in the infected tissues (joints in particular) [52,98] and demonstrated that depletion of macrophages improved the rheumatic disease [52]. As reviewed above, CHIKV, RRV and SINV can infect human macrophages and it has been shown that particularly the macrophage-derived proinflammatory factors triggered by the infection are critical factors in the development and persistence of joint and muscle manifestations [52,108]. It was recently shown that upregulation of macrophage migration inhibitory factor (MIF), which also has a recognized role in the pathogenesis of rheumatoid arthritis (RA), is associated with severe inflammatory disease in RRV-infected mice [70]. MIF-deficient mice, however, developed only a mild disease. Elevated levels of proinflammatory cytokines and chemokines including tumour necrosis factor-α (TNF-α), IFN-γ, and macrophage chemoattractant protein-1 (MCP-1) have been detected in mouse model of RRV and in synovial fluid of RRV-infected patients [108]. These factors as well as interleukin- 6 (IL-6) were also found to be increased in CHIKV mouse model [52]. In patients with acute CHIKV infection, an increase in IL-1β, RANTES and IL-6 was associated with the disease severity [132] and in a longitudinal case-control study, persistent arthralgia was found to

be connected with high levels of IL-6 and granulocyte macrophage colony-stimulating factor [24]. Furthermore, secretion of MIF, TNF-α, IL-1β and IL-6 was induced by SINV infection in human macrophages [10].

4.5.3 Role of adaptive immunity

CD4+ and CD8+ T lymphocytes have been detected in inflammatory infiltrates of RRV-infected and CHIKV-infected mice, although to a lesser extent than macrophages and NK cells [52,125]. In addition, CD4+ and CD8+ T cells have been detected in human synovial biopsies taken from patients with RRV-infection [48]. The role of adaptive immune response in the development of RRV-induced disease was investigated with RAG-1-deficient mice lacking functional T and B cells [125]. It was found that such mice developed a similar inflammatory disease characterized by a more widespread myositis than wild-type mice. Therefore, it is believed that the adaptive immunity does not play a major role in the immunopathogenesis of arthritogenic alphaviruses.

4.6 Host genetic factors in arthritogenic alphavirus infection

Genetic factors are important in the predisposition to several diseases. Regarding viral diseases in general, the susceptibility as well as the course of disease is often determined by a combination of environmental, pathogen-related and host-genetic factors. Some studies have found robust human leukocyte antigen (HLA) associations to viral diseases such as the human immunodeficiency virus (HIV) infection [22] and Puumala hantavirus infection [91,129] but only a few studies on the role of host genes in alphavirus infections have been conducted. It was recently shown that persons with rhesus (Rh) positive blood are more susceptible to CHIKV infection than individuals with Rh-negative blood [92,113]. Furthermore, a few studies have investigated the possible HLA association to SINV, CHIKV and RRV infections. These studies and the function of the HLA system are reviewed in detail below.

4.6.1 Major histocompatibility complex (MHC)

MHC gene region, also called HLA in humans, on the chromosome 6, consist of approximately 240 genes of which roughly 130 are functional [87]. Of these functional genes, more than 40% contribute to immune response [1]. When a pathogen invades the human body, the immune system recognizes the intruder and initiates an adaptive immune response. MHC molecules are needed for presenting peptides of viral antigens, which is the initial step for the activation of T cells. There are two classes of MHC molecules that play a major role in antigen presentation, namely MHC class I and class II. The genes encoding for the MCH class I and II are extremely polymorphic, which ensures that each individual can present a broad repertoire of antigenic peptides to T cells. It is thought that selection for resistance to infections drives the generation of MHC variation [196]. The classical MHC class I molecules, *HLA-A, -B,* and *-C*, present products derived from endogenous proteins in the cytosol to cytotoxic CD8+ T cells. MHC class II molecules, *HLA-DR,-DQ,* and and *-DP*, present exogenous antigens, taken from the extracellular environment by phagocytosis or endocytosis, to CD4+ T cells, which leads to cytokine and antibody production [86,87] (Figure 7). However, cross-presentation of antigens also occurs [121]. Each MHC class I molecule consist of α-chain, encoded by the respective *HLA* genes, and β2-microglobulin originating from chromosome 15. MHC class II molecules include one α-and β-chain but *HLA-DR* β-chain is encoded by 4 loci unlike *HLA-DP* and *HLA-DQ* (Figure 7). The class I molecules are expressed on the surface of all cells, except erythrocytes, but the expression of class II proteins is limited to certain antigen-presenting cells such as DCs, B cells and macrophages [86,87]. In addition, the MHC region also includes class III molecules that are mostly proteins with immune functions. The most polymorphic genes within the class III molecules are complement *C4A* and *C4B* genes that encode complement C4 protein, which has an important role in the activation of the classical pathway of the complement system [23]. The majority of humans have two C4 loci coding for C4A and C4B proteins, respectively (Figure 7). C4A and C4B proteins differ in their chemical

reactivities. C4B displays higher affinity for antigens containing hydroxyl group, whereas C4A shows stronger affinity for amino-group-containing antigens [23].

Strong associations with MHC genes have been found in more than 100 diseases, of which many are autoimmune diseases and only a few are infectious diseases [53,176,196]. Regarding arthritogenic alphavirus infections, the role of *HLA* alleles in the occurrence and consequence of RRV infection has been reported in one study. The prevalence of HLA-DR7 antigen was found to be higher among RRV patients than controls indicating a probable association [50]. A study on patients with chronic CHIKV infection showed that *HLA-DRB1*01* and *DRB1*04* alleles were frequently found among patients who also developed RA post infection [14]. The role of HLA alleles *B*27* and *DRB1*04* was earlier investigated in a study on 21 patients with SINV infection but no association was found [99].

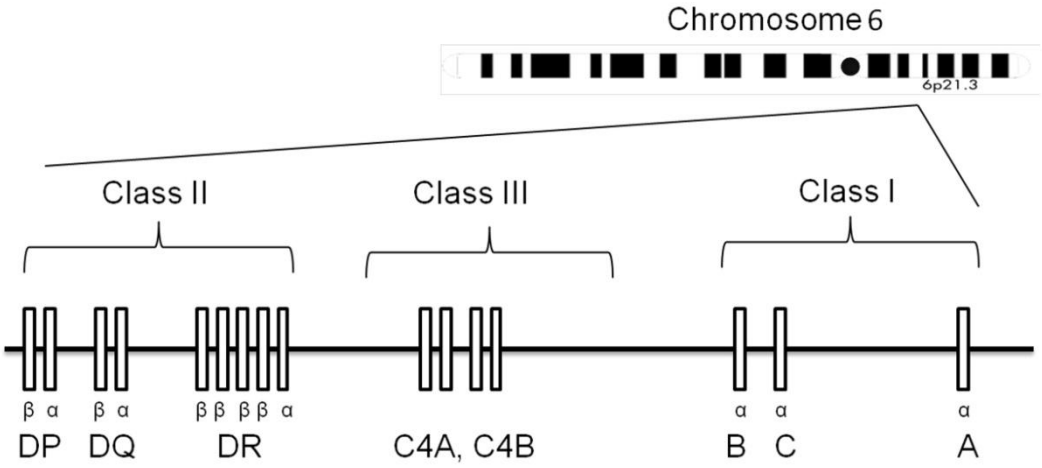

Figure 7. Localisation of the MHC genes on chromosome 6. The major HLA genes as well as the C4 genes are shown.

5. Aims of the study

The specific aims of the study were:

- To characterise epidemiology and to identify risk factors for SINV infection in Finland
- To study the molecular epidemiology of Finnish SINV strains isolated from patients with SINV infection and from mosquitoes
- To develop and evaluate a real-time RT-PCR assay for the detection and diagnostics of SINV infection
- To study factors behind pathological processes leading to myalgia in SINV infection
- To study the role of genetic factors in the susceptibility and outcome of SINV infection
- To study the autoreactivity in SINV infection

6. Materials and methods

The materials and methods used in the study are described below.

6.1 Study materials

6.1.1 Ethics statement

Ethical approval for this study was obtained from the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (permission nr 127/13/03/00/2009). A written informed consent was received from the patients. For in vitro experiments in V, informed consent was obtained from all patients or legal representatives prior to the tissue being donated to the tissue bank, in accordance with the French legislation on bioethics.

6.1.2 Patients and controls in a case-control study (II)

A case-patient was defined as a person with rash-arthritis in whom the diagnosis of acute SINV infection was confirmed by serology and notified to National Infectious Disease Registry (NIDR) between July 15 and October 22, 2002. The criteria for confirmed laboratory diagnosis were seroconversion in paired serum samples and/or positive IgM result in EIA [118]. The presence of SINV antibodies was also confirmed using HI test in some cases. Notified cases were excluded from the study if they reported a previous physician-diagnosed SINV infection in a questionnaire or were absent from their permanent place of residence during the 10-day exposure period before the onset of first symptoms of SINV. Cases reported from the health district of North Karelia were excluded due to another ongoing study on SINV infection in the area [95].

Healthy control subjects were selected among the general population. For every enrolled case-patient, persons who matched the patient according to year of

birth, sex, and postal code or residency were listed from the National Population Information System. Control subjects who reported in a questionnaire rash illness or arthralgia during the two months prior to data collection, or a previous physician diagnosed SINV infection, were excluded from the study. Furthermore, persons who were absent from their permanent place of residence for more than a day during the 10-day period before the respective case-patient's s onset of illness were excluded.

6.1.3 Blood specimens (IV and VI)

Patient serum samples (n=44) from the acute phase of SINV infection and 3 years p.i. as well as whole-blood samples from the acute phase (n=35) collected during the earlier follow-up cohort study [93] were used in the original publications IV (24 of the 44 serum samples) and/or VI. Additionally, acute-phase serum samples from SINV-infected patients (n=34) and SINV-negative controls (n=24) collected during an outbreak in 2009 were used in the original publication IV. The serum samples were obtained from the Department of Virology and Immunology of Helsinki University Hospital Laboratory (HUSLAB), which is a major diagnostic laboratory in Finland. The sera were aliquoted and stored at -70°C or -20°C.

6.1.4 Patient case with persistent SINV infection (V)

The patient participated in the study by personally contacting the investigators during an ongoing study recruitment. The study patient was a previously healthy 51-year old male from eastern Finland with prolonged symptoms of SINV infection. In September 2009 the patient had rash and swelling and prominent tenderness in wrists and ankles as well as in knees, elbows and shoulders at a later stage. Other symptoms of acute SINV infection included fever, lower back pain, headache, dizziness, and fatigue. The patient had been exposed to mosquitoes earlier during outdoor activities. The musculoskeletal symptoms resulted in diminished walking and hand function. SINV IgM and IgG antibody seroconversion, determined with EIA [118], was seen between paired sera in the

acute phase. At six months p.i. the following data and specimens were obtained from the patient: serum samples, leukocyte samples, muscle biopsy specimen taken from deltoid muscle, questionnaire, and results from blood parameters. Monocytes were further isolated from blood by a previously described method based on Percoll density gradient [157]. IgM and IgG titres in serum were determined with end-point titration.

6.1.5 Database material (I,VI)

The notifications of laboratory-confirmed cases of SINV infection reported to the NIDR included information on date of sample collection, date of birth, sex, and place of treatment. Multiple notifications of persons with identical birth date, sex and place of treatment received within a 12-month period were combined as one case. Data were analysed by sex, age, week and month of disease onset and by hospital district of treatment.

Data available on 90 Finns from bone marrow donor registry [4] and on healthy 150 voluntary individuals from an occupational health survey [173] were used as reference populations for *HLA* allele and *C4* gene number frequency comparisons (VI).

6.1.6 Cell lines (III, IV, V)

Cells lines including Vero and Vero E6 cells lines (African green monkey kidney epithelial cells), grown in cell culture flasks in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics, were obtained from the American Type Culture Collection (ATCC). The human myogenic precursor cells, myoblasts, were initially isolated from the quadriceps from three different healthy donors as previously described [31]. The cells were provided by the AFM Tissue Bank (Paris, France). Myoblasts were grown in Ham's F-10 nutrient mixture with Glutamax medium (Gibco/Invitrogen, Carlsbad, CA, USA) including gentamycin (50 µg/ml) and 20% FCS. Myoblasts were differentiated into myotubes by substituting the growth medium by differentiation

medium, including D-MEM with Glutamax supplemented with gentamycine, insulin and transferring, for 3 to 5 days.

6.1.7 Viruses (III,IV, V)

SINV strains Ilomantsi-2002A, -2002B, -2002C, Kiihtelysvaara-2002 and Johannes-2002, previously isolated from human skin biopsies and whole-blood sample in Finland, [96] as well as one SINV strain isolated from mosquitoes in Russia [117], were used in the study. Partial sequences of these strains have been published earlier (1268nt within the nsP3-nsP4 region) [96]. Viruses were passaged two or three times in Vero cells except for LEIV-9298, which had been passaged two times in suckling mice and once in Vero cells.

6.1.8 Mosquito samples (III)

Mosquitoes (approximately 8100) were collected by our research group using hand nets and BG-Sentinel trapping system (Biogents, Regensburg, Germany) during the years 2005-2009 in Central, Southern and Eastern Finland. The mosquitoes were snap frozen at -20°C, placed in tubes and transported on dry ice to storage at- 70°C.

6.2 Methods

6.2.1 Questionnaires (II, V)

All case-patients and their five randomly selected controls who met the selection criteria, received a self-administered, standard-questionnaire by mail. A reminder was mailed to non-responders twice. The questionnaire included questions regarding demographic and household information, symptoms and treatment of illness, physician visits, occupational (logging, gardening, farming) and leisure

(hiking, camping, hunting) outdoor activities, animal contacts, untreated water exposure, insect bites and measures used to protect against insect bites. For the case-patients, the questions addressed the 10 days before the onset of SINV infection (the exposure period) and for the control subjects, the 10-day period before the date when the first serum sample was obtained from the respective case-patient. Study subjects were asked to use a calendar as a memory aid to recall their activities and potential exposures. Epidemiological data using a similar questionnaire as described above was also collected from the patient with persistent SINV infection in V.

6.2.2 Statistical analyses (II, IV, VI)

Cochran and Mantel-Haenzel statistics were used to calculate matched odds ratios (mOR) and 95% confidence intervals (CI) in univariate analyses (II). Conditional logistic regression models were developed to identify independent risk factors associated with SINV infection (II). Due to the survey design, the variables in the model had varying proportions of missing responses. The assumption in the univariate analyses was that the missing data were approximately missing at random [166,183]. Therefore, only cases with complete information were included in the analyses. P-value of 0.15 was used as the screening criterion for selection of variables for the multivariable analysis. The initial multivariable analysis (Model 1) was performed using frequentist conditional logistic regression in which missing data were assumed to be missing completely at random [26]. The likelihood ratio test was used to determine the statistical significance of each variable, and backward elimination was utilized for determining the best model.

We addressed the problem of missing data by means of Bayesian full likelihood modelling, (conditional logistic regression, Model 2), in which the missing data become an additional parameter and the influence of missing data is taken into account. The variable selection indicators were also included in the model and Gibb´s variable selection was performed [26]. The significant variables with >50% probability for inclusion were included in the final model.

The significant variables in the multivariable Model 2 were further subjected to dose-response analyses. The dose was defined as the average number of insect bites or hours of outdoor activity per day multiplied by the number of exposure days. The variables were considered ordinal and quartiles of doses were used as cut-offs. The dose-response analyses were done by frequentist (univariate) and Bayesian (multivariable) conditional logistic regression.

The statistical analysis in the original publication IV included Probit regression model with 95% probability endpoint [40], which was used to estimate the analytical sensitivity. Two tailed Chi-square or Fisher's exact test was used for comparison of HLA and C4 gene as well as autoantibody frequencies between different groups (VI). Mc Nemar's test was done to compare paired proportions. P value was corrected with Holm-Bonferroni algorithm for multiple testing when appropriate (VI).

Statistical significance was considered at 5% level and P values were two-tailed in all the analyses. The analyses including descriptive, univariate and frequentist multivariable analyses were completed using SPSS software (version 17 or 18, USA). Winbugs software (version 1.4.3, UK) was used for creating Bayesian models and adjusted population-attributable risk (PAR) was calculated as reported earlier [19,130] (II).

6.2.3 Virus isolation (III, IV, V)

Virus isolation was attempted from serum samples (IV), muscle biopsy (V) and mosquitoes (III). The isolation trial on serum samples or muscle specimen was performed using a modification of a previously described protocol [96]. The Vero cells were rinsed with phosphate buffered saline (PBS) containing antibiotics to remove the culture media and subsequently 50 µl of serum was added to cells in 25-cm^2 flasks. After 1 hour incubation, fresh MEM + 2% FCS with a mixture of glutamine, ampicillin and penicillin, was added. Frozen muscle tissue was first cut into small pieces, homogenized in a mortar with sterile sand and suspended in 150 µl Dulbecco´s MEM+ 0.2% bovine serum albumin. A volume of 100 µl of this suspension diluted in 500 µl of MEM was added to confluent Vero cells. The cells

were then incubated for 1 h and fresh MEM was added. The cell cultures were examined daily for CPE. To detect viral antigens, the cells were studied further with IFA using SINV IgG-positive serum as previously described [96]. The cell culture supernatants were also subject to RNA extraction in order to attempt viral RNA detection with real-time reverse transcriptase (RT) PCR (developed in original publication IV).

The mosquitoes were homogenised in pools consisting of 8-10 mosquitoes. After homogenisation using sterile sand in PBS containing 0.2% bovine serum albumin and antibiotics, mosquito homogenates were added on confluent cells. The cells were rinsed with PBS before the infection with mosquito pool filtrates. After 1 h of incubation, fresh MEM was added. The virus isolation was performed on mosquito C6/36 (*Aedes albopictus*) and mammalian Vero E6 cells in 25-cm^2 bottles. When cytopathic CPE was observed, the cells were further studied by IFA and the supernatant was stored at -70 ºC for further studies.

6.2.4 RNA extraction (III, IV, V)

RNA was extracted from cell culture supernatants and sera with QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and from monocytes and muscle biopsy using RNAeasy mini kit (QIAGEN) according to the manufacturer's instructions.

6.2.5 RT-PCR (III, IV)

The cDNA synthesis was mainly performed with RevertAid™ H Minus M-MuLV Reverse Transcriptase 451 (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 20 µl containing 11.5 µl of template RNA, 4 µl of reaction buffer, 20 pmol of SINV specific primers, 20 units RiboLock™ RNase inhibitor,1 mM of each dNTP and 200 units of M-MuLV Reverse Transcriptase. The mixture was incubated at 42ºC for 60 minutes and the reaction was completed by heating at 70°C for 10 minutes. The cDNA was in some cases synthesized with random hexamers in a reaction containing 8 µl of template RNA using Superscript III First-

Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

PCR was done with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) in a final volume of 50 µl including 5 µl of template cDNA, 10 µl of 5Xphusion HFbuffer, 200 µM of each dNTP, 0.5 µM of forward and reverse primer and 0.02 U/µl of Phusion DNA Polymerase. After 30s initial denaturation at 98°C, the PCR reactions were amplified in 30 cycles with the following cycling parameters: 10s at 98°C, 30s at 64°C, 30s or 3.5 min (long amplicons) at 72°C followed by a final extension of 10 min at 72°C. Annealing temperature was based on the melting temperature (Tm) of the lowest Tm primer calculated by a Tm calculator using nearest-neighbour method specified by the manufacturer (Thermo Fisher Scientific). Nested RT-PCR targeting the E2 gene region was performed on patient serum samples (IV) using a previously published protocol [95].

Attempt to determine mosquito species in the pool of mosquitoes, positive for SINV, was performed with PCR (performed with Phusion High-Fidelity DNA Polymerase) targeting the mitochondrial cytochrome *c* oxidase subunit I genes (COI) using previously described primers UEA3/FLY10 [116,163] and LCO1490/HCO2198 [42]. DNA from mosquitoes was extracted with Qiagen DNeasy Blood& Tissue kit (QIAGEN). The PCR products were directly sequenced and the sequences recovered from the mosquito pool were compared to GenBank and Barcode of Life Data Systems (www.barcodinglife.com) databases.

6.2.6 Sequencing and sequence analyses (III, IV)

PCR products were purified with Qiaquick Gel extraction kit or Qiaquick PCR purification kit (QIAGEN). The sequencing of Ilomantsi-2005M and Ilomantsi-2002A was performed by amplifying 15 overlapping fragments covering the full-length protein coding region of SINV. The sequencing was carried out with ABI PRISM dye terminator sequencing kit (Applied Biosystems, Foster City, CA, USA).

The strains Ilomantsi-2002B, -2002C, Kiihtelysvaara, Johannes and LEIV-9298 were sequenced using high-throughput pyrosequencing. PCR products amplified by 3 primer pairs (Table 1; III) resulting in amplicon sizes of 4067 nt, 3591 nt and 5214 nt, and covering the complete protein coding area, were used as material for pyrosequencing. Pyrosequencing was performed with 454 GS FLX genome sequencer (Roche, Basel, Switzerland) at the DNA sequencing and Genomics laboratory of the Institute of Biotechnology, University of Helsinki.

Sequences were aligned with MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm [38]. The alignments were displayed and the nucleotide and deduced amino acid differences calculated with Bioedit Sequence Alignment editor [59]. Maximum likelihood (ML) phylogenetic tree was constructed using RAxML program with bootstrap analysis on 100 replicates. As a phylogenetic model that best described the data, general time reversible model with gamma distribution of rate heterogeneity was employed. The model was selected based on Akaike information criteria in FindModel [5]. The ratio of synonymous to non-synonymous (ds/dn) substitutions was calculated with the program SNAP (www. hiv.lanl.gov/content/hiv-db/SNAP) using the Nei–Gojobori model of evolution.

Median joining network analysis (Network version 6.0 software) [11] was performed to visualize genetic relationships based on single mutations between the SINV strains. The software connects existing sequences within the network with median vectors that are hypothetical (ancestral) sequences and represents specific mutational steps. The DNA alignment prior to Network analysis was performed with DNA alignment software and the output figure from Network was modified with Network Publisher.

6.2.7 One-step real-time RT-PCR (IV,V)

A one-step real-time RT-PCR was developed for the detection of SINV RNA as part of this study (IV). The primers and the probe were designed using Primer Express Software version 3.0 (Applied Biosystems) and chosen within the nsP1 gene region (Table 3). The real-time RT-PCR was done with Quantitect One Step

Probe RT-PCR Kit (QIAGEN) in a volume of 25 µl containing 5 µl of template RNA, 400 nM of primers and 250 nM of probe. The TaqMan® probe (Applied Biosystems) was labeled at the 5'-end with reporter 6-carboxyfluorescein (FAM) and at the 3'-end with minor groove binder (MGB)-non-fluorescent quencher (NFQ). The ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used for the assay with the following parameters: 50°C for 30 min, 95°C for 15 min, 45 cycles of 95°C for 15 s and 60°C for 1 min. Alternative primers and a probe labeled with quencher tetramethyl-6-carboxyrhodamine (TAMRA) were additionally designed within the E1 protein region and a subset of samples was analysed with this test format.

Table 3. Primers and the probe in the nsP1 gene region used in SINV real-time RT-PCR. Genome position according to SINV Ockelbo strain (GenBank accession number M69205).

Name	Sequence 5' to 3'	Genome position
Forward primer	GGTTCCTACCACAGCGACGAT	227-247
Reverse primer	TGATACTGGTGCTCGGAAAACA	280-301
Probe	FAM-TTGGACATAGGCAGCGCA-MGB-NFQ	249-266

The specificity of the assay was evaluated using viral RNA from supernatants of the five cell cultured Finnish SINV strains. Additionally, RNA from SFV (expression vector VA7) [199] and CHIKV alphaviruses, tick-borne encephalitis flavivirus (TBEV, strain Kumlinge A52), and Puumala (PUUV) hantavirus-positive (assessed with PCR) spleen and liver tissue from a patient with PUUV infection were tested. Serum samples (n=24) from patients suspected with SINV infection but confirmed SINV-antibody negative were also analysed to evaluate the specificity. The precise analytical sensitivity was determined by ten-fold dilutions of in vitro transcribed SINV RNA in 8 replicates at each concentration.

6.2.8 Plaque titration assay (IV)

The sensitivity of PCR assay was also approximated in plaque forming units (PFU) by determining the lowest concentration at which a positive result was

attained. Plaque titration assay for Ilomantsi-2002A virus strain was performed. In brief, serial dilutions of virus were added to Vero E6 cells. After 1 hour incubation at 37°C, agarose (0.5%) overlay medium was added to the cells and the plates were incubated for 4 days. The cells were then stained with crystal violet and the plaques were counted. RNA was extracted from ten-fold dilutions of this virus stock and analysed with the real-time RT-PCR assay.

6.2.9 Production of SINV RNA transcript (IV)

The real-time RT-PCR target region was amplified from Ilomantsi-2002C and Ilomantsi-2002B strain and cloned into pGEM®-T cloning vector (Promega, Madison, WI, USA). The presence of the insert was confirmed by sequencing and restriction enzyme analysis. Following linearization of the plasmid by digestion with BsaI, RNA was generated using RiboMAX™Large Scale RNA production system with SP6 polymerase (Promega) according to the manufacturer´s instructions. The transcribed RNA was then treated with DNAse and purified with RNeasy Mini Kit (QIAGEN). RNA was quantified by spectrophotometer and RNA copy number was calculated based on its concentration, length, and the molecular weight.

6.2.10 HLA and complement C4 genotyping (V, VI)

DNA extracted (NucleoSpin® Tissue kit, Macherey-Nagel) from whole blood (n=35) was genotyped for HLA alleles using genomic real-time PCR. Unlabeled primers with SYBR green QPCR (Stratagene, Cedar Creek, TX, USA) or Absolute QPCR SYBR Green Mix (Abgene, Epsom, UK) were used according to the manufacturers' instructions with minor modifications. Complement C4 allotypes were determined with electrophoresis followed by immunofixation [119]. Copy numbers of *C4A* and *C4B* genes were determined using isotype-specific genomic real-time PCR amplification. The typing analyses were performed in an EFI-accredited (European Federation for Immunogenetics) laboratory (HaartBio, Helsinki, Finland).

6.2.11 *Cryosectioning and staining of muscle biopsy (V)*

The muscle specimen was snap frozen at -170°C in isopentane pre-cooled with liquid nitrogen. Frozen biopsy was stored at -80°C prior to cryosectioning. Cryosections were stained with routine histological [haematoxylin and eosin (H&E), Herovici, PAS and OilRedO] and histochemical methods [for fibre typing ATPase with preincubations at pH 4.3 and 10.4, and for oxidative enzymes NADH-tetrazolium reductase (NADH-TR) and cytochrome-C-oxidase + succinate dehydrogenase (COX-SDH)]. Immunohistochemical staining for fetal, neonatal, fast and slow myosin heavy chains (MyHCd, MyHCn, MyHCf and MyHCs) and inflammatory cells (CD20 for B-cells, CD3 for T-cells and CD68 for histiocytes; DAKO, Glostrup, Denmark) was performed. A mouse monoclonal anti-Semliki Forest virus directed against a conserved region of the alphavirus nucleocapsid protein [56] or a mouse polyclonal anti-SINV (National Reference Centre for Arbovirus, Pasteur Institute, Paris, France) was used for the detection of SINV.

6.2.12 *Electronmicroscopy (V)*

A selected part of the muscle biopsy was fixed in 3% phosphate-buffered glutaraldehyde and processed to be embedded in Epon. Toluidine blue stained semithin sections were used to choose the regions for thin sectioning. Thin sections were examined in a JEOL JEM 1400 electron microscope.

6.2.13 *In vitro infection of muscle cell cultures (V)*

These experiments were performed by the collaborator at Institut Pasteur in Paris, France. The sensitivity of primary human myoblasts and myotubes to SINV infection was assessed at different multiplicity of infection (MOI) 10, 1, and 10^{-1}. Ilomantsi-2002B and Ilomantsi-2002C strains were used for the experiments. At different days post-seeding, cells were incubated for 2 h with viral strains, washed once in PBS after which the culture medium was added. At certain days p.i., cultures were processed for SINV antigen detection by IF or viral titration. For IF, cells were fixed for 20 min in 4% paraformaldehyde, incubated for 30 min in PBS

with 10% normal goat serum (Vector, Burlingame, CA, USA) and then permeabilised with 0.1% Triton X-100 (Sigma,St Louis,MO,USA). Permeabilisation medium was removed and primary antibodies (anti-alphavirus nucleocapsid in dilution 1/50 or polyclonal anti-SINV in dilution 1/100) were incubated on cells for 90 min at room temperature. Then, secondary antibody (horse anti-mouse antibody, coupled to FITC,1/100; Vector) was incubated for 90 min at room temperature after 3 washes with PBS. Muscle cell identification was done using a rabbit polyclonal anti-desmin antibody (dilution 1/1000) and a goat anti-rabbit serum (dilution 1/100, Vector). F-actin cytoskeleton was visualized using Rhodamine-Phalloidine (dilution 1/200; Molecular Probes, Eugene, OR, USA). After 3 washes, cultures were mounted in Fluoromount G medium (Southern Biotech Birmingham, USA). Preparations were observed with a Zeiss Axiovision fluorescence microscope and image acquisition was done with a Zeiss Axiocam camera. Supernatants from cell cultures were collected and plaque-titrated on Vero cells at different timepoints p.i. Culture supernatants were also collected at 8 and 24 h p.i. for cytokine studies. The concentration of 5 cytokines and 1 chemokine (IL-6, IL-8, TNF-α, IFN-α, IFN-γ and MCP-1) was assessed using Luminex® assay (Cytokine Human Singleplex; Invitrogen) according to the manufacturer's instructions.

6.2.14 Analyses of autoantibodies (VI)

Autoantibodies were measured (at the Department of Virology and Immunology, HUSLAB) in serum of SINV-infected patients (n=44) in the acute phase and 3 years p.i. Nuclear (ANA), mitochondrial (AMA), smooth muscle (SMA), and parietal cell (PCA) antibodies were determined by the indirect immunofluorescence assay. For ANA, sera were screened at 1:80 dilution and titrated further at four fold dilution steps using HEp-2 slides (Nova Lite HEp-2 assay, INOVA Diagnostics Inc, San Diego, CA, USA) as substrates. For tissue antibodies, unfixed 5 µm cryostat sections of tissue blocks of rat kidney, rat stomach, mouse liver, and mouse stomach were used as previously described [97]. As secondary antibodies, anti-human IgG coupled with FITC (DAKO) were

used. Extractable nuclear (ENA) and cyclic citrullinated peptide antibodies (CCP) were measured by fluorescence enzyme immunoassay (ImmunoCap250, Phadia, Uppsala, Sweden) and rheumatoid factor (RF) using N LatexRf Kit and BN ProSepc nephelometer (Siemens, Munich, Germany). The following reference titres or units were used as cut-offs (based on the accredited methods of HUSLAB): ANA (<320), AMA (<50), RF (<20 IU/ml), CCP (<7 U/ml), SMA (<50), ENA (<0.7U).

7. Results and discussion

The main results of the original publications of the study are summarised and discussed below. In addition, some unpublished data are presented.

7.1 Descriptive epidemiology–2009 SINV outbreak (I)

Since the first documented outbreak of SINV infection in 1974, larger epidemics have occurred approximately every seven years. Previously it has been suggested that tetraonid birds, which also have been found SINV-antibody positive [18,94], may contribute to the cyclic appearance of human epidemics. There is evidence that the grouse population crashed concurrently with SINV outbreaks in the 1970s–80s [111]. However, the bird cycles have been less regular since the 1980s. The latest major outbreak occurred in 2002 when 597 human cases were reported. We described the epidemiology of SINV infection in Finland in 2009 when another major epidemic was again expected to occur and compared the epidemic pattern to the previous ones.

A total of 105 laboratory-confirmed cases (incidence of 2.0/100,000/year) were reported to NIDR from June through October, 2009 (Fig.1; I). Most of the cases occurred in September (n=60) and August (n=33). The majority of the patients were females (60%) and the highest incidence was reported among the persons aged 50–59. North Karelia had the highest incidence, followed by East Savo, Central Ostobothnia and Central Finland together with Southern Ostrobothnia (Fig.2; I and Table 4).

Health Care District	2009 No of cases (Incidence rate*)	2002 No of cases (Incidence rate*)
1 - Helsinki and Uusimaa	7 (0.5)	59 (4.2)
2 - Varsinais-Suomi	0 (0.0)	10 (2.2)
3 - Satakunta	1(0.4)	12 (5.2)
4 - Kanta-Häme	0 (0.0)	5 (3.0)
5 - Pirkanmaa	12 (2.5)	59 (13.0)
6 - Päijät-Häme	5 (2.4)	6 (2.9)
7 - Kymenlaakso	3 (1.7)	3 (1.7)
8 - South Karelia	0 (0.0)	9 (7.0)
9 - South Savo	4 (3.9)	31 (29.0)
10 - East Savo	4 (6.9)	11 (16.7)
11 - North Karelia	14 (8.2)	140 (81.2)
12 - North Savo	9 (3.6)	95 (37.8)
13 - Central Finland	15 (5.5)	72 (27.2)
14 - Southern Ostrobothnia	11 (5.5)	32 (16.4)
15 - Vaasa	7 (4.2)	11 (6.6)
16 - Central Ostrobothnia	5 (6.4)	10 (12.9)
17 - Northern Ostrobothnia	8 (2.1)	27 (7.3)
18 - Kainuu	0 (0.0)	3 (3.6)
19 - Länsi-Pohja	0 (0.0)	1 (1.5)
20 - Lapland	0 (0.0)	1 (0.8)
21 - Ahvenanmaa	0 (0.0)	0 (0.0)
Total	105 (2.0)	597 (11.5)

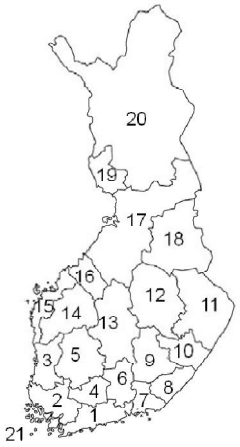

Table 4. Number and annual incidence rates of laboratory confirmed SINV infections in different health care districts (HD) in 2009 and 2002. The geographical location of each HD is shown in the inserted map. * Incidence rate per 100,000 population.

The number of cases was the highest in Central Finland (n=15) where most of the cases occurred in July–August. In the traditional endemic area of North Karelia, only one case was reported during this time period. However, the majority of the cases in North Karelia were reported in September–October (n=13) whereas only 5 cases were confirmed in Central Finland during these months (Fig. 3a and 3b; I and Figure 8).

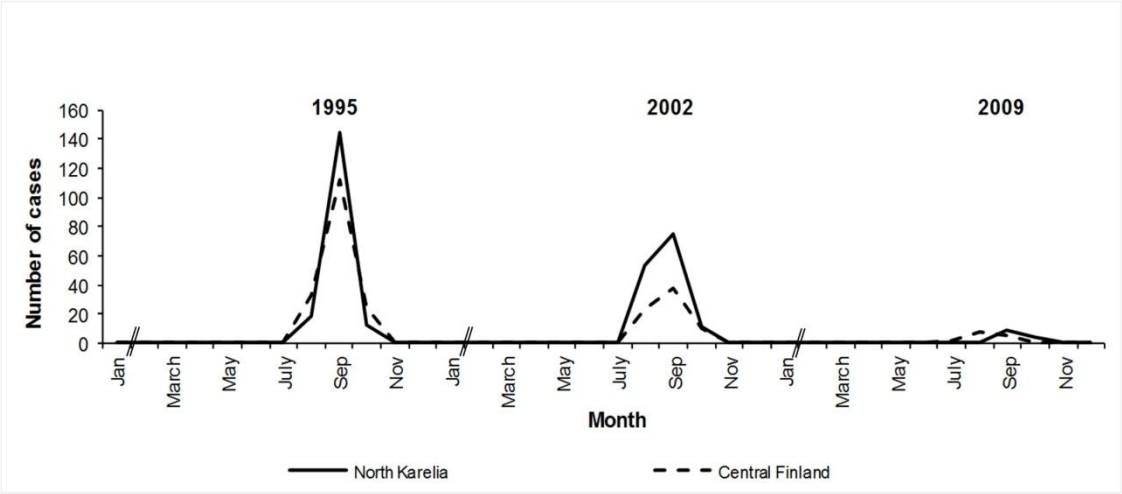

Figure 8. Laboratory confirmed SINV infections in North Karelia and Central Finland in 1995, 2002 and 2009. Data obtained from NIDR.

The epidemic season in 2009 was considerably milder than the previous outbreaks in 1995 and 2002. Nevertheless, the number of cases was higher than the average number (n=57) reported during the non-epidemic years. To compare, only 5 cases were reported in Sweden in 2009. Regarding the role of grouse, it was reported that the density of grouse in 2007 was well above average but dropped in 2008 [2]. The population decline continued unexpectedly in 2009 and the density reached the lowest figures ever. It is possible that the further decline in grouse population in 2009 could have diminished their role as amplifying hosts resulting in a milder epidemic than expected. Thus, this descriptive data indicates that grouse may play a significant role in the human epidemiology of SINV infection. As in previous epidemics, the highest incidence was reported in North Karelia. However, the difference to other regions in central and northwestern Finland was smaller than before possibly reflecting the increased human seroprevalence towards SINV in the region.

There were differences in the peak month of incidence between North Karelia and Central Finland, not observed during the last epidemic in 2002 or 1995 (Fig. 3a and 3b; I and Figure 8). This phenomenon could be associated with variation in mosquito activity and population size due to differences in weather conditions in these areas. It was observed that May was drier than normally in Joensuu (the

largest city in North Karelia) but the precipitation in June–July was significantly higher than on average [3]. The dry May possibly contributed to fewer cases in July–August whereas the high rainfall in June and July created suitable environmental conditions for mosquitoes to develop and thus, more SINV infections were reported in early autumn.

The role of environmental factors is supported by a recent study on the climatic, ecological and socioeconomical factors as predictors of SINV infection in Finland [75]. This study, utilising an advanced time series regression model, showed that high mean temperatures in May and June as well as high monthly precipitation in June were significantly (positively) associated with both the occurrence and incidence of SINV infection. It was also found that hatch-year black grouse density was positively significant for the occurrence of the SINV infection, further highlighting the role of grouse as amplifying hosts for SINV infections in Finland. The model was able to give quite accurate predictions for the number of cases, for example the prediction for 2009 was 85 cases (95% CI; 2-1187).

7.2 Description of case-patients and analytical epidemiology (II)

Few controlled studies have previously been conducted to assess epidemiological risk factors for arthropod-borne viral diseases [61,78,128] and only one such study on alphaviruses has been reported [64]. Factors associated with clinical SINV infection have not been previously evaluated in controlled studies. We conducted a population-based case-control study to determine factors associated with acquiring acute SINV infection and to identify opportunities for its prevention. The study also collected information on the clinical features of the disease and patient characteristics.

A total of 597 laboratory-confirmed cases of SINV infection were reported in 2002. There were 140 cases in North Karelia health district, which was excluded from the study. The questionnaires were returned by 369 of 391 (94.4%) eligible case-patients and 1216 of 1832 (66.4%) control subjects. As a number of case-

patients and control subjects were excluded according to the study protocol, 337 cases and 934 controls in total were included in the analyses. The median age for case-patients was 49 years (range, 1–94 years) and 58% were females.

The typical symptoms of SINV-infected case-patients included papular rash (96%), joint symptoms (96%), fatigue (77%), muscle pain (62%), and headache (49%) (Table 1; II). Fever and, surprisingly, upper respiratory tract symptoms were reported by 36% of case-patients. Regarding underlying illnesses or previous injury affecting joint or connective tissue, joint injury was reported significantly more often by cases (P=0.02). Current or previous osteoarthritis and bacterial joint infection were also reported more commonly by cases than controls (P=0.06 and 0.05, respectively) (Table 1; II). Rash was mostly located in the upper and lower limbs and in the torso and the most often affected joints were ankles, wrists and knees. The median duration of rash and joint symptoms among the cases who were able to report the date when symptoms subsided was 6 (range; 1–80 days) and 10 days (range; 1–47), respectively. Of the cases, 69% reported ongoing symptoms at the time they completed the questionnaire. The median time between the onset of symptoms and completing the questionnaire was 32 days in the case-patients. The estimated median incubation period of SINV infection was 4 days (range; 2–18) based on data from 13 cases who reported a probable day of exposure. Previous estimation (8–9 days) was based only on a single case report [95].

Twenty cases (6%) required hospitalization for a median duration of 4 days (range; 1–19 days). Prescription medicines, primarily NSAIDs and antihistamines, were consumed by 62% of cases and 61% had taken over-the-counter drugs. Median time between the onset of symptoms and the first medical contact was 2 days (range; 0–102 days).

Univariate analyses on risk factors showed that cases were significantly more likely to report bites by any insect as compared to controls (mOR=31.8; 95% CI 11.5–87.8) but of the specific arthropods, only mosquito bites were significantly associated with SINV infection (mOR=19.7; 95% CI 9.0–43.1) (Table 2; II). Deer fly bites appeared to be inversely associated with SINV infection, a finding that may be by chance and related to the high degree of missing responses for this

variable. The reported protective measures against insect bites including insect repellents, mosquito coils and nets were not associated with a decreased likelihood for SINV infection (Table 2; II). Most outdoor activities (Table 3; II) as well as handling sick or dead animals or having observed them near the residence were associated with SINV infection.

In the multivariable Model 1, exposure to mosquito bites was the only variable significantly associated with SINV infection but in the Model 2, spending time outdoors in the woods/marshland also remained independently associated with increased odds of the disease (mOR=1.8; 95% CI 1.3–2.5), in addition to mosquito bites (mOR=16.7; 95% CI 9.1–33.4) (Table 4; II). The number of case-patients and control-subjects included in the analysis was substantially greater in the Bayesian model where the missing data were taken into account (Table 4; II). No significant interaction was found between the variables in the final model. The adjusted PAR for mosquito bites in the multivariable model was 87.2% (0.872; 95% CI 0.78–0.94). Furthermore, significant dose-response relations were found for insect bites overall and time spent outdoors in woods or marshland (Fig 2; II and Table 5).

Table 5. Dose-response associations for number of insect bites and time spent in woods or marshland and odds of SINV infection. CI= Bayesian credible interval.

Exposure variable	**mOR (95%CI)**			
	1st dose quartile	**2nd dose quartile**	**3rd dose quartile**	**4th dose quartile**
Exposure to insect bites	1	23.8 (7.6-63.4)	29.9 (10.4-81.4)	72.5 (25.4-187)
Spending time in woods or marshland	1	1	1.3 (0.8-2)	2.2 (1.5-3.3)

As the time spent in woods or marshland was zero hours for 50% of participants, the first quartile and median (2nd quartile) were equal (mOR=1). The dose was defined as the average number of insect bites (or hours of outdoor activity) per day multiplied by the number of days of exposure.

To our knowledge, our study represents the largest report of risk factors for arthropod-borne viral diseases. The study increased knowledge about risk factors for SINV infection and provided a comprehensive description of its clinical

characteristics. SINV infection was strongly associated with the number of insect bites, reflecting primarily mosquito bites, with no evidence for other vectors to be involved. Although mosquitoes have been suspected to be the probable vectors of SINV in Finland [45,135], SINV has also been isolated earlier from ticks in Italy [57] and it has been uncertain whether other arthropods could transmit the disease, particularly since other viruses like WNV circulating between mosquitoes and birds can also be transmitted by ticks [104]. Time spent outdoors in woods or marshland was independently associated with SINV infection in Model 2 also with a significant dose-response. Although the involvement of an unknown vector cannot be excluded, it is plausible that some participants may not have noticed being bitten by mosquitoes while spending time outdoors.

The study had limitations, such as possible misclassification and recall bias, but the strength of association with dose-response relations increased the confidence in our findings. The problem of missing information, common in self-reported surveys, was tackled by using Bayesian full likelihood modelling approach, which is less sensitive for bias caused by missing data [26]. It was observed that for some covariates data were not missing completely at random, as assumed in frequentist conditional logistic regression. By using the Bayesian approach somewhat different results compared to the frequentist model were obtained due to increased statistical power. These results highlight the advantage of using current statistical methods for dealing with missing information in self-reported surveys to increase the validity of the results.

Reported protective measures against insect bites were not significantly associated with reduced likelihood of SINV infection. In contrast, a study on RRV in tropical Australia [64] concluded that protective measures significantly reduced the disease incidence. In tropical countries, the use of insect repellents and nets to protect against mosquito-borne viruses is often promoted by public health authorities [69,138]. The reasons for lack of association in the study may be related to the problems in measuring the extent of protection and the lack of statistical power due to a small number of study subjects reporting protective measures. Use of protective measures could also be less common in Finland than

in tropical regions where the awareness of mosquito-borne pathogens is high and life-threatening illnesses are present.

The findings of this study add to the knowledge-base of clinical features of SINV infection and are generally consistent with previous studies [95,99,134,135,197]. However, in contrast with previous reports, one third of case-patients reported upper respiratory tract infection symptoms. It is unclear whether these symptoms were caused by SINV infection or resulted from simultaneous infection with respiratory viruses as has been reported for CHIKV infection [165]. Our data also further highlight that persistent joint symptoms are common among persons infected with SINV as only 32% of cases were able to report the time when joint symptoms ended at the time of completing the questionnaire.

The finding that past/active joint injury (significantly) and ongoing/past bacterial joint infection or osteoarthritis were more commonly reported among cases than controls raises interesting questions. The presence of underlying damage to the joint tissue may predispose to more severe SINV infection symptoms. Common genetic factors which are associated with both an increased susceptibility for rheumatic diseases and symptomatic SINV infection are also a possibility. This issue is further discussed later in this section and in original publications V and VI.

7.3 Molecular epidemiology (III)

In addition to classical epidemiology, we performed a molecular epidemiological study to investigate the genetic relationships and characteristics of different SINV strains. We isolated one SINV strain from a pool of mosquitoes, observed as a strong CPE and a positive IFA result in the Vero cells, collected from an endemic area for human SINV infections in Eastern Finland (Ilomantsi municipality). We sequenced the full-length protein coding region, 11292nt, of this novel SINV strain, which was designated as Ilomantsi-2005M. This strain represented the first SINV strain isolated from mosquitoes in Finland. Additionally, we sequenced the full-length protein coding region of five SINV strains isolated from humans in Finland (Ilomantsi-2002A, -2002B, -2002C, Kiihtelysvaara-2002 and Johannes-2002) in 2002 [96] and of a strain isolated from mosquitoes in Russia in 1983

(LEIV-9298) [117]. As only 8 full-length sequences of SINV were available in the GenBank prior to our study, this study significantly increased the data on SINV sequences allowing for more detailed phylogenetic analyses.

Unfortunately the species of the mosquito vector of the mosquito-isolated SINV could not be determined in this study with adequate accuracy as individual mosquito homogenates were not available and mosquitoes were not identified by entomologist prior to pooling. However, we were able to amplify COI sequences of Ochlerotatus spp (98-99% identity to *Ochlerotatus annulipes)* in the virus isolation pool. These mosquitoes are known to be anthropophilic and endemic in Finland [198] and thus, could potentially act as vectors for SINV. Prior to year 2000, *Ochlerotatus* genus was classified as a member of *Aedes* genus [155] from which LEIV-9298 strain was isolated in Russia, close to the border of Finland.

Analysis of nucleotide and deduced amino acid identities (Supplementary Table 1; III) showed that Ilomantsi-2005M shared very high nucleotide and amino acid homology with all the Finnish strains isolated from humans and with the strains isolated from mosquitoes in Russia and Sweden in the 1980s. These Northern European SINV strains were further shown to cluster together sharing a common ancestor in the phylogenetic analyses based on full-length protein-coding region (Fig.1A; III and Figure 9).

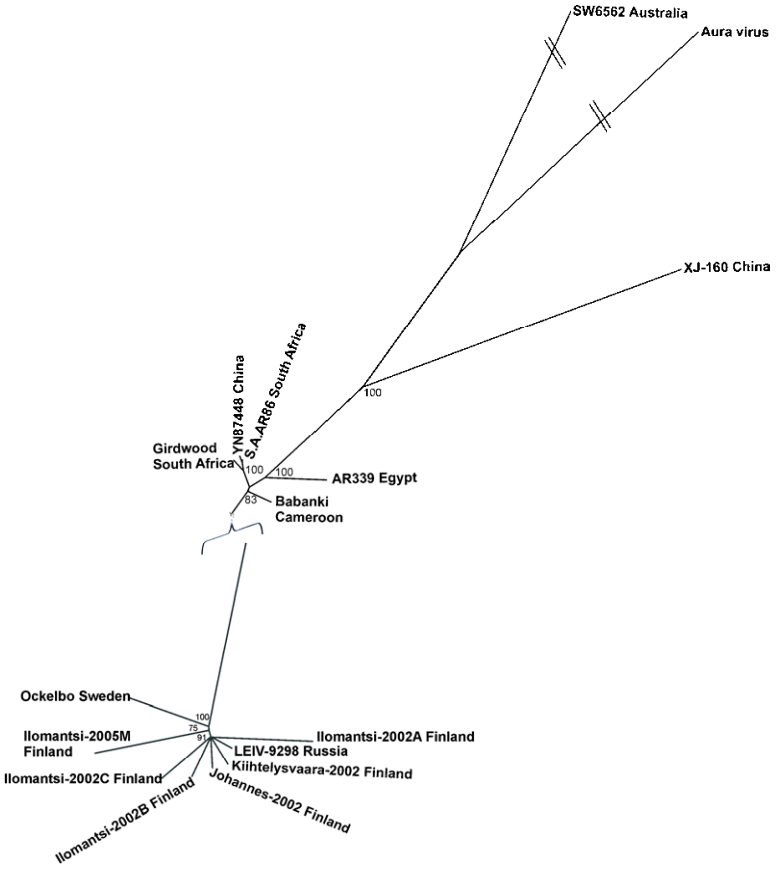

Figure 9. The radial layout of maximum likelihood phylogenetic tree based on full-length protein coding nucleotide sequences of SINV strains. For clarity, the cluster including the Northern European strains is shown enlarged. The bootstrap support values >70% are shown.

Similar clustering was observed in the phylogenetic tree based on partial structural polyproteins (2189 nt), which also included SINV strains recently isolated from mosquitoes in Germany (Fig.1B; III). These German SINV strains showed high genetic relatedness to Northern European strains. The median joining network also demonstrated the clustering of the German strains observed in the phylogenetic tree. Overall, the data on full-length sequences of the coding region further support the presumption that SINV has a local circulation in the endemic regions in Northern Europe. The observed separate clustering of strains from Northern Europe and Africa additionally suggest that new SINV strains are not frequently being introduced to Finland or Northern Europe, although SINV has probably been originally imported from Africa by migratory birds as suggested earlier [96,115]. It is plausible that the introduction of SINV to Northern Europe has occurred in the late 1960s since antibodies to SINV were not found in large

seroprevalence studies in humans and birds conducted in the early 1960s in Finland [18] and since the first clinical cases of SINV infections have been reported in Sweden in 1967 [135] and in Finland in 1974 [18].

The sequence analyses did not indicate significant differences between the endemic, isolated from mosquitoes, and epidemic, isolated from humans, SINV strains in Finland and no amino acid changes were shared exclusively by the strains isolated from mosquitoes. The average ds/dn ratio for the full-length protein-coding sequences of SINV strains was 20.9 indicating that positive selection is not acting significantly on SINV. When comparing individual SINV genes, the lowest ds/dn ratio was observed in the nsP3 gene (ds/dn=11.2) and the highest in the nsP4 gene (ds/dn=57.7) (Fig.S2; III).

Alignment of the deduced amino acid sequences of the complete SINV polyprotein showed that Finnish, Swedish and Russian SINV strains shared 9 identical amino acid changes. Interestingly, 6 of these changes occurred in the nsP3 protein and 5 of these changes were found in the C-terminal region of the nsP3 protein (Fig.S3; III). The C-terminal region of nsP3, the function of which is not well understood, encodes a cluster of serines and threonines that are heavily post-translationally phosphorylated [101,106]. Two amino acid changes in the C-terminus of nsP3 protein shared by the Northern European strains were mutations from serine or threonine to other amino acids. It has been shown that reduced phosphorylation in SINV nsP3 leads to decreased production of minus-strand RNA [30]. Also, a deletion of the phosphorylated residues in the nsP3 of SFV decreases the level of RNA synthesis [201]. It would be of interest to further investigate whether these mutations shared by the Northern European strains are associated with a decreased level of RNA synthesis and/or with the adaptation of the virus into new vectors or host species when SINV was first introduced to Northern Europe.

7.4 The presence of viraemia in SINV infection (IV)

The laboratory diagnosis of SINV is based on serology, and IgM antibodies become detectable within the first week of illness only in approximately 40% of the

patients [95]. We developed a one-step real-time RT-PCR assay for the detection of SINV and evaluated its applicability as a diagnostic tool for human SINV infection in acute-phase serum samples. Prior to our study, a few conventional PCR [73,95] and real-time RT-PCR assays [65,77] for SINV detection have been published but the validity and performance of these methods have not been properly evaluated with clinical samples.

The assay detected viral RNA of all Finnish SINV strains (Fig.2; IV) in cell culture supernatants. The 95% detection limit determined by probit analysis was 9 copies/reaction (Fig 2; IV). The lowest template concentration that gave positive signal was 1.4 copies/reaction (120 copies/ml) and using plaque-titrated virus stock, 0.001 PFU/reaction (0.09 PFU/ml). Cell culture supernatants of SFV, CHIKV and TBEV, as well as PUUV-infected tissue samples did not give any false-positive signals. All the repeatedly SINV-seronegative (n=24) control sera were non-reactive in the test.

Of the acute-phase serum samples from SINV-infected patients, 7/58 (12%) were positive in the real-time RT-PCR assay. All the SINV-RNA positive samples were negative for SINV IgM and IgG. In 4/7 (57%) of the samples only one of the duplicates was positive in repeated runs indicating a very low viral load and uneven Poisson distribution of template in the replicates. The viral load of the samples approximated from the in vitro RNA standard dilutions (Figure 10) was in the range 130–660 viral copies/ml of serum. The detection sensitivity was not increased by using higher primer concentration (up to 900 nM) or longer reverse transcription time (45 min).

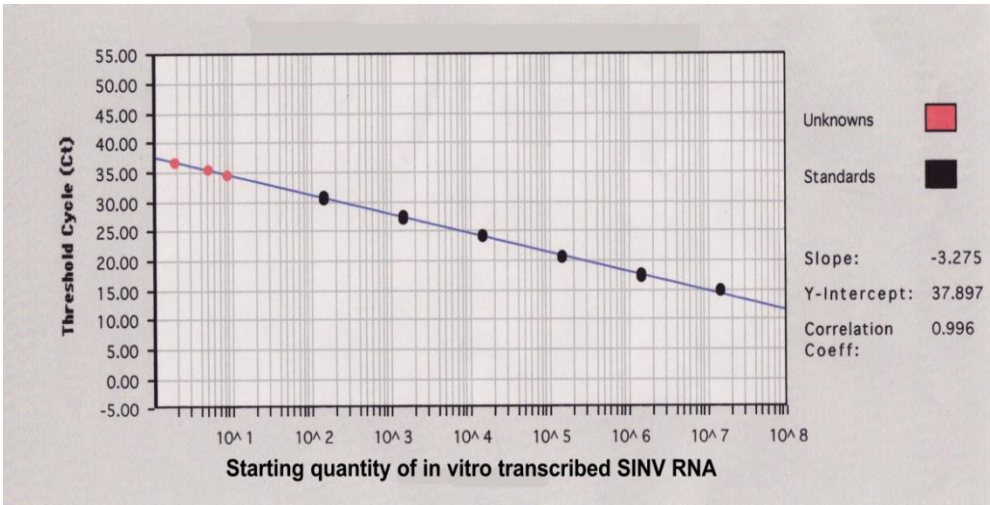

Figure 10. Standard curve generated from ten-fold dilutions of in vitro transcribed SINV RNA.

The positive samples were also tested with primers and TAMRA-labelled probe designed within the E1 protein but an inferior sensitivity was obtained with this test format. SINV RNA was detected by conventional nested PCR in 2/7 of the samples positive with the real-time RT-PCR. These samples originated from patients whose skin biopsies were previously found positive with nested PCR and from whom two SINV strains were isolated [96]. No infectious virus could be recovered from any of the seven serum samples in virus isolation trials.

Previous studies have suggested that acute SINV infection is characterised by a narrow viraemic window and low level of viraemia [96,118]. This assumption is supported by our data as only 12% of the serum samples were positive with high Ct values in the novel real-time RT-PCR assay corresponding to levels of $<10^3$ SINV copies/mL of sample. On the contrary, high viral loads, up to 10^9 copies/mL of plasma, have been reported in acute CHIKV infection [141]. Our findings evidently suggest that real-time RT-PCR from serum samples is not a practical approach for the laboratory diagnosis of SINV but may be used for screening the virus for example in mosquitoes and potential vertebrate hosts. Due to highly conserved primer binding region of the nsP1 protein, the assay can potentially detect all SINV strains.

The sensitivity of the assay may have improved by increasing the extraction volume and concentrating the samples. However, the volume used in RNA extraction (140 µl) has been found optimal for the kit (QIAGEN) and generally

works well for real-time RT-PCR assays used in the routine diagnosis of CHIKV infection for example, where viral RNA is frequently found during the first week of infection [39,141]. Moreover, a protocol requiring considerably larger volumes would likely diminish the applicability of the assay for clinical purposes as extra time-consuming steps would be required.

The low viral load in serum may be linked to the pathogenesis of SINV infection. Since SINV can infect human macrophages, it is possible that after inoculation, SINV targets cells circulating in blood such as monocytes that further spread the virus to different tissues and the presence of SINV in serum remains transient. SINV RNA was previously detected in 5 out of 73 acute-phase whole blood samples with nested RT-PCR [95]. Our preliminary data show that SINV RNA can be detected in whole blood with our novel real-time assay but comprehensive comparative analyses of whole blood and serum have not yet been performed due to the lack of sample material. The specific role of viral load in the pathogenesis and the mechanisms behind the considerable difference in the viral load between CHIKV and SINV (although both replicate to high titres in cell cultures) remains to be determined. In addition, the low viraemia in serum probably affects the epidemiology of SINV infection since the probability that mosquitoes become infected with SINV when they feed on an infected person and subsequently spread the virus to other humans is markedly decreased.

Isolation of CHIKV has been successful only from serum samples containing $>10^7$ RNA copies/ml [141], which significantly exceeds the levels of SINV RNA found in our study. Regardless of frequent attempts by us (unpublished data) and others [73,80], only one isolate has ever been recovered from human serum in China [218]. Nevertheless, doubts have been raised about the authenticity of the isolate due to very high sequence similarity with the widely used laboratory strain AR86 [115].

7.5 Clinical description of a patient with persistent SINV infection (V)

The patient who had a serologically confirmed acute SINV infection earlier was examined by a rheumatologist 6 months p.i. when the symptoms, arthralgia and myalgia, were still present. This patient was unable to work due to the symptoms. In the clinical examination, tenderness was observed in the right metacarpophalangeal joints. The right wrist was swollen, and dorsal swelling on the wrist within the tendon sheaths of the extensor muscles was observed. Prominent tenderness was observed during extension and flexion, with a considerably weakened handgrip. Tenderness was also observed in acromioclavicular joint and myalgia particularly in the right supraspinatus insertion area. Otherwise the rheumatological status was normal. The blood parameters (detailed in V) were otherwise within normal range except for leukocyte count, neutrophil count, and C4 complement level, which were slightly elevated. A persistently positive SINV-IgM antibody level was observed, suggestive of active virus replication in some parts of the body (likely in the joints), and the end-point titres of IgM and IgG antibodies were 77 and 434, respectively. The first acute-phase serum sample of the patient was part of the study material in IV and found negative for SINV RNA.

7.6 Factors behind the pathogenesis of myalgia (V)

Since the studies on tissue tropism and pathophysiological mechanisms of arthritogenic alphavirus infection have primarily focused on RRV and CHIKV infections, the data on SINV are scarce. We investigated the factors behind the pathogenesis of myalgia in SINV infection by studying a unique muscle biopsy obtained from the SINV patient described above, and by *in vitro* experiments on primary human myoblasts and myotubes.

Histology and immunohistochemistry on the muscle biopsy showed no signs of necrosis or inflammatory cell infiltrates (phagocytes, B- and T-cells). However, ATPase staining used for fibre typing showed several medium dark type 2C

(immature) fibres (Fig 1A; V). Also, increased number of internal nuclei, indicative of regeneration of muscle cells, were observed (Fig.1B: V). In the COX-SDH staining, five COX-negative fibres were encountered (Fig.1C;V). Immunohistochemistry staining to show regeneration revealed several MyHCn-positive fibres, also indicative of regeneration (Fig 1D; V). SINV antigen was not detected immunohistochemically in the muscle biopsy. Structures resembling viral particles were not seen in EM but an increased quantity of glycogen in the myofiber sarcoplasm was observed further demonstrating an ongoing regeneration process. SINV RNA was not detected in the muscle biopsy and no infectious virus could be recovered from the biopsy by virus isolation. Blood monocytes were also negative for SINV RNA.

SINV antigens (Ilomantsi-2002B strain) were detected in primary human myoblasts and myotubes (from three different donors) using an antibody targeting the capsid of alphaviruses at 24h p.i. (Fig 2B and 2C; V). Immunoreactivity was, however, already observed 12h p.i. (data not shown). Desmin staining confirmed the myogenic origin of the cells positive for SINV and the specificity of viral immunoreactivity was confirmed by the absence of staining in mock-infected cells (Fig.2A; V) or by omitting the primary antibody. Infected cells were found positive for SINV proteins also by using the SINV-specific polyclonal mouse antibody (Fig.2D; V). Similar results were obtained when using the Ilomantsi-2002C strain (data not shown). The experiments were performed with MOI of 10, but immunoreactivity was also detected with lower MOI, although with less positive muscle cells. The infection rate stayed around 50% for both viral strains in myoblasts and in myotubes, even at later time points, suggesting the existence of muscle cells refractory to infection (Fig.2E and F; V). Viral infection of the muscle cells was productive reaching a peak in viral yield at 48h p.i. After this time point, the virus titre started to decrease (Fig.3; V) and cell rounding and detachment was observed. At later time points, for example 20 days p.i., only some individual cells were found immunoreactive. Studies on cytokine secretion showed that infected muscle cells did not exhibit any significant changes in the production of IFN-α, IFN-γ, TNF-α, IL-6, IL-8 or MCP-1 at 8 h or 24h post-infection, although a small

increase in MCP-1 production was observed 24h p.i. in myotubes (data not shown).

Only a single study performed with CHIKV has previously investigated the susceptibility of primary human muscle cells to alphavirus infection. The study showed that CHIKV can infect and cause CPE in human skeletal muscle satellite cells. In addition, CHIKV antigens were detected by immunohistochemistry in the satellite cells of muscle biopsies from two patients with myositis [139]. Access to muscle biopsies from SINV-infected patients is constrained and difficult to obtain but in this study we were able to extensively investigate this unique tissue specimen. In the biopsy we found evidence of muscle regeneration due to previous necrotic lesions. The necrosis was likely caused by previous SINV infection in muscle cells but no active viral replication was detectable at 6 months p.i. as demonstrated by negative result from virus isolation, PCR and immunohistochemistry. The regeneration process was already in an advanced stage due to the long time since the infection. However, instead of direct virus-induced damage, the necrosis of muscle tissue may also have been caused by secondary immune-mediated response triggered by the SINV infection. We also acknowledge that other factors apart from SINV infection as a cause of the findings cannot be excluded and further studies on muscle biopsies, if available, should be performed.

The *in vitro* experiments showed that differentiated (myotubes) and non-differentiated (myoblasts) human muscle cells were susceptible for SINV infection and were able to produce infectious viruses. These findings support our speculation that an earlier SINV infection could have caused necrosis resulting in regeneration evident in the muscle biopsy. Our *in vitro* findings are somewhat different from a study on CHIKV as myotubes were found refractory to CHIKV infection [139]. Based on this finding and the fact that some myotubes and myoblasts cells remained refractory to infection in our study suggest that cell differentiation may have an effect on the susceptibility.

The biopsies in the study on CHIKV were taken during the acute phase of infection and 3 months p.i. Fewer immunoreactive satellite cells but more infiltrating inflammatory cells were detected in the biopsy taken in the chronic

phase as compared to the biopsy taken earlier [139]. Since the muscle biopsy from the SINV-infected patient was taken considerably later, it seems probable that the virus had already been eliminated and the inflammation had faded at this time. Interestingly, a study on RRV-infected mice showed that internal nuclei, indicating regeneration, were abundant in muscle cells when inflammation and pathology had already resolved 30 days p.i. [125].

The finding on COX-negative fibres, indicating mitochondrial DNA deletions, was unexpected. These deletions commonly increase in number with age but the finding was pathological as the patient was relatively young. COX-negative fibres are frequently seen in a condition called sporadic inclusion body myositis (sIBM) and studies have speculated that chronic persistent viral infection may be a triggering factor for sIBM [29]. However, whether the mtDNA deletions result from previous SINV infection remains unclear and definitely warrants further studies.

7.7 Genetic factors in the susceptibility of clinical SINV infection (V, VI)

As only a minority of SINV infections are symptomatic [18] with a defined geographical distribution and earlier chronic joint symptoms possibly predispose to progressive symptoms in arthritogenic alphavirus disease (II and [13]), we aimed to characterise the genetic predisposition factors influencing the occurrence and/or consequence of SINV infection.

We first determined the *HLA* and *C4* genes in the patient with persistent symptoms of SINV infection from whom the muscle biopsy was obtained (V). DNA extracted from whole blood was genotyped for *HLA-A, -B, -C* and *-DR.* The patient was found homozygous for *HLA-A*03* and- *B*35* alleles and heterozygous for *HLA-DRB1*01* and- *DRB1*03* alleles. The *C4* gene and protein analyses showed that the patient had a total deficiency of *C4B* genes and proteins but an increased quantity of C4A proteins. As the total C4 level in the patient was slightly elevated, these results showed that the lack of C4B protein is compensated by an increased concentration of C4A proteins.

Previous studies have identified *HLA-B*35* as a risk factor for infectious diseases, most importantly for the progression of HIV to AIDS [196]. In addition, it has been shown that *DRB1*01* allele and C4B deficiency are associated with rheumatic diseases, particularly with RA [53,140,158]. Encouraged by the results of this single case, we aimed to determine *HLA* and *C4* genes in a cohort of SINV-infected patients from which blood samples were available. The cohort had been followed over three years and at 3 years p.i., the patients were classified into four clinical categories (A to D) based on objective findings in a clinical examination performed by a rheumatologist, as well as subjective joint symptoms reported in an interview [93]. The clinical categories were defined as follows: (A) Arthritis, defined as swelling, pain on palpation or tenderness in joint movement assessed by a rheumatologist (n=2) ; (B) Objective joint pain, defined as pain on palpation or tenderness in joint movement assessed by a rheumatologist (n=5); (C) Subjective joint pain, defined as joint pain reported in the standardized interview (n=5); (D) No joint symptoms that could be associated with SINV infection (n=37) [93]. Thus, as detailed earlier [93], 12/49 (24.5%) of the patients had persistent joint pain attributable to the previous SINV infection.

A set of HLA alleles that have been reported to have an association with rheumatic and infectious diseases [196] (Table 6), including the alleles determined in the patient case described above, as well as the copy number of *C4* genes were determined.

Table 6. HLA alleles determined in the study VI and examples of significant autoimmune and infectious disease associations reported with these alleles. [53,196]

HLA allele	Disease associations
*B*27*	Ankylosing spondylitis
*B*35*	Rapid progression from HIV to AIDS
*DRB1*01*	Rheumatoid arthritis
*DRB1*03*	Systemic lupus erythematosus
*DRB1*04*	Rheumatoid arthritis
*DRB1*15*	Multiple sclerosis

The allele frequency of *DRB1*01* was significantly higher in SINV-infected patients than in the reference population (32.9% vs. 12.8%, corrected P=0.003, OR= 3.3; 95% CI 1.7-6.5) (Table 1; VI). Overall, 18/35 (51.4%) patients were carrying *DRB1*01* allele, of whom five were homozygous for *DRB1*01*. Among those positive for *DRB1*01* allele, 12 had also *HLA-B*35* allele and *C4B* deficiency (0 or 1 allele). It is known that these alleles are often detected together [32]. The frequency of *C4B* or *C4A* deficiency did not significantly differ between those with SINV infection and the reference group (51.4% vs, 39% and 8.6% vs. 18% for C4B and C4A deficiency, respectively). Patients with SINV infection had more often three copies of *C4A* gene but the difference to reference group was not significant. The *HLA* and *C4* frequencies were also compared between the clinical categories. Patients who experienced persistent joint pain 3 years p.i. and were classified into category A, B or C were considered one group and patients from category D another group. The differences in the *HLA* allele or *C4B* deficiency distribution between the groups were not statistically significant (Table 2; VI) but there was a trend towards *DRB1*01* being more common (allele frequency of 50%) among patients in categories A-C (P=0.07). The patients (n=2) from category A (arthritis), had *B*35* and *DRB1*01* alleles (one of them was homozygous for *DRB1*01*) and partial *C4B* deficiency.

