Surveillance of Hantaviruses in Poland: A Study of Animal Reservoirs and Human Hantavirus Disease in Subcarpathia

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Abstract

The first cluster of hemorrhagic fever with renal syndrome (HFRS) in Poland was identified in 2007 in the Subcarpathian region. The natural environment of this area is a key habitat for hantavirus vectors. The animal reservoir of existing human HFRS clusters was studied to assess the occurrence of viruses (including Tula virus, Puumala virus, and Dobrava–Belgrade virus) among rodents. We examined 70 suspected human cases with symptoms corresponding to the clinical picture of HFRS. Serological analysis (indirect immunofluorescence assay and immunoblot) confirmed the presence of anti-hantavirus antibodies in 18 patients, which were surveyed with regard to developed symptoms and presumed rodent contact. Seroepidemiological analysis of newly confirmed human cases was performed, putative areas of human exposure were studied, and 194 rodents were subsequently captured from identified areas. Internal organs (lungs, heart, spleen, bladder, and kidneys) were collected from 64 *Apodemus flavicollis*, 55 *Apodemus agrarius*, 40 *Myodes glareolus*, 21 *Mus musculus*, and 14 *Microtus arvalis* and tested for the presence of hantavirus RNA by reverse transcription and subsequent real-time PCR. Positive samples were also tested by indirect immunofluorescence. Animal reservoir surveillance enabled the first detection of Puumala virus and Dobrava–Belgrade virus among animals in Poland. Furthermore, some places where rodents were captured correlated with areas of residence of laboratory-confirmed human cases and likely detected virus species. Moreover, three species of hantaviruses coexisting in a relatively small area were identified.

Key Words: Hantavirus—Rodent-borