

## Expansion of spatial and host range of *Puumala* virus in Sweden: an increasing threat for humans?

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### SUMMARY

Hantaviruses are globally distributed and cause severe human disease. *Puumala* hantavirus (PUUV) is the most common species in Northern Europe, and the only hantavirus confirmed to circulate in Sweden, restricted to the northern regions of the country. In this study, we aimed to further add to the natural ecology of PUUV in Sweden by investigating prevalence, and spatial and host species infection patterns. Specifically, we wanted to ascertain whether PUUV was present in the natural reservoir, the bank vole (*Myodes glareolus*) further south than Dalälven river, in south-central Sweden, and whether PUUV can be detected in other rodent species in addition to the natural reservoir. In total, 559 animals were collected at Grimsö (59°43'N; 15°28'E), Sala (59°55'N; 16°36'E) and Bogesund (59°24'N; 18°14'E) in south-central Sweden between May 2013 and November 2014. PUUV ELISA-reactive antibodies were found both in 2013 (22/295) and in 2014 (18/264), and nine samples were confirmed as PUUV-specific by focus reduction neutralization test. Most of the PUUV-specific samples were from the natural host, the bank vole, but also from other rodent hosts, indicating viral spill-over. Finally, we showed that PUUV is present in more highly populated central Sweden.

**Key words:** Bank vole, *Bunyaviridae*, disease emergence, hantavirus, *Myodes glareolus*, *Puumala* virus, Sweden, zoonosis.

### INTRODUCTION

Hantaviruses are single-stranded, negative-sense RNA viruses belonging to the family *Bunyaviridae* [1]. These

constitute a widespread group of viruses, and several are zoonotic agents with great impact on public health [2]. Hantaviruses are the major causative agents of two severe human diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) [3, 4]. Geographically, HFRS is mainly limited to Eurasia while HCPS is restricted to the Americas. Approximately, 10 000 cases of human HFRS are diagnosed annually in

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Europe [4, 5], about 150 000–200 000 cases throughout the world, although there are likely thousands of cases that are never reported [6–8]. Further, the number of HFRS cases are increasing, although the drivers of this phenomenon are unclear. Factors may range from increased surveillance to climatic factors [9, 10], including a shift in host distribution and behavior as a result of climate change [5]. The clinical manifestation differs between hantaviruses, where Puumala virus (PUUV) causes less severe human diseases compared with other more pathogenic hantavirus species [11]. However, all pathogenic hantavirus infections have a similar initial clinical presentation; mainly influenza-like illness, with symptoms including myalgia, malaise and high fever [12]. Virus transmission to humans occurs through inhalation of virus-contaminated aerosol from rodent excreta. Humans are most likely exposed to virus-contaminated aerosol through dust or handling hay/timber that has been in close contact with the hosts. Furthermore, there is a strong correlation between human infections and the number of infected rodents circulating in the same area [13, 14]. Rodent-to-rodent transmission occurs through both indirect (aerosol) and direct (contact) transmission [13–15].

Hantaviruses constitute a large group of viruses with global distribution, reflecting the distribution of host reservoirs. There has been an increased focus on wild rodents as reservoirs for hantaviruses in Europe due to recent detections of Seoul virus (SEOV) in wild rats combined with severe SEOV-caused human HFRS cases. Specifically, SEOV has recently been detected in England [16], France [17], and the Netherlands [18]. Furthermore, SEOV was found in Swedish pet rats that originated from England [16]. Globally, more than 20 distinct species of hantaviruses have been described, and each virus species is spread by one specific mammalian host as a result of long-term co-evolution [19–21]. This hypothesis is supported by phylogenetic studies, whereby the genetic relationship between host and virus diversification is mirrored [22–24]. Although rodents constitute the majority of hosts, hantaviruses might have first appeared in *Chiroptera* (bats) or *Soricomorpha* (moles and shrews), before emerging in rodent species [25].

