SEROLOGIC EVIDENCE OF PUUMALA VIRUS INFECTION IN WILD MOOSE IN NORTHERN SWEDEN

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Abstract. Puumala (PUU) virus is the causative agent of nephropathia epidemica, the Scandinavian form of hemorrhagic fever with renal syndrome. The infection is acquired by airborne transmission of PUU virus from its rodent reservoir, the bank vole. Besides serologic data indicating that the virus may spread also to heterologous rodents, there is little information on the susceptibility of wild living animals to PUU virus. We studied the occurrence of antibodies to PUU virus in serum samples from 427 wild-living moose, of which 260 originated from the PUU virus-endemic northern and central parts of Sweden and 167 originated from the southern, nonendemic part of Sweden. Samples from 5 animals showed reactivity in an ELISA for recombinant PUU virus nucleocapsid protein, an immunofluorescent assay, and a neutralization test. These 5 animals all originated from the PUU virus-endemic northern part of Sweden. In conclusion, 5 of 260 moose from the endemic region showed convincing serologic evidence of past PUU virus infection. The seroprevalence was low, suggesting that the moose is subjected to endstage infection rather than being part of an enzootic transmission cycle.

Members of the genus Hantavirus (family Bunyaviridae) are associated with zoonotic diseases, which are grouped in 2 clinical syndromes. Hemorrhagic fever with renal syndrome (HFRS), which occurs in Europe and Asia, is caused by Hantaan (HTN), Seoul (SEO), Dobrava (DOB), and Puumala (PUU) virus. The severity of HFRS varies with the causative agent, with the fatality rate ranging from 5% to 15% in HTN to <0.5% in PUU virus infection. Hantavirus pulmonary syndrome (HPS) occurs in North and South America and is caused by Sin Nombre virus (SNV). This syndrome is a fulminant disease with a fatality rate of ~50%. Due to an increased awareness of the heterogeneity of viruses within the genus, new hantaviruses are currently identified and linked to the syndromes.

Hantaviruses are associated with rodents. The association is strictly regulated insofar as each virus tends to be adapted preferentially to one given species of rodents. The animals are held to be persistently infected with the virus and transmission among the animals seems to occur mainly via aerosolized excretions. This host restriction suggests that the evolution of hantaviruses is more or less restricted by the evolution of rodents.

In addition to its circulation within a rodent reservoir, a hantavirus may also infect heterologous wild living rodents and other mammals. However, data on such spread is not yet comprehensive. The information is based largely on surveys comprising limited numbers of mammals of various species and less on thorough investigations of specific virus-mammal associations. The obvious exception is humans, in which the spread of hantavirus infection is well elucidated. Human infection is believed to be acquired by inhalation of aerosols containing contaminated rodent secreta. Except for single cases described of person-to-person transmission, there is no evidence to indicate that infected humans may contribute to further spread of hantaviruses.

In Scandinavia, PUU virus has been the only hantavirus isolated so far. It is the causative agent of nephropathia epidemica (NE), the mild form of HFRS. In Sweden, NE occurs only in the northern and central parts of the country. During rodent-rich years in the region, the annual incidence may exceed 30 cases per 100,000 inhabitants. In a randomized and stratified study from northern Sweden, a prevalence of PUU virus antibodies of 9% was found in the adult population.

The reservoir of PUU virus is the bank vole Clethrionomys glareolus. This is apparent from successful attempts to isolate virus from tissue samples as well as from serologic investigations. Moreover, serologic investigations verify that rodent species other than the bank vole may become infected. In studies of small mammals captured in northern Sweden, bank voles were found to have antibodies to PUU virus much more frequently than did rodents of other species.

As is true for hantaviruses in general, there is only limited information on the susceptibility to PUU virus infection of mammals other than humans and rodents. We attempted to find serologic evidence of hantavirus infection in the Swedish moose (Alces alces), an animal that is well characterized with regard to living habitat and migration. The study comprised animals from PUU virus-endemic regions as well as nonendemic regions, and due to the lack of serologic background data in moose, the observations were validated by use of different serologic techniques.

MATERIALS AND METHODS

Sampling of blood from wild-living moose. During 1995–1997, 427 moose (205 females, 202 males, and 20 sex undetermined) were investigated. As appears from the age distribution (Figure 1), a large proportion of the animals were calves. Six geographically separated areas were represented (Figure 2), each measuring 2,500–5,000 km². The moose were darted from a helicopter (Hughes 500E; The Boeing Company, Seattle, WA) during the winter. They were
injected with a mixture of an anesthetic and a tranquilizer (Immobilon®; Veterinary Products, Leyland, United Kingdom and Rompun®; Bayer AG, Leverkusen, Germany). Venous blood was sampled from the jugular vein into Vacutainer® test tubes (Becton Dickinson, Franklin Lakes, NJ). The study was approved by the Ethical Committee of Animal Research in Umeå (A10/91, A101-A103/93).

**Preparation of monoclonal antibodies to moose IgG.** Immunoglobulin G was purified from normal moose serum essentially as described with slight modifications according to standard operation procedures of Svanova Biotech (Uppsala, Sweden). Six BALB/c mice were immunized. First, 300 μg of moose IgG in 0.5 ml of Freund’s complete adjuvant were given subcutaneously. Six weeks later, the mice were given booster immunizations with 300 μg of moose IgG in 0.5 ml of Freund’s incomplete adjuvant. Four days after the second immunization, the mice were killed and spleen cells were collected and fused with the myeloma cell line SP2/0 as previously described. Supernatants from the hybridoma cell clones were tested by an ELISA for the presence of anti-moose IgG antibodies, using purified IgG (3 μg/ml) as antigen for coating. Positive clones were subcloned 4 times, retested in an ELISA, and checked for cross-reactions with IgG of other mammalian species. The clone 33A/B10 was found to react strongly with moose IgG but not with bovine, caprine, ovine, or roe deer IgG. This clone was selected for purification of monoclonal antibody (Merza M, unpublished data) and stored at −20°C in 50% glycerol.

**Hantavirus recombinant nucleocapsid protein ELISA.** Wells of microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with *Escherichia coli*-expressed recombinant fusion proteins containing amino acids 1–117 of the SEO virus, SNV, and PUU virus nucleocapsid (N) proteins as described previously. Each serum sample was diluted 1/100 in phosphate-buffered saline (PBS) containing *E. coli* antigen and incubated at 4°C overnight with all 3 hantavirus antigens. Control wells contained no antigen. After 4 washes in deionized water, mouse anti-moose monoclonal antibodies were diluted 1/400 in PBS (0.2 μg/ml), added to the wells, and the plates were incubated at 37°C for 2 hr. After 4 washes in deionized water, horseradish peroxidase-conjugated goat anti-mouse IgG (γ-chain specific; Sigma, St. Louis, MO) at a dilution of 1/2,000 in PBS was added to each well and the plates were incubated at 37°C for 1 hr. After 4 washes in deionized water and incubation for 15 min with 100 μl of 2 M H₂SO₄, Antibody activity was expressed as the net absorbance value at 450 nm (mean absorbance of antigen-coated wells—mean absorbance of control wells). A net absorbance ≥0.2 was considered to show the presence of antibodies. Absorbance values of control wells were 0.05–0.14 (mean = 0.08). For control purposes, samples of positive and negative human serum were included, using appropriate conjugates.

**Immunofluorescent assay.** An indirect immunofluorescent antibody assay (IFA) was performed essentially as previously described. A local isolate of PUU virus (Umeå/305/human/95 strain) was propagated in Vero E6 cells, applied to slides, and used as antigen. In standardization experiments, this strain was found to give results identical to those of the Sotkamo strain of PUU virus. Serum diluted 1/8 in PBS was added to the slides and incubated overnight at 4°C. The slides were then washed with PBS-Tween, and mouse anti-moose monoclonal antibody diluted 1/40 in PBS was added and incubated for 1 hr at 37°C. The slides were then washed with PBS-Tween, and a fluorescein-labeled rabbit
anti-mouse IgG (F0313; Dako, Glostrup, Denmark), diluted 1/20 in PBS, and 0.003% Evans blue were added and incubated for 1 hr at 37°C. Samples were read blindly in a fluorescence microscope and a characteristic cytoplasmic fluorescence at a serum dilution ±1/8 was considered to show antibody reactivity. Serum samples with reactivity to SNV and/or SEO virus proteins in the ELISA were tested also by IFA with SNV (strain CC107), HTN virus (strain 76–118), and SEO virus (strain SCF1) virus antigens. Two-fold titration was performed to the endpoint.

