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

3

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25 Running Head: Puumala hantavirus in Sweden

26 **Summary**

27 Hantaviruses are globally distributed and cause severe human disease. Puumala hantavirus
28 (PUUV) is the most common species in Northern Europe, and the only hantavirus confirmed
29 to circulate in Sweden, restricted to the northern regions of the country. In this study, we
30 aimed to further add to the natural ecology of PUUV in Sweden by investigating prevalence,
31 and spatial and host species infection patterns. Specifically, we wanted to ascertain whether
32 PUUV was present in the natural reservoir, the bank vole (*Myodes glareolus*) further south
33 than Dalälven river, in south-central Sweden, and whether PUUV, in addition, can be
34 detected in other rodent species in addition to the natural reservoir. In total, 559 animals were
35 collected at Grimsö (59°43'N; 15°28'E), Sala (59°55'N; 16°36'E) and Bogesund (59°24'N;
36 18°14'E) in south central Sweden between May 2013 and November 2014. PUUV ELISA-
37 reactive antibodies were found both in 2013 (22/295) and in 2014 (18/264), and 9 samples
38 were confirmed as PUUV-specific by focus reaction neutralization test. Most of the PUUV-
39 specific samples were from the natural host, the bank vole, but also from other rodent hosts,
40 indicating viral spill-over. Finally, we showed that PUUV is present in more highly populated
41 central Sweden.

42

43 **Keywords:** Bank vole; *Bunyaviridae*; Disease emergence; Hantavirus; *Myodes glareolus*;

44 Puumala virus; Sweden; Zoonosis

45 **Introduction**

46 Hantaviruses are single-stranded, negative-sense RNA viruses belonging to the family
47 *Bunyaviridae* [1]. These constitute a widespread group of viruses, several are zoonotic agents
48 with great impact on public health [2]. Hantaviruses are the major causative agents of two
49 severe human diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus
50 cardiopulmonary syndrome (HCPS) [3, 4]. Geographically, HFRS is mainly limited to
51 Eurasia while HCPS is restricted to the Americas. Approximately, 10 000 cases of human
52 HFRS are diagnosed annually in Europe [4, 5], about 150,000 to 200,000 cases throughout
53 the world, although there are likely thousands of cases that are never reported [6-8]. Further,
54 the number of HFRS cases are increasing, although the drivers of this phenomenon are
55 unclear. Factors may range from increased surveillance to climatic factors [9, 10], including a
56 shift in host distribution and behaviour as a result of climate change [5]. The clinical
57 manifestation differs between hantaviruses, where Puumala virus (PUUV) causes less severe
58 human diseases compared to other more pathogenic hantavirus species [11]. However, all
59 pathogenic hantavirus infections have a similar initial clinical presentation; mainly influenza-
60 like illness, with symptoms including myalgia, malaise and high fever [12]. Virus
61 transmission to humans occurs through inhalation of virus-contaminated aerosol from rodent
62 excreta. Humans are most likely exposed to virus-contaminated aerosol through dust or
63 handling hay/timber that has been in close contact with the hosts. Furthermore, there is a
64 strong correlation between human infections and the number of infected rodents circulating
65 in the same area [13, 14]. Rodent-to-rodent transmission occurs through both indirect
66 (aerosol) and direct (contact) transmission [13-15].

67 Hantaviruses constitute a large group of viruses with global distribution, reflecting the
68 distribution of host reservoirs. There has been an increased focus on wild rodents as
69 reservoirs for hantaviruses in Europe due to recent detections of Seoul virus (SEOV) in wild

70 rats combined with severe SEOV-caused human HFRS cases. Specifically, SEOV has
71 recently been detected in England [16], France [17], and the Netherlands [18]. Furthermore,
72 SEOV was found in Swedish pet rats that originated from England [16]. Globally, more than
73 20 distinct species of hantaviruses have been described, and each virus species is spread by
74 one specific mammalian host as a result of of long term co-evolution [19-21]. This hypothesis
75 is supported by phylogenetic studies, whereby the genetic relationship between host and virus
76 diversification is mirrored [22-24]. Although rodents constitute the majority of hosts,
77 hantaviruses might have first appeared in *Chiroptera* (bats) or *Soricomorpha* (moles and
78 shrews), before emerging in rodent species [25].
79 The natural reservoir host for PUUV, the most common hantavirus circulating in central and
80 northern Europe, is the bank vole *Myodes glareolus*. PUUV is currently the only hantavirus
81 known to circulate in Sweden, and is endemic in the northern parts of the country [13, 26].
82 The current hypothesis is that PUUV is endemic only north of the river Dalälven, located
83 north of the most urbanised regions of Sweden [26, 27]. This is reflected by the lack of
84 human cases of south of the river Dalälven, however, recent sampling of rodents has
85 suggested this may no longer be correct [13, 28]. In this study, we aimed to further add to the
86 ecology of PUUV in Sweden by investigating prevalence, spatial, and host species infection
87 patterns. Specifically, we wanted to ascertain the prevalence and distribution of hantaviruses
88 in Swedish rodents south of the river Dalälven, and assess the host range of PUUV in rodent
89 species in addition to the natural reservoir in this region.

90

91 **Materials and Methods**

92 ***Sampling strategy and ethics statement***

93 All trapping and sampling was carried out in accordance with Swedish and European law and
94 regulations provided by the Swedish Board of Agriculture. The capture and sampling

95 protocols were approved by an ethical permission from the Animal Experiments Ethical
96 Committee, Umeå, (Reference: A13-14). All trapping and sampling was conducted by trained
97 biologists.

98 *Study sites and sample collection*

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

111 *Serological screening*

112 *Enzyme Linked Immunosorbant Assay*

113 Tissues were subdivided into smaller pieces of approximately 25 g, and homogenized in PBS
114 (using a beater for 3 minutes in PBS). The homogenate was initially assayed using a
115 hantavirus IgG ELISA, based on baculovirus-expressed PUUV nucleocapsid protein antigen
116 [29], as previously described for use in sera [30]. This method has been validated and
117 successfully used previously with organ homogenates [eg. 16, 18].

118 *Focus reaction neutralization test*

119 To confirm hantavirus-specificity, the ELISA positive samples were further evaluated by
120 focus reaction neutralisation test (FRNT), the gold standard for hantavirus serology [31].
121 Briefly, a new subsection of tissue was homogenised as described above, initially extracted in
122 PBS (1:25). The homogenate was further diluted (1:2) in 1x Hanks balanced serum solution
123 (Corning, New York, USA), mixed with diluted virus (PUUV strain Kazaan-E6) [31] and
124 added to confluent Vero E6 cell monolayers in six-well tissue culture plates. After 7 days, a
125 solution of monkey anti-PUUV polyclonal serum in 5% Fetal Calf Serum (Gibco,
126 ThermoFisher, Boston, USA) and wash buffer (0,15% Tween- 20 in PBS) was added and
127 incubated. Virus-infected cells were visualized by addition of peroxidase-labelled goat anti-
128 human IgG (BioRad Laboratories, Hercules, CA), followed by terminative 3, 3', 5, 5'-
129 tetramethylbenzidine substrate (Sigma, Stockholm, Sweden). The FRNT-positive samples
130 from 2014 were further titrated (1:50 to 1:800) to ascertain the minimal dilution of rodent
131 tissues to avoid non-specific inhibition. FRNT results are presented in percentages,
132 representing the percentage reduction of the number of foci. A dilution series of infected
133 Vero E6 cells were used as a positive control, and, 80% reduction of the number of foci was
134 selected as the cut-off for the virus neutralization titre.

135

136 **Results**

137 A total of 559 animals were screened for PUUV reactive antibodies across three locations,
138 south of the putative PUUV geographical boarder. Roughly similar numbers of organs were
139 screened in 2013 and 2014, however in 2013 all 295 samples were homogenates from
140 spleens, as compared to 187 hearts and 77 spleens in 2014. More than 50% of samples
141 collected were from bank vole (n= 342), and PUUV reactive antibody prevalence in bank
142 vole was 7.6% with no significant difference in prevalence between 2013 and 2014 (Fisher

143 Exact Test; $X^2=1.237$, $df=1$, $p=0.266$). However, a number of other species were also positive
144 including pygmy shrew (*Sorex minutus*, 25%), common shrew (*S. araneus*, 3.1%), yellow-
145 necked mouse (*Apodemus flavicollis*, 11.6%), wood mouse (*A. sylvaticus*; 16.7%) and a
146 neonate roe deer (*Capreolus capreolus*, 9%). While antibody prevalence appeared higher in
147 yellow-necked mouse and pygmy shrew as compared to bank vole, sample size for these
148 species was much smaller. Species tested but not positive included Eurasian water shrew
149 (*Neomys fodiens*), field vole (*Microtus agrestis*), wood lemming (*Myopus schisticolor*), and
150 three avian species. Different locations appeared to have different importance for different
151 species, however sampling bias did not allow for comparisons except for bank voles and
152 yellow-necked mouse. For bank vole, PUUV antibody prevalence was higher in Bogesund
153 (Fisher Exact Test; $X^2=8.787$, $df=1$, $p=0.003$) and Grimsö (Fisher Exact Test; $X^2=4.26$, $df=1$,
154 $p=0.04$) than Sala. In contrast, yellow-necked mice in Sala had a higher prevalence (18.2%)
155 than Bogesund (0.5%), however due to small sample sizes this is not significant (Fisher Exact
156 Test; $X^2=3.634$, $df=1$, $p=0.056$) (Table 1).

157 Subsequently, all ELISA positives were assayed by FRNT to confirm hantavirus-specificity.
158 Diluting homogenates prior to FRNT analysis proved crucial; homogenates from 2014 were
159 serially diluted and revealed that a minimal dilution for a reliable result was 1:100 for this
160 sample type (antibodies extracted from rodent spleens and hearts). The dilution 1:50, used in
161 2013, was insufficient to avoid the possibility of non-specific inhibition, which would result
162 in false positive outcomes. Thus, FRNT confirmation from the 2013 samples is tentative,
163 however we infer that 5 of the 22 ELISA positives in 2013 reacted at 1:50 by FRNT dilution;
164 roe deer ($n=1$), common shrew ($n=1$) and bank voles ($n=3$). In 2014, 9 ELISA positives were
165 confirmed by FRNT, limited to bank voles from Bogesund (5/56 tested), a wood mouse in
166 Bogesund (1/2 tested) and yellow-necked mice in Sala (3/22 tested). Interestingly, one
167 yellow-necked mouse (Sample 134, 2014) had an end-point titre of $\geq 1:800$ (Table 2)

168

169 **Discussion**

170 Emerging and re-emerging pathogens are among the greatest challenges of the twenty-first
171 century, and present a large economic burden to society. Further, most emerging and
172 reemerging pathogens are zoonotic viruses; viruses with natural hosts in the animal reservoir
173 [32-34]. European studies indicate that hantaviruses are not only spreading to new areas [17,
174 18], but also to new hosts [35]. In this study, we aimed to assess the dynamics of hantaviruses
175 in Sweden, by assessing virus diversity and prevalence, spatial distribution, and host species
176 fidelity through antibodies. Spatially, the current working hypothesis is that PUUV in
177 Sweden is endemic north of the river Dalälven [26, 27], however both this study and Löhmus
178 et al (2016) clearly demonstrated PUUV infections in bank voles south of this boarder. We
179 found positive rodents from Grimsö, Sala and Bogesund, captured in both 2013 and 2014,
180 however, different areas were more important for different species. Reactive antibody
181 prevalence was highest in Grimsö and Bogesund in bank vole; the Sala landscape, which is
182 mostly agricultural was devastated by a large fire during the sampling period of this study.
183 How this affects PUUV antibody prevalence is uncertain. In contrast, Sala was more
184 important for yellow-necked mouse. The role of habitat for disease risk is complex, but a
185 recent review suggests that there is a strong correlation between habitat and disease
186 prevalence. Specifically, factors such as forest cover, fires, fragmentation and barrow space
187 influence the dispersal of voles (and in this case mice), consequently affecting the
188 epidemiology of PUUV [4, 19, 36, 37].The Bogesund site is particularly interesting as it is
189 the southern most location of both this study, where PUUV prevalence in bank voles was
190 high. At this southern location Löhmus et al 2016 detected PUUV in a more southern
191 location, but in yellow-necked mice [28]. This range expansion of PUUV in wildlife
192 reservoirs has yet to result in numerous human causes. A similar trend is evident in France,

193 where PUUV has been detected in voles in populated regions with no human cases of HFRS,
194 however in this case it is suggested to be driven by specific amino acid differences in the
195 viruses [38]. Regardless, expansion of PUUV into areas with a higher human population is
196 concerning in context of public health.

197 Not only did we detect an expansion in the known PUUV geographic range, we also illustrate
198 an increase in host range following detection of PUUV reactive antibodies in a number of
199 permissive species. Yellow-necked mouse, wood mouse, common shrew and pygmy shrew
200 were found among the ELISA positive samples; in total 37% of ELISA reactive samples
201 were from species other than bank vole, indicating PUUV spill-over to other rodent and
202 shrew species, or the presence of to date unknown hantaviruses causing cross-reacting
203 antibodies detected by ELISA. Yellow-necked mouse has previously been shown to be a
204 permissive host for PUUV in Sweden [28], but we found ELISA reactive antibodies in most
205 species tested (given a large enough sample size), with the exception of field vole. While
206 rodents, specifically mice are plausible spill over hosts, detection of PUUV reactive
207 antibodies from a roe deer is unusual. The actual hantavirus species infecting Swedish
208 shrews awaits further investigations. Given the numerous shrew-carried hantaviruses
209 discovered during the last decade [6, 8], it is likely that one or several of these species are
210 circulating also in Sweden, although also PUUV spill-over events can not be excluded at this
211 stage. Given the deviation from known hantavirus host range, a more in depth analysis of
212 shrews and ungulates ranging from sampling to virus sequencing is warranted. Indeed, Ahlm
213 et al. (2000), described hantavirus-infected moose from northern Sweden [39], thus ungulates
214 appear permissive to PUUV infection, but whether they are dead-end hosts or not is
215 unknown. Hantaviruses are considered to be host-specific [21], however, this study revealed
216 unexpected spill-over to a spectrum of different rodents, corroborating the hypothesis that
217 PUUV epidemiology may be more complex [30, 40, 41].

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

231 **Conclusions**

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

242 excreta, creating a large reservoir for potential hantavirus infections in humans.

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255 **Conflict of interest**

256 The authors declare no conflict of interest

257 **Ethical standards**

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

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355

356 **Table Legends**

357 Table 1: ELISA prevalence and number of samples collected from locations south of the river

358 Dalälven in 2013-2014 in Sweden.

Species	Prevalence (# ELISA positive/# samples collected)					Total
	Year		Location			
	2013	2014	Bogesund	Grimsö	Sala	
Bank vole (<i>Myodes glareolus</i>)	9.3% (15/162)	6.1% (11/180)	13.4% (11/82)	8.1% (13/160)	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% (0/19)	<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)	3.4% (3/89)	<0.001% (0/4)	3.1% (3/96)
Eurasian Pygmy shrew (<i>Sorex minutus</i>)	25% (2/8)	NT	NT	25% (2/8)	NT	25% (2/8)
Eurasian Water shrew (<i>Neomys fodiens</i>)	<0.001% (0/1)	NT	NT	<0.001% (0/1)	NT	<0.001% (0/1)
Wood lemming (<i>Myopus schisticolor</i>)	NT	<0.001% (0/1)	NT	<0.001% (0/1)	NT	<0.001% (0/1)
Wood mouse (<i>Apodemus sylvaticus</i>)	NT	16.7% (3/18)	50% (1/2)	NT	12.5% (2/16)	16.7% (3/18)
Yellow-necked mouse (<i>Apodemus flavicollis</i>)	25% (1/4)	10.2% (4/39)	0.5% (1/20)	<0.001% (0/1)	18.2% (4/22)	11.6% (5/43)
Unknown mouse species	NT	<0.001% (0/8)	<0.001% (0/7)	NT	<0.001% (0/1)	<0.001% (0/8)
Roe deer (<i>Capreolus capreolus</i>)	9% (1/11)	NT	12.5% (1/8)	<0.001% (0/3)	NT	9% (1/11)
Great tit (<i>Parus major</i>)	<0.001% (0/1)	<0.001% (0/2)	NT	<0.001% (0/1)	<0.001% (0/2)	<0.001% (0/3)
Eurasian nuthatch (<i>Sitta europaea</i>)	<0.001% (0/1)	NT	NT	NT	NT	<0.001% (0/1)
European robin (<i>Erithacus rubecula</i>)	<0.001% (0/1)	NT	NT	<0.001% (0/1)	NT	<0.001% (0/1)
Total	295	264	123	285	151	559

359

360

361 Table 2: FRNT neutralization of ELISA positive samples from small mammals collected in
 362 2014.

Sample ID	Year	Organ	Area	Species	FRNT ^{a,b}				
					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 ^c	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%

363 a. FRNT result at 1:100 dilution of less than 20% indicates a positive result

364 b. Percentage of foci as compared to virus control

365 c. Not tested

366

367 **Figure Legend**

368 Figure1: Locations from which small mammals were collected in this study. Sample sites are
369 indicated in black. Stockholm, the largest city in Sweden, and Uppsala, Sweden's fifth largest
370 city are indicated with a grey marker have been included for reference. The river Dalälven,
371 the assumed Swedish PUUV border, is indicated.

372