The natural reservoir host for PUUV, the most common hantavirus circulating in central and northern Europe, is the bank vole *Myodes glareolus*. PUUV is currently the only hantavirus known to circulate in Sweden, and is endemic in the northern

parts of the country [13, 26]. The current hypothesis is that PUUV is endemic only north of the river Dalälven, located north of the most urbanized regions of Sweden [26, 27]. This is reflected by the lack of human cases of south of the river Dalälven, however, recent sampling of rodents has suggested this may no longer be correct [13, 28]. In this study, we aimed to further add to the ecology of PUUV in Sweden by investigating prevalence, spatial, and host species infection patterns. Specifically, we wanted to ascertain the prevalence and distribution of hantaviruses in Swedish rodents south of the river Dalälven, and assess the host range of PUUV in rodent species in addition to the natural reservoir in this region.

## METHODS

### Sampling strategy and ethics statement

All trapping and sampling was carried out in accordance with Swedish and European law and regulations provided by the Swedish Board of Agriculture. The capture and sampling protocols were approved by the Animal Experiment Ethical Committee, Umeå, (Reference: A13–14). All trapping and sampling was conducted by trained biologists.

### Study sites and sample collection

Rodents were captured between May 2013 – November 2014 from three geographical locations south of the river Dalälven: Sala (59°55'N, 16°36'E), Grimsö (59°43'N, 15°28'E), and Bogesund (59°24'N, 18°14'E) (Figure 1). These geographic locations represent three different ecotypes. Both Sala and Grimsö are inland, however where Grimsö is more forested, the area around Sala is mostly agricultural. Furthermore, at the time of sampling the area around Sala had been heavily affected by a large fire, resulting in a disturbed landscape. Bogesund is in close proximity to the Baltic Sea and has a more rocky terrain. Rodents were captured using commercially available snap-traps. Following capture, carcasses were frozen to  $\leq -20^{\circ}\text{C}$  within 2 h of collection. In the laboratory, the rodents were defrosted and were dissected. Partial spleen and heart tissues were collected and frozen in  $-80^{\circ}\text{C}$  until required for analysis. Other tissues were collected from the rodents for a number of other studies, and the carcasses were appropriately disposed following dissections.



**Fig. 1.** Locations from which small mammals were collected in this study. Sample sites are indicated in black. Stockholm, the largest city in Sweden, and Uppsala, Sweden's fifth largest city are indicated with a gray marker have been included for reference.

### Serological screening

#### *Enzyme-linked immunosorbent assay (ELISA)*

Tissues were subdivided into smaller pieces of approximately 25 g, and homogenized in phosphate buffered saline (PBS) (using a beater for 3 min in PBS). The homogenate was initially assayed using a hantavirus IgG ELISA, based on baculovirus-expressed PUUV nucleocapsid protein antigen [29], as previously described for use on serum samples [30]. This method has been validated and successfully used previously with organ homogenates [e.g. 16, 18].

#### *Focus reduction neutralization test (FRNT)*

To confirm hantavirus-specificity, the ELISA-positive samples were further evaluated by FRNT, the gold standard for hantavirus serology [31]. Briefly, a new subsection of tissue was homogenized as described above, initially extracted in PBS (1:25). The homogenate was further diluted (1:2) in 1x Hanks balanced

serum solution (Corning, New York, USA), mixed with diluted virus (PUUV strain Kazaan-E6) [31] and added to confluent Vero E6 cell monolayers in six-well tissue culture plates. After 7 days, a solution of monkey anti-PUUV polyclonal serum in 5% Fetal Calf Serum (Gibco, Thermo Fisher, Boston, USA) and wash buffer (0.15% Tween 20 in PBS) was added and incubated. Virus-infected cells were visualized by addition of peroxidase-labelled goat anti-human IgG (Bio-Rad Laboratories, Hercules, CA), followed by terminative 3, 3', 5, 5'-tetramethylbenzidine substrate (Sigma, Stockholm, Sweden). The FRNT-positive samples from 2014 were further titrated (1:50 to 1:800) to ascertain the minimal dilution of rodent tissues to avoid non-specific inhibition. FRNT results are presented in percentages, representing the percentage reduction of the number of foci. A dilution series of infected Vero E6 cells were used as a positive control, and, 80% reduction of the number of foci was selected as the cut-off for the virus neutralization titer.

### RESULTS

A total of 559 animals were screened for PUUV reactive antibodies across three locations, south of the putative PUUV geographical boarder. Roughly similar numbers of organs were screened in 2013 and 2014, however, in 2013 all 295 samples were homogenates from spleens, as compared with 187 hearts and 77 spleens in 2014. More than 50% of samples collected were from bank vole ( $n = 342$ ), and PUUV reactive antibody prevalence in bank vole was 7.6% with no significant difference in prevalence between 2013 and 2014 (Fisher Exact Test;  $\chi^2 = 1.237$ ,  $df = 1$ ,  $P = 0.266$ ). However, a number of other species were also positive including pygmy shrew (*Sorex minutus*, 25%), common shrew (*Sorex araneus*, 3.1%), yellow-necked mouse (*Apodemus flavicollis*, 11.6%), wood mouse (*Apodemus sylvaticus*; 16.7%) and a neonate roe deer (*Capreolus capreolus*, 9%). While antibody prevalence appeared higher in yellow-necked mouse and pygmy shrew as compared with bank vole, sample size for these species was much smaller. Species tested but not positive included Eurasian water shrew (*Neomys fodiens*), field vole (*Microtus agrestis*), wood lemming (*Myopus schisticolor*), and three avian species. Different locations appeared to have different importance for different species, however sampling bias did not allow for comparisons except for bank voles and yellow-necked mouse. For bank vole, PUUV antibody prevalence was higher in

Table 1. ELISA prevalence and number of samples collected from locations south of the river Dalälven in 2013–2014 in Sweden

Species	Prevalence (# ELISA positive/# samples collected)				
	Year		Location		
	2013	2014	Bogesund	Sala	Total
Bank vole ( <i>Myodes glareolus</i> )	9.3% (15/162)	6.1% (11/180)	13.4% (11/82)	2.0% (2/100)	7.6% (26/342)
Field vole ( <i>Microtus agrestis</i> )	<0.001% (0/17)	<0.001% (0/9)	<0.001% (0/1)	<0.001% (0/6)	<0.001% (0/26)
Common shrew ( <i>Sorex araneus</i> )	3.4% (3/89)	<0.001% (0/7)	<0.001% (0/3)	<0.001% (0/4)	3.1% (3/96)
Eurasian pygmy shrew ( <i>Sorex minutus</i> )	2.5% (2/8)	NT	NT	NT	2.5% (2/8)
Eurasian water shrew ( <i>Neomys fodiens</i> )	<0.001% (0/1)	NT	NT	NT	<0.001% (0/1)
Wood lemming ( <i>Myopus schisticolor</i> )	NT	<0.001% (0/1)	NT	NT	<0.001% (0/1)
Wood mouse ( <i>Apodemus sylvaticus</i> )	NT	16.7% (3/18)	50% (1/2)	12.5% (2/16)	16.7% (3/18)
Yellow-necked mouse ( <i>Apodemus flavicollis</i> )	25.0% (1/4)	10.2% (4/39)	0.5% (1/20)	18.2% (4/22)	11.6% (5/43)
Unknown mouse species	NT	<0.001% (0/8)	<0.001% (0/7)	<0.001% (0/1)	<0.001% (0/8)
Roe deer ( <i>Capreolus capreolus</i> )	9% (1/11)	NT	12.5% (1/8)	NT	9% (1/11)
Great tit ( <i>Parus major</i> )	<0.001% (0/1)	<0.001% (0/2)	NT	<0.001% (0/2)	<0.001% (0/3)
Eurasian nuthatch ( <i>Sitta europaea</i> )	<0.001% (0/1)	NT	NT	NT	<0.001% (0/1)
European robin ( <i>Erithacus rubecula</i> )	<0.001% (0/1)	NT	NT	NT	<0.001% (0/1)
Total	295	264	123	151	559

NT, not tested.

Bogesund (Fisher Exact Test;  $\chi^2 = 8.787$ ,  $df = 1$ ,  $P = 0.003$ ) and Grimsö (Fisher Exact Test;  $\chi^2 = 4.26$ ,  $df = 1$ ,  $P = 0.04$ ) than Sala. In contrast, yellow-necked mice in Sala had a higher prevalence (18.2%) than Bogesund (0.5%), however due to small sample sizes this is not significant (Fisher Exact Test;  $\chi^2 = 3.634$ ,  $df = 1$ ,  $P = 0.056$ ) (Table 1).