**Focus-reduction neutralization test.** Endpoint titers of neutralizing antibodies were determined by the focus-reduction neutralization test (FRNT) as previously described. Briefly, serum was serially diluted and each sample was incubated for 1 hr at 37°C and subsequently inoculated into wells of 96-well tissue plates containing confluent Vero E6 cell monolayers. After absorption for 1 hr at 37°C, the wells were overlaid with a mixture of agarose and basal Eagle’s medium and incubated 9 (SEV virus) or 12 days (PUU virus). Virus-infected cells were detected with hantavirus-specific polyclonal rabbit antisera, followed by peroxidase-labeled goat antibodies and substrate. An 80% reduction in the number of foci was considered to show virus neutralization.

**Immunoblotting.** Immunoblotting was performed essentially as described previously. Briefly, whole extracts of hantavirus-infected Vero E6 cells were separated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–15% gel) and transferred to nitrocellulose filters. Filters were pre-absorbed with 5% non-fat dry milk in PBS and incubated overnight at 4°C with serum samples diluted 1/200. The mouse anti-mouse monoclonal antibody 33A/B10 (10 μg/ml) was incubated for 2 hr at 20°C, followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) for 2 hr at 20°C. Specific antibody binding was detected by 5-bromo-4-chloro-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) according to the manufacturer’s instructions (Sigma).

**Data processing and statistical analyses.** The software SAS® (SAS, Cary, NC) was used for data processing and statistical analyses. Comparison of groups of animals with regard to the presence of antibodies to PUU virus was made by the chi-square test.

**RESULTS**

Twenty-seven of 427 moose showed reactivity in the ELISA towards recombinant PUU virus nucleocapsid protein (Table 1). Serum from 5 of these 27 animals reacted also in IFA with PUU virus-infected cells and neutralized PUU virus as determined by the FRNT. Neutralization occurred at titers ranging from 80 to 2,560. The ELISA values of serum from 4 of the 5 animals were higher than those from any of the 22 ELISA-reactive, IFA-nonreactive animals. All 5 animals originated from the 2 most northern areas studied (Figure 2). There was cross-reactivity in the ELISA between PUU virus and SNV proteins (Table 1), a finding that is

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**Table 1**

<table>
<thead>
<tr>
<th>Geographic area</th>
<th>Individual</th>
<th>ELISA*</th>
<th>IFA†</th>
<th>FRNT‡</th>
<th>Immunoblot§</th>
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<tr>
<td>Gaddede</td>
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<td></td>
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<tr>
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<td>0.16</td>
<td>0.46</td>
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* Recombinant nucleocapsid protein of Seoul virus (SEO), Sin Nombre virus (SNV), or PUU virus was used as antigen. Serum dilution = 1/100.
† Immunofluorescent assay (IFA) using cells infected with PUU or Hantaa (HTN) virus. Reciprocal titer values are given and – denote a negative result using a screening dilution of 1/8.
‡ Focus-reduction neutralization test; serum titration from 1/20.
§ Immunoblotting towards native PUU and SEO virus antigen was performed in selected reactive serum samples. ND = not done; – = negative result.
¶ Net absorbance value at 450 nm.
consistent with observations in humans. Also consistent with data on humans, cross-reactivity of a lower degree was observed to the SEO virus nucleocapsid protein.

Selected moose serum samples were tested by immunoblotting. A complete correlation with the FRNT data was obtained. Five of five sera examined reacted strongly with native PUU virus nucleocapsid protein in immunoblotting whereas 4 FRNT-negative sera were also negative also by immunoblotting (Table 1).

These data provided strong serologic evidence of previous PUU virus infection in 5 (2%) of 260 wild-living moose in the PUU virus-endemic region. As a group, these 5 animals did not differ with regard to sex, age, or weight from the other 255 animals from endemic areas. No close connection was demonstrated between seropositive animals. The distance between locations of capture of seropositive animals within an area was 15–31 km. A calf of one of the seropositive females (B54) was negative for antibodies to PUU virus.

