

# A small-scale survey of hantavirus in mammals from eastern Poland

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## Abstract

Samples of 30 dead small mammals each were collected on area 'A' located in eastern Poland which is exposed to flooding by the Vistula river, and on the area 'B', also located in eastern Poland but not exposed to flooding. Kidneys and livers of the mammals were examined by the PCR and nested PCR methods for the presence of hantavirus RNA. Out of 7 species of small mammals examined, the presence of hantaviruses was detected in 4 of them. Hantavirus prevalence was low in *Apodemus agrarius* (2.6%), the most numerous mammal species, whereas in the remaining 3 positive species (*Microtus agrestis*, *Myodes glareolus*, *Sorex araneus*) this was 12.5–100%. The presence of hantaviruses was detected only in the animals found on area 'A' exposed to flooding, and their prevalence was statistically greater compared to area 'B' not exposed to flooding (16.7% vs. 0%,  $p=0.0345$ ). The overall positivity of the examined small mammals population from the areas 'A' and 'B' was 8.3%. The sequence analysis of the samples positive for hantavirus proved that the amplified products showed 77–86% homology with the L segment sequence of hantavirus Fusong-Mf-731 isolated from *Microtus fortis* in China. The presented study is the first to demonstrate the occurrence of hantavirus infection in small mammals from eastern Poland, and the first to demonstrate the significant relationship between flooding and the prevalence of hantaviruses in small mammals.

## Key words

hantaviruses, epidemiology, small mammals, PCR, floods, eastern Poland

## INTRODUCTION

Hantaviruses are enveloped, tri-segmented, single-stranded RNA viruses classified within the family Bunyaviridae. These viruses, distributed worldwide, are maintained by different species of rodents and insectivores, in which they produce chronic, non-apparent infections. Humans become infected through contact with urine, saliva or faeces from infected rodents, mainly via the aerosol route [1, 2]. In Eurasia, two forms of the disease are distinguished: 1) severe Haemorrhagic Fever with Renal Syndrome (HFRS), caused by Hantaan virus (HTNV), Dobrava virus (DOBV) and Seoul virus (SEOV), with the mortality rate amounting up to 20%, and 2) considerably milder Nephropathia Epidemica (NE), caused mainly by the Puumala virus (PUUV), Saaremaa virus (SAAV) and Tula virus (TULV) [3, 4, 5, 6]. Infections caused by hantaviruses (HDV) pose an increasing global health problem and are regarded as 'emerging infectious diseases' [1, 4, 5, 7].

Specific kinds of hantaviruses are associated with specific species of small mammals which are both reservoirs and vectors of the disease. PUUV is mainly transmitted by the bank vole (*Myodes glareolus*), DOBV the by yellow-necked mouse (*Apodemus flavicollis*), SAAV by the striped field mouse (*Apodemus agrarius*) and TULV by the European common vole (*Microtus arvalis*) [6, 7, 8, 9, 10]. Global warming may stimulate the growth of small mammals populations and risk of disease [11, 12, 13, 14].

Recently, the first focus of hantavirus disease (HDV) was established in the Carpathian mountains in southeast Poland, close to foci described earlier in Slovakia [3, 4, 15]. A total of 13 serologically-confirmed clinical cases of HDV were described, of which 10 were HFRS cases caused by DOBV and 3 were NE cases caused by PUUMV [3, 4]. In seroepidemiological studies of forestry workers in eastern Poland, Knap *et al.* [16] and Grygorczuk *et al.* [17] found a positive response to hantaviruses in respectively 2.5% and 8.7%, which indicates the possibility of occupational exposure.

To date, no small mammals in eastern Poland have been tested for the presence of hantaviruses. Therefore, the PCR method was carried out for the examination of dead small mammals found on the two territories within the Lublin province of eastern Poland: territory 'A' in western part of the province exposed to floods by the Vistula river, and territory 'B' in the central part of the province which is not exposed to floods.

## MATERIALS AND METHODS

**Collection of mammals.** Samples of 30 dead small mammals in each area – 'A' and 'B' – were collected during summer/autumn season. After collection, the mammals were placed in plastic containers and stored at -80°C for further investigation. The total collection of 60 mammals consisted of the following rodent and insectivore species: strip field mouse (*Apodemus agrarius*) – 39 specimens, common shrew (*Sorex araneus*) – 8 sp., bank vole (*Myodes glareolus*) – 5 sp., wood mouse (*Apodemus sylvaticus*) – 5 sp.,

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common vole (*Microtus arvalis*) – 1 sp., field vole (*Microtus agrestis*) – 1 sp., and yellow-necked mouse (*Apodemus flavicollis*) – 1 sp.

**RNA isolation.** After thawing, the small mammals were subjected to necropsy and the organs placed in the separate vials. Total RNA was extracted from homogenized organs using the RNeasy Mini Kit (Qiagen, USA) according to the producer's instructions. The amounts of extracted RNA measured with NanoDrop ND1000 spectrophotometer (USA) were in the range of 10–60 ng.

**Reverse Transcription.** Reverse transcription was carried out using the QuantiTect Reverse Transcription kit (Qiagen, USA), according to the producer's instructions.

**PCR and nested PCR reaction.** The reactions were performed according to Arai *et al.* [18].

To amplify a hantavirus S- and L-segment sequences, the polymerase chain reactions (PCR) were carried out with the following primers:

- for the region of the S-segment: outer OSM55: 5'-TAGTAGTAGACTCC-3' and HTN-S6: 5'-AGCTCIGGATCCATITCATC-3'; inner Cro2R: 5'-AIGAYTGRTARAAIGAIGAYTTYTT-3' and PHS-5F: 5'-TAGTAGTAGACTCCTTRAARAGC-3';
- for the region of the L-segment: outer HAN-L-F1: 5'-ATGTAYGTBAGTGCWGATGC-3' and HAN-L-R1: 5'-AACCADTCWGTYCCRTCATC-3'; inner HAN-L-F2: 5'-TGCWGATGCHACIAARTGGTC-3' and HAN-L-R2: 5'-GCRTCRTCWGARTGRTGDGCA-3'.

The first PCR reaction was carried out in a 50 µl reaction volume which contained the following mix of reagents: 2.5 U *Taq* DNA polymerase (Qiagen, USA), 1 × PCR buffer containing 15 mM MgCl<sub>2</sub>, additional 1.25 mM MgCl<sub>2</sub> (all from Qiagen, USA), 250 mM each of dNTP (Fermentas, Lithuania), 0.25 µM of each of primer (Eurogentec, Seraing, Belgium), nuclease-free water (Applied Biosystems, USA) and 2 µl of cDNA (10 × diluted in nuclease-free water). The reaction was performed in a C1000 Thermal Cycler (BioRad, USA) under the following conditions: initial denaturation at 94°C for 30 sec, two-degree step-down annealing from 46 °C to 38 °C for 40 sec, and elongation at 72 °C for 1 min; then 30 cycles (denaturation 94 °C for 30 sec, annealing at 42 °C for 40 sec, elongation at 94 °C for 30 sec); final extension step at 72 °C for 7 min.

Nested PCR reaction was carried out under the same conditions with 2 µl of the first amplification product. For detection of the specific reaction products (1,083 bp for the S-segment and 347 bp for the L-segment), electrophoresis was performed in 2% agarose gel under standard conditions. After ethidium bromide staining, the strips were read under UV light.

As a positive control, the antigens of 6 hantaviruses (Hantaan, Sin Nombre, Puumala, Dobrava, Seoul, Saremaa) were retrieved from slides of the commercial kit for detection of anti-hantavirus IgG antibodies by the immunofluorescence method (Euroimmune, Germany). RNA was isolated using Qiampl Viral RNA Mini Kit (Qiagen, USA) according to manufacturer's procedure. RNase-free water was used as a negative control.

Precautions to exclude contamination were as follows: One-use tips with filters (PCR-clean/dualfilter/sterile) and one-use Eppendorf test tubes (PCR-clean, free of detectable human DNA, DNase, RNase, PCR inhibitor) were used. The glass and plastic surfaces were treated with RNase Zap (Ambion, the RNA company, USA) which completely removes contamination with RNase.

## RESULTS

The results are presented in Table 1. Out of 7 species of small mammals examined, the presence of hantaviruses was detected in 4 of them. Hantavirus prevalence was low in *Apodemus agrarius* (2.6%), the most numerous mammal species, whereas in the remaining 3 positive species (*Microtus agrestis*, *Myodes glareolus*, *Sorex araneus*) it was 12.5–100%. The presence of hantaviruses was detected only in the animals found on area 'A' exposed to flooding, and their prevalence was statistically greater compared to area 'B' not exposed to flooding (16.7% vs. 0%,  $p=0.0345$ ). The overall positivity of the examined small mammals population from the areas 'A' and 'B' was 8.3%.

The sequence analysis of the samples positive for hantavirus proved that the amplified products showed 77–86% homology with the L segment sequence of hantavirus Fusong-Mf-731 isolated from *Microtus fortis* in China (Accession No. FJ170808).

