

SHORT COMMUNICATION

## First evidence of Seoul hantavirus in the wild rat population in the Netherlands

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We report the first detection of Seoul hantavirus (SEOV)-specific antibodies in the wild brown rat population in the Netherlands. SEOV-reactive antibodies were found in three rats out of 16 in a repeated series of tests including immunofluorescence assay, immunoblot, and enzyme-linked immunosorbent assay. Focus reduction neutralization test confirmed the presence of SEOV-specific antibodies, and reverse-transcription polymerase chain reaction (RT-PCR) confirmed the presence of hantaviral RNA. This discovery follows the recent findings of SEOV infections in wild and pet brown rats and humans in England, Wales, France, Belgium, and Sweden, indicating an even higher importance of this hantavirus for public health in large areas of Europe.

Keywords: *Seoul virus; wildlife diseases; hantavirus; rodent-borne diseases*

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U nie van Waterschappen (Dutch water management) catches muskrats at vulnerable locations all over the Netherlands on a regular basis. However, as a by-catch, brown rats (*Rattus norvegicus*) are also frequently caught in the traps. In this study, 16 rats trapped by the regional water authority Rijn and IJsseland in the Eastern province of Gelderland in the Netherlands in 2013 were screened for Seoul hantavirus (SEOV) antibodies by using three different screening methods. Three rats showed positive screening results, and samples from these three rats were confirmed for SEOV-specific antibodies by focus reduction neutralization test (FRNT), the gold standard for hantavirus serology, and by reverse-transcription polymerase chain reaction (RT-PCR) for the presence of hantaviral RNA. Several reports about SEOV emergence in Europe have appeared recently (1–5), which has raised concerns about the health and safety of people who are involved in rodent control programs or who work in rat-infested environments. The screening of SEOV in Dutch

brown rats was conducted as part of a rat pathogen collaboration project between the Zoonosis Science Center at the Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden, and the Symbiant Pathology Expert Centre, the Netherlands.

### Background

Hantaviruses are a globally distributed group of rodent- and insectivore (*Eulipotyphla*)-borne RNA viruses. Most commonly, hantaviruses are transmitted to humans via aerosols of infectious excreta (urine, feces, saliva) from chronically infected wild rodents (6). The Old World hantaviruses are most often associated with hemorrhagic fever with renal syndrome (HFRS), while the New World hantaviruses cause hantavirus pulmonary syndrome (HPS). This dichotomy, however, is not completely clear in all cases (7). The natural reservoirs of hantaviruses are small rodents or insectivores, and the virus is believed to cause asymptomatic life-long infection in these animals.

Hantaviruses are usually species-specific, although spill-overs have been described (8).

Puumala hantavirus (PUUV) causes the majority of hantavirus-related clinical cases in Europe. PUUV causes a mild form of HFRS and is carried and shed by the bank vole (*Myodes glareolus*) (9). Other HFRS-causing hantaviruses circulating in Europe include Dobrava and Saaremaa hantaviruses, which are carried by the yellow-necked mouse (*Apodemus flavicollis*) and the striped field mouse (*A. agrarius*), respectively (6). SEOV is the only known hantavirus with a potentially world-wide distribution due to the ubiquitous occurrence of its reservoir hosts, the brown and the black rat (*Rattus norvegicus* and *R. rattus*, respectively) (10). Nevertheless, prior to the last decade, human and wild rat infections of SEOV were, for the most part, detected only in Asia with human infections in Europe limited to laboratory rat handling (4). During recent years, however, SEOV has been detected in wild rats in France, Belgium, and the United Kingdom (2, 3), and in pet rats in Sweden, England, and Wales (1, 4). Human cases of severe SEOV-caused HFRS have been reported in France and the United Kingdom (3, 5). In neither of these severe SEOV cases were there any connections to pet or laboratory rats, strongly indicating that the infections were contracted from nature. This study proves the occurrence of SEOV in the wild rat population in the Netherlands.

## Virus investigation

Analyses were conducted at the Zoonosis Science Center at the Department of Medical Biochemistry and Microbiology, Uppsala University, The Public Health Agency of Sweden, Stockholm, Sweden, and at the Department of Microbiology, Graduate School of Medicine, Hokkaido University, Japan.

Rat hearts were vortexed together with 1 ml of phosphate-buffered saline (PBS) and centrifuged. These samples were assumed to have a dilution equivalent to serum dilution of 1:25. The samples were initially screened by 1) enzyme-linked immunosorbent assay (ELISA), 2) indirect immunofluorescence assay (IFA), and 3) immunoblotting (IB):

1. An in-house SEOV ELISA (will be described elsewhere, Verner-Carlsson, unpublished) was used for initial screening. Briefly, ELISA-plates were coated by the hantavirus-reactive bank vole Mab 1C12 (4 µg/ml), followed by post-coating (3% bovine serum albumin (BSA) in PBS), and incubation of SEOV (strain 80–39) native antigen. Rat samples were diluted 1:16 (equivalent to a final serum dilution of 1:400) and rat control sera were incubated at a dilution of 1:400, followed by alkaline phosphatase (ALP)-conjugated goat anti-rat antibodies (Jackson ImmunoResearch). P-Nitrophenyl phosphate substrate was added after washing. The reactions were measured after 30 min at 405 nm. All incubations lasted 1 h at 37°C, and the plates were

washed four times between each step. Cut-off was set to OD 0.100 at 405 nm.

2. IFA using SEOV (strain SR-11) infected Vero E6 cells was used as described earlier (11). Briefly, rat blood collected on filter paper was diluted in PBS and used for IFA analyses and IB analyses. Serum from a Wistar rat experimentally infected with SEOV (strain SR-11) was used as positive control. Rat samples were diluted 1:50, and control rat serum was diluted 1:100 and put onto glass slides with prefixed SEOV-infected Vero E6 cells for 1 h at room temperature. After washing, FITC-conjugated goat anti-rat antibodies were incubated for 1 h at room temperature. Fluorescence staining was visualized by an immunofluorescence microscope.
3. The samples were further subjected to IB using SEOV (strain SR-11) infected Vero E6 cells and uninfected Vero E6 cells as antigens, as described earlier (12). Briefly, after transfer onto PVDF membranes, the membranes were blocked in BLOCK ACE buffer (DS Pharma Biomedical) for 1 h at RT. The membranes were subsequently incubated with the rat samples diluted 1:50 and positive/negative control rat serum at 1:100 dilutions, for 1 h RT, using a microwell system, followed by the addition of horseradish peroxidase-conjugated goat anti-rat IgG for 1 h at RT. Antibody binding was visualized using a colorimetric assay (4-chloro-1-Naphthol method).

Two out of 16 samples, #22 and #33, were found clearly positive by all three screening methods; rat #84 was positive by ELISA and IFA, but negative in IB (Table 1). To finally confirm the hantavirus reactivity and a SEOV-specificity, we tested the three positive samples #22, #33, and #84 by FRNT, as previously described (13). Briefly, the rat samples were serially diluted and mixed with diluted viruses containing 30–70 focus forming units/100 µl. Confluent Vero E6 cell monolayers in six-well tissue culture plates were used and incubated at 37°C for 1 h after addition of the virus/antibody solution. To create a secluded environment, a mixture of tissue culture and agarose medium was subsequently added to the wells, which were then incubated for 8 days. To ensure visibility of the results, the agarose was removed from the wells, and the cells were fixed with methanol. To indicate virus-infected cells, the anti-hantavirus bank vole Mab 1C12 followed by peroxidase-labeled goat antibodies to mouse IgG (Jackson) were added. 3, 3', 5, 5'-Tetramethylbenzidine substrate (Sigma) was used as substrate, and foci were enumerated. The samples #22, #33 and #84 all showed high titers of neutralizing antibodies (Table 1).

A hantavirus genus-specific nested RT-PCR was applied as described earlier (14). Samples #22, #33 and #84 all showed positive results, while the serologically negative sample #31 was found negative (Table 1).

Table 1. Summary of ELISA, IFA, IB, FRNT, and PCR results

Sample	ELISA (OD)	IFA	IB	FRNT (end-point titer)	PCR
19	NT	–	–	NT	NT
20	NT	–	–	NT	NT
21	0.00	–	–	NT	NT
22	0.91	+	+	3,200	+
29	0.00	–	–	NT	NT
30	0.00	–	–	NT	NT
31	0.00	–	–	< 100	–
32	0.00	–	–	NT	NT
33	0.87	+	+	3,200	+
34	0.00	–	–	NT	NT
35	0.00	–	–	NT	NT
36	0.02	–	–	< 100	NT
37	0.00	–	–	NT	NT
38	0.00	–	–	NT	NT
84	1.19	+	–	3,200	+
85	0.02	–	–	NT	–
Pos. control	1.00	+	+	1,600	+
Neg. control	0.00	–	–	< 100	–

ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescence assay; IB: immunoblotting; FRNT: focus reduction neutralization test; PCR: polymerase chain reaction; NT: not tested; OD: optical density at 405 nm, adjusted to the positive control = 1.00.

## Discussion

This is the first discovery of SEOV-specific antibodies in wild rats in the Netherlands. In previous attempts to discover SEOV, during 2008–2011, 161 brown rats and 61 black rats were trapped at different types of environments (farmlands, urban locations, and nature reserves) in Rijn and IJssel regions in the Netherlands. Despite initial findings of SEOV-reactive antibodies in several animals based on an adapted commercial IFA, and two different ELISAs, SEOV-infection could never be confirmed by neutralization test or viral sequence data (data not shown). It is therefore possible that the dispersal of SEOV in the area has happened relatively recently.

Whereas the detection of antibodies can, in case of a number of pathogens, indicate a previous infection that is no longer an infectious threat, similar conclusions cannot be made concerning hantaviruses. As demonstrated in the case of PUUV infections, hantaviruses can be secreted by bank voles for several months after the initial infection (15, Lundkvist et al., unpublished observations). Furthermore, even at room temperatures, the virus is stable and infectious over a few weeks outside the rodent (16) making virus transmission control a huge challenge.

As seen from the example of West Nile virus emergence in the United States, the existence of wildlife reservoirs makes eradication of a disease that has had time to establish itself unrealistic. Thus, the recommendations for

handling the situation must be made with a long-term perspective. Since aerosols, contaminated by virus-infected rodent excreta, are the main source for human infection with hantaviruses, actual physical contact between rodent and man is not necessary for disease transmission (6). Consequently, in many cases it will be difficult for both patients and medical specialists to suspect transmission of SEOV. Therefore, disseminating information about the occurrence of SEOV in wild rat populations in the Netherlands has great importance for both medical workers and the general public. In addition, tailored messages are needed for people exposed to SEOV due to their working environments. Transmission-preventive measures in the form of gloves and protective facemasks may be necessary for people working in rat-infested environments, particularly for professional rat controllers. Steps have been taken by the Netherlands Centre for Infectious Disease Control (RIVM) to provide this type of information. Further studies are planned to collect evidence on the circulation of this hantavirus among the wild rat population in other geographical areas in the Netherlands, to generate and compare Dutch SEOV sequences to SEOV from other countries, while the occurrence of human infections will be assessed through increased surveillance of clinical cases with HFRS-like symptoms and serosurveillance of, for example, blood donors.

The recent discoveries of SEOV circulating in wild rat populations in France, Belgium, and the United Kingdom (2, 3), and now also in the Netherlands, together with the cases of severe human SEOV-infections so far confirmed in the United Kingdom and France (1, 4), strongly highlight the importance of further studies all over Europe.

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