Subsequently, all ELISA positives were assayed by FRNT to confirm hantavirus-specificity. Diluting homogenates prior to FRNT analysis proved crucial; homogenates from 2014 were serially diluted and revealed that a minimal dilution for a reliable result was 1:100 for this sample type (antibodies extracted from rodent spleens and hearts). The dilution 1:50, used in 2013, was insufficient to avoid the possibility of non-specific inhibition, which would result in potentially false positive outcomes. Thus, FRNT confirmation from the 2013 samples is tentative, however we infer that 5 of the 22 ELISA positives in 2013 reacted at 1:50 by FRNT dilution; roe deer ( $n = 1$ ), common shrew ( $n = 1$ ) and bank voles ( $n = 3$ ). In 2014, nine ELISA positives were confirmed by FRNT, limited to bank voles from Bogesund (5/56 tested), a wood mouse in Bogesund (1/2 tested) and yellow-necked mice in Sala (3/22 tested). Interestingly, one yellow-necked mouse (Sample 134, 2014) had a FRNT end-point titer of  $\geq 1:800$  (Table 2).

## DISCUSSION

Emerging and re-emerging pathogens are among the greatest challenges of the 21st century, and present a large economic burden to society. Further, most emerging and re-emerging pathogens are zoonotic viruses; viruses with natural hosts in the animal reservoir [32–34]. European studies indicate that hantaviruses are not only spreading to new areas [17, 18], but also to new hosts [35]. In this study, we aimed to assess the dynamics of hantaviruses in Sweden, by assessing virus diversity and prevalence, spatial distribution, and host species fidelity through antibodies. Spatially, the current working hypothesis is that PUUV in Sweden is endemic north of the river Dalälven [26, 27], however both this study and Lohmus *et al.* [28] clearly demonstrated PUUV infections in bank voles south of this border. We found positive rodents from Grimsö, Sala and Bogesund, captured in both 2013 and 2014, however, different areas were more important for different species. Reactive antibody prevalence was highest in Grimsö and Bogesund in bank vole; the Sala landscape,



Table 2. FRNT neutralization of ELISA-positive samples from small mammals collected in 2014

Sample ID	Year	Organ	Area	Species	FRNT**†				
					Interpretation	(1:50)	(1:100)	(1:200)	(1:800)
2	2014	Heart	Bogesund	Bank vole	POS	3%	1·70%	10%	53%
11	2014	Heart	Bogesund	Bank vole	POS	8%	0%	17·5%	92·50%
22	2014	Heart	Bogesund	Bank vole	POS	5%	1·70%	18%	56·70%
28	2014	Heart	Bogesund	Bank vole	POS	1·70%	3%	25%	51·70%
40	2014	Heart	Bogesund	Bank vole	NEG	10%	32%	85%	110%
43	2014	Heart	Bogesund	Bank vole	NEG	5%	30%	52·50%	70%
47	2014	Heart	Bogesund	Wood mouse	POS	0%	1·70%	6·70%	45%
51	2014	Heart	Bogesund	Bank vole	POS	8%	20%	77·50%	117·50%
72	2014	Heart	Sala	Yellow-necked mouse	NEG	10%	30%	47·50%	135%
129	2014	Heart	Sala	Bank vole	NEG	8%	35%	67·50%	110%
130	2014	Heart	Sala	Wood mouse	NEG	10%	62·50%	112·50%	137·50%
132	2014	Heart	Sala	Wood mouse	NEG	31%	NT‡	NT	NT
134	2014	Heart	Sala	Yellow-necked mouse	POS	11·70%	12·50%	2·50%	15%
135	2014	Heart	Sala	Bank vole	NEG	13%	62·5%	52·50%	60%
142	2014	Spleen	Sala	Yellow-necked mouse	POS	5%	5%	25%	90%
145	2014	Spleen	Sala	Yellow-necked mouse	POS	10%	10%	22·50%	55%
249	2014	Heart	Grimsö	Bank vole	NEG	5%	50%	75%	90%
252	2014	Spleen	Bogesund	Bank vole	NEG	48%	52·5%	80%	90%

\* FRNT result at 1:100 dilution of <20% indicates a positive result.

† Percentage of foci as compared with virus control.

‡ Not tested.

which is mostly agricultural was devastated by a large fire during the sampling period of this study. How this affects PUUV antibody prevalence is uncertain. In contrast, Sala was more important for yellow-necked mouse. The role of habitat for disease risk is complex, but a recent review suggests that there is a strong correlation between habitat and disease prevalence. Specifically, factors such as forest cover, fires, fragmentation and barrow space influence the dispersal of voles (and in this case mice), consequently affecting the epidemiology of PUUV [4, 19, 36, 37]. The Bogesund site is of particular interest as it is both the most southern site of this study and a location with high PUUV prevalence in bank voles. This range expansion of PUUV in wildlife reservoirs has yet to result in numerous human cases. A similar trend is evident in France, where PUUV has been detected in voles in populated regions with no human cases of HFRS, however in this case it is suggested to be driven by specific amino acid differences in the viruses [38]. Regardless, expansion of PUUV into areas with a higher human population is concerning in context of public health.

Not only did we detect an expansion in the known PUUV geographic range, we also illustrate an increase in host range following detection of PUUV reactive antibodies in a number of permissive species. Yellow-

necked mouse, wood mouse, common shrew and pygmy shrew were found among the ELISA-positive samples; in total 37% of ELISA-reactive samples were from species other than bank vole, indicating PUUV spill-over to other rodent and shrew species, or the presences of an unknown hantavirus causing cross-reacting antibodies detected by ELISA. Yellow-necked mouse has previously been shown to be a permissive host for PUUV in Sweden [28], but we found ELISA reactive antibodies in most species tested (given a large enough sample size), with the exception of field vole. While rodents, specifically mice are plausible spill-over hosts, detection of PUUV-reactive antibodies from a roe deer is unusual. The actual hantavirus species infecting Swedish shrews awaits further investigations. Given the numerous shrew-carried hantaviruses discovered during the last decade [6, 8], it is likely that one or several of these species are also circulating in Sweden, although PUUV spill-over events cannot be excluded at this stage. Given the deviation from known hantavirus host range, a more in depth analysis of shrews and ungulates ranging from sampling to virus sequencing is warranted. Indeed, Ahlm *et al.*, described hantavirus-infected moose from northern Sweden [39], thus ungulates appear permissive to PUUV infection, but whether they are dead-

end hosts or not is unknown. Hantaviruses are considered to be host-specific [21], however, this study revealed unexpected spill-over to a spectrum of different rodents, corroborating the hypothesis that PUUV epidemiology may be more complex [30, 40, 41].

Based upon our results, and emerging evidence [30, 40, 41], strict host fidelity in this system seems unlikely. The role that these spill-over hosts play in the epidemiology is, however unclear; they are indeed permissive to infection, and given the detection levels in this study, these spill-over events are not rare. In order to reveal the role of putative spill-over hosts play in the epidemiology of PUUV we need to ascertain whether they are dead-end hosts, spill-over hosts, or are able to transmit infection. Regardless, it is likely that PUUV potentially has lower fitness in species other than bank voles, which may in turn limit frequency of infections. This potentially expanded model of PUUV (and hantavirus) epidemiology has large implications for the mitigation of human hantavirus-derived disease cases, as more hosts increase the risk for human transmission. This is further compounded with range expansion into more populated regions of Sweden. If these phenomena result in endemicity in new hosts or geographic regions, the health burden caused by hantaviruses will certainly increase.

## CONCLUSIONS

Studies such as these are imperative in ascertaining PUUV prevalence in wildlife hosts to better inform risk areas for human infections. Given an expansion of PUUV range in the wildlife host, surveillance in humans is prudent. PUUV hantavirus is a putatively emerging virus in Sweden, with detections of antibodies against PUUV in both the reservoir and other small mammals farther south than previously described. Specifically, PUUV is now detected in more densely populated areas, as described here, in close proximity to large cities such as Uppsala and Stockholm. Moreover, rodents such as yellow-necked mouse utilize anthropogenic buildings 10 times more frequently than bank voles [28]. These two factors rapidly decrease distance, and thus increase interactions, between humans and the wildlife reservoir. This may have large implications, as it increases the probability of human contact with infected rodent excreta, creating a large reservoir for potential hantavirus infections in humans.

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## DECLARATION OF INTEREST

None.

## ETHICAL STANDARDS

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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