**Table 1.** Prevalence of hantaviruses in the organs of small mammals found on the territory of Lublin province, eastern Poland.

Area Species	'A'	'B'	Total
	Infected/Examined (Percent)	Infected/Examined (Percent)	Infected/Examined (Percent)
<i>Apodemus agrarius</i>	0 K + 1 L + 0 (K+L)/21 = 1/21 (4.8%)	0 K + 0 L + 0 (K+L)/18 = 0/18 (0)	0 K + 1 L + 0 (K+L)/39 = 1/39 (2.6%)
<i>Apodemus flavicollis</i>	Not found	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)
<i>Apodemus sylvaticus</i>	0 K + 0 L + 0 (K+L)/4 = 0/4 (0)	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)	0 K + 0 L + 0 (K+L)/5 = 0/5 (0)
<i>Microtus agrestis</i>	1 K + 0 L + 0 (K+L)/4 = 1/1 (100%)	Not found	1 K + 0 L + 0 (K+L)/1 = 1/1 (100%)
<i>Microtus arvalis</i>	Not found	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)	0 K + 0 L + 0 (K+L)/1 = 0/1 (0)
<i>Myodes glareolus</i>	1 K + 0 L + 1 (K+L)/2 = 2/2 (100%)	0 K + 0 L + 0 (K+L)/3 = 0/3 (0)	1 K + 0 L + 0 (K+L)/5 = 2/5 (40.0%)
<i>Sorex araneus</i>	0 K + 1 L + 0 (K+L)/2 = 1/2 (50.0%)	0 K + 0 L + 0 (K+L)/6 = 0/6 (0)	0 K + 1 L + 0 (K+L)/8 = 1/8 (12.5%)
Total	2 K + 2 L + 1 (K+L)/30 = 5/30 (16.7%)*	0 K + 0 L + 0 (K+L)/30 = 0/30 (0)	2 K + 2 L + 0 (K+L)/60 = 5/60 (8.3%)

1 K = infected kidney; 1 L = infected liver; 1 (K+L) = infected kidney and liver. \*Prevalence significantly greater compared to area "B" ( $p<0.05$ ).

## DISCUSSION

The detection for the first time of the presence of hantaviruses in small mammals from the territory of eastern Poland is in concordance with the results of the studies by Knap et al. [3, 15, 16], Nowakowska et al. [4], and Grygorczuk et al. [17], who have described the clinical cases of HFRS and NE from eastern Poland, and recorded positive findings in the serological screening of forestry workers from this part of the country.

So far, small mammals in Poland were examined for the presence of hantaviruses only by Song et al. [8] in the central part of the country. The authors found in *Microtus arvalis* and *Myodes glareolus* the presence of anti-Puumala antibodies and in *M. arvalis* also the presence of RNA similar to that of Tula virus. Thus, the finding in the presented study of the presence of hantaviruses in *Apodemus agrarius*, *Microtus agrestis* and *Sorex araneus* is the first detection of these small mammal species as hantavirus carriers on the territory of Poland.

To the best of our knowledge, to date, no Fusong-type hantaviruses have been reported from Europe. The isolates of RNA in the presented study are partially in accord with that of Fusong, which suggests that in Europe an undetermined hantavirus may occur which is closely related to the Far Eastern specimen.

The total score of positive results recorded for the small mammals examined in the presented work (8.3%) was similar to other studies on the small mammal reservoir of hantaviruses conducted in Slovakia [19, 20], Greece [21], Hungary [22], the United States [23] and China [24] (respectively 6%, 10.3%, 8.2%, 7.25%, 8.7%, 6.3%). By contrast, the low prevalence of hantaviruses among *Apodemus agrarius* does not correspond with the high seropositive reactions to Hantaan virus (22.3–26.9%) noted in Korea for this rodent species [25, 26]. The common occurrence of hantaviruses in bank voles (*Myodes glareolus*) is in agreement with reports indicating bank voles as abundant reservoirs and vectors of Puumala virus, causing high morbidity of Nephropathia Epidemica (NE) in the northern and central European countries, such as Sweden [27, 28, 29] and Germany [5, 11, 13]. The growth of NE cases could be predicted on the basis of bank vole propagation, increasing with global warming and other climate changes [11, 12, 13, 14, 28, 29].

Until recently, no relationships between humidity and hantaviral infections have been discovered, with the exception of the study by Verhagen et al. [30] who observed that the infections were more common in wet habitats. The results of the presented work suggest that periodic flooding could be a significant factor increasing the probability of hantaviral infection, similar to that proved for leptospiral infection [31].

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