Our data showed that symptomatic, clinical SINV infection shows strong association with the HLA system. The frequency of *HLA-DRB1*01* in patients with SINV infection was remarkably high. It was particularly frequent in patients who at 3 years p.i. experienced joint manifestations associated with the previous SINV infection [93] implying that HLA association may be particularly related to the persistence of joint manifestations in SINV infection.

The main strength of this study was the access to a unique, prospective follow-up material from patients with SINV infection. The reference populations, however, were not from the same area of residence as our patients with SINV infection. Although *HLA-B*35* seems to be more common in eastern Finland [177], the *DRB1*01* allele does not display similar frequency deviations and thus, the

validity of our findings is not threatened. In addition, the reference populations were not tested for SINV antibodies, resulting in a possible misclassification bias, which however would have made the detection of an association more difficult. Unfortunately we could not perform more detailed analyses of HLA genes due to the limited amount of DNA available from the only whole blood samples collected in the beginning of the study.

Twelve patients (34%) in the study cohort (VI), including the two patients in category A diagnosed with arthritis, as well as the separate patient case with persistent arthralgia/myalgia (V) had *HLA-B*35- DRB1*01* alleles and partial *C4B* deficiency. Interestingly, patients in category A were previously shown to have persisting IgM antibodies 3 years p.i. The persistence of IgM for 6 months p.i. was also observed in the patient described earlier (V). Thus, the data suggest that the combination of the *HLA-B*35- DRB1*01* alleles and *C4B* deficiency may be associated with a more prominent or persistent form of the disease although only *DRB1*01* remains as a statistically significant risk factor in the overall patient population.

7.8 Autoreactivity in SINV infection (VI)

In addition to genetic analyses on MHC region, we aimed to study the association between SINV infection and autoimmunity by measuring various autoantibodies in the acute phase and 3 years p.i. from the same cohort of patients. Autoantibodies were determined in serum of 44 patients.

We observed significant seroconversion of RF in 11/44 (25%) of the patients (P=0.022) (Table 3; VI). RF levels 3 years p.i. ranged from 21–179 IU/mL (median 32). One patient had CCP antibodies, highly specific markers of RA, both in the acute phase and 3 years p.i. The frequency of seroconversion for AMA and ANA was not significant within the cohort although one patient became positive for AMA and 3 patients for ANA during the 3-year follow up period. Antibodies to pyruvate dehydrogenase, indicative of primary biliary cirrhosis were not found in any of the AMA-positive patients. Three years p.i. all AMA-positive patients had titres of 250, and the titres of ANA positive patients ranged from 320 to >5000. In

the two patients positive for ANA already at the acute phase, the titre increased over 3 years from 320 to 1280, and 1280 to 5000, respectively. The patients in category A were both positive for ANA 3 years p.i and one was positive for RF. Overall, the differences in the frequency of autoantibodies between the patient categories (A-C vs. D) were not statistically significant. Data on both *HLA* and *C4* genes and autoantibodies were available for 31 patients. Patients with *C4B* deficiency had seroconversion of RF more often than patients without *C4B* deficiency (P=0.05). Amongst the RF-positive patients 3 years p.i., 8/10 had *C4B* deficiency and 7/10 had *DRB1*01* allele.

The frequencies of RF, AMA and ANA in the SINV-infected patients 3 years p.i. were 29.5%, 6.8% and 15.9%, respectively (Table 2; VI and Table 7). These prevalences are clearly higher than those reported in normal healthy individuals (Table 7) although a proper statistical comparison cannot be made due to lack of appropriate Finnish reference populations. In addition, the methods used to determine the prevalences in the reference populations cited in Table 7 were not in all cases identical to the ones used in our study.

Table 7. Patients with autoantibodies (AMA, RF and ANA) at acute phase and 3 years after SINV infection and the prevalence of autoantibodies in healthy reference population.

Autoantibodies	Acute phase n (%)	3 y p.i. n (%)	Reference population (%)
Mitochondrial antibody (AMA)	2/44 (4.5)	3/44 (6.8)	0.5-0.9 [137]
Rheumatoid factor (RF)	4/44 (9)	13/44 (29.5)	2 [90]
Antinuclear antibody (ANA)	4/44 (9)	7/44 (15.9)	3.3 [189]

7.9 SINV-induced and autoimmune arthritides – similarities? (VI)

One of the hallmarks of autoimmune diseases is association with HLA [21]. The risk allele identified in this study, *DRB1*01*, has been linked to rheumatic diseases

such as juvenile idiopathic arthritis [143] and RA [82,212]. Interestingly, the same allele is also associated with symptomatic parvovirus B19 infection [84]. Parvovirus B19 infection shares the main clinical features with SINV infection such as rash and arthralgia and has been implicated as the causative agent of autoimmune disorders including RA [114]. Another hallmark of autoimmune diseases includes the presence of autoantibodies in serum, demonstrating ongoing tissue destruction and autoinflammation. Autoantibodies, particularly RF, are regularly detected in acute viral infections [46,164,167] as well, but the appearance is often transient. In some serum specimens of patients with SINV infection, autoantibodies were still detected at 3 years p.i. and in fact several patients seroconverted to RF during the follow-up. Furthermore, a characteristic feature of autoimmune diseases is the female predominance [213], which has also been observed in SINV-infected patients.

Thus, our combined data on host genetics and autoantibodies suggest that similar genetic predisposing factors may contribute to the development of SINV-induced and autoimmune arthritides resulting in a disease with similar features although different etiologies are involved. It remains, however, unclear whether SINV or other arthritogenic alphaviruses in general are able to trigger the development of autoimmune diseases. It would be interesting to study the expression profile and kinetics of proinflammatory cytokines/chemokines in SINV-infected patients, since recent studies have shown that RA and alphavirus-induced arthritis share notable similarities in cytokine/chemokine profiles such as upregulation of MIF expression [10,70].

8. Concluding remarks and future prospects

Many viruses in the genus *Alphavirus* are medically important arboviruses widely distributed throughout the world and responsible for encephalitic and arthritogenic disease. Due to the ongoing changes in the climate and the environment as well as increased transportation of goods and human travel, the emergence and re-emergence of these viruses to new geographical areas is conceivable. The massive outbreak of CHIKV in 2004–2009, which also reached the Western world, has in recent years increased interest into research particularly on arthritogenic alphaviruses. This study focused on SINV and aimed in an interdisciplinary fashion to increase knowledge on (molecular) epidemiology and risk factors, to develop an assay for detection as well as to study the pathogenesis and genetic susceptibility of SINV infection.

The findings on SINV epidemiology were compatible with the presumption that changes in the grouse population and weather factors contribute to the human epidemiology of SINV infection, which was further supported by a recent, larger statistical study [75]. The population-based case-control study identified risk factors for SINV infection, e.g. confirmed the role of mosquitoes in the transmission, and expanded data on clinical features and patient characteristics. It is central to identify the factors, and their interactions, associated with the emergence of outbreaks to be able to better predict future outbreaks and design intervention strategies. Detailed knowledge of epidemiological risk factors and case-patient characteristics also helps generate hypotheses that can further be investigated in intervention and laboratory studies. Future studies should further address the public health and economic impact of SINV epidemics in Finland, particularly the long-term effects of persistent arthralgia on the quality of life.

The evolutionary history of viruses can be constructed and possible genetic markers of pathogenicity found by studying the viral genomes and the molecular epidemiology. Our molecular epidemiological study on full-length coding sequences increased the SINV sequence data substantially, established hypotheses regarding the endemic circulation of SINV in Northern Europe and

further detected distinct amino acid signatures in Northern European strains that could be associated with vector or host species adaptation. The sensitive and specific real-time quantitative PCR assay developed in this study serves as an important molecular tool in further studies where sensitive detection of SINV is needed. Since our results indicated that the viraemia in serum is low and of short duration, the virus may be more easily detected in a whole-blood sample. It would be helpful to study the viraemia and the tissue distribution of SINV in animal models to better understand the life cycle of the virus in the acute phase of the infection.

The mechanisms of musculoskeletal disease caused by arthritogenic alphaviruses have been inadequately understood and histopathological studies in humans are a rarity. Our findings on the muscle biopsy and the *in vitro* susceptibility of human muscle cells for SINV infection provided new insights concerning pathological processes leading to myalgia in SINV infection. To further study the pathobiology of SINV-induced myalgia, more human biopsy samples are needed but in addition, animal models of chronic infection would be desired. These studies could also facilitate the development of novel antiviral and/or anti-inflammatory treatment modalities.

The data on MHC genes and autoantibodies showed that SINV-induced arthritis shares similar features and/or predisposing genetic determinants with autoimmune diseases. The role of host genes in SINV infection, and in alphavirus infections in general, has been relatively unknown. We demonstrated that symptomatic SINV infection shows strong association with the markers of the HLA system and showed that autoantibody titres, RF in particular, are elevated in serum of patients 3 years p.i. Due to high linkage disequilibrium between MHC genes, further typing should be performed to address the influence of other MHC genes in the locus. As the number of clinical SINV infections in Finland greatly exceeds the incidence rates from other countries where SINV evidently circulates, host genetics may also partly explain this discrepancy in incidence. The mechanisms of autoimmunity are yet unknown but e.g. molecular mimicry may be involved, and it would therefore be important to determine the specific SINV T-and B-cell epitopes.

Since the crucial role of macrophage-induced cytokines/chemokines has been highlighted in the immunopathogenesis of RA and in alphavirus-induced arthritis, studies on the cytokine/chemokine profiles both in sera of SINV-infected patients and in macrophages originating from these patients are indeed needed. The potential causality between SINV infection and autoimmune diseases definitely warrants further investigations, and the possibilities of employing therapeutic strategies used for autoimmune diseases in the treatment of SINV-induced arthritis could be considered.

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