We also used an ELISA to study the occurrence of moose antibodies to recombinant nucleocapsid protein of SEO virus, an agent that occurs world-wide, as well as to SNV, the HPS-causing virus isolated in the United States. Seoul virus protein was chosen as a representative for the interrelated HTN/DOB/SEO group of viruses. When using a cut-off value defined for humans (≥0.2), 16 animals showed a selective reactivity to the recombinant nucleocapsid protein of SEO virus and 3 animals showed reactivity to that of SNV (Table 2). Serum from those 5 animals that showed the highest ELISA values to the SEO viral protein were subjected to the FRNT. None of these 5 sera neutralized SEO virus (Table 2). Four of these sera were negative by immunoblotting against SEO virus nucleocapsid protein. Only serum G39, which reacted with an optical density of 2.44 to recombinant SEO virus antigen in the ELISA, and which was also reactive to SEO virus and HTN virus by the IFA, although negative by the FRNT, reacted strongly with native SEO virus nucleocapsid protein by immunoblotting (Table 2). This serum was nonreactive to PUU virus by all applied methods. This animal originated from southwestern Sweden.

**DISCUSSION**

Convincing evidence of past PUU virus infection was obtained in 5 of 427 moose investigated. The conclusion was based on concordant results in the ELISA, IFA, and neutralization assays. The pattern of reactivity in the ELISA, including a strong cross-reaction with SNV and a weaker reaction with SEO virus, was the same as found in humans subjected to PUU virus infection. All 5 animals originated from the PUU virus-endemic northern part of Sweden.

Besides these 5 animals, serum from 46 other animals showed ELISA reactivity to one or more hantavirus recombinant proteins. A remarkably strong reactivity to SEO virus antigen, although without neutralizing activity, was found in serum from one of the animals (G39, Table 2) originating from southern Sweden. Due to its association with the rat, SEO virus shows a world-wide distribution. There is, however, no direct evidence to indicate an occurrence of this serotype in Sweden. Recent studies of farmers, as well as a randomized adult population, have identified individuals seroergic to SEO virus but not to PUU virus.

Moreover, the neutralization test or immunoblotting towards
the homologous hantavirus did not verify these aberrant ELISA reactivities. Finally, there is no evidence from clinical or epidemiologic data indicating that hantaviruses other than PUU virus might exist in the region. On the other hand, novel hantaviruses are currently identified in various countries and associated with rodent species. Thus, aberrant patterns might justify an awareness on the possible presence of new hantaviruses in the region that are cross-reactive with known serotypes.

There is little information on the occurrence of PUU virus infection in mammals other than rodents and humans. An examination in The Netherlands of sera from 829 feral and 2,025 domestic animals representing 34 different species showed antibodies to hantavirus only in rodents. Investigations of cats in European countries disclosed antibodies to Hantaan virus, antibodies that may have been cross-reactive and actually induced by PUU virus infection or possibly by infection with the rat-associated SEO virus. In surveys of studies from regions where the HTN and SEO viruses, but not PUU virus, occur endemically, antibodies to hantavirus has been reported in samples from small and medium-sized wild mammals such as moles, weasels, hares, foxes, roe deer, and also among domestic animals such as cats, dogs, and pigs. Furthermore, in an outbreak of HFRS among laboratory staff, IFA reactions of low titer towards HTN virus were found in rabbits, guinea pigs, dogs, and cats.

In line with those studies on the HTN/SEO virus group, the present data may reflect a more general susceptibility to hantavirus infection among mammals. Obviously, such susceptibility does not necessarily imply that the infection rate should be high. According to the present results, the risk of infection in moose seems to be low, and this is consistent with studies of rodents other than the primary reservoir species of various hantaviruses. Also consistent with studies of heterologous rodents are infections with persistent infection and enzootic spread among the group, 37–43 the present data may reflect a more general suscepti-

bility to hantavirus infection among mammals. Moreover, 263 (62%) of the animals were less than 3 years old (Figure 1), limiting the time of exposure.

In conclusion, convincing evidence of past PUU virus infection was found in wild living moose in the northern PUU virus-endemic region of Sweden. The results provide a piece of information relevant to the question of whether wild-living mammals other than rodents may be susceptible to the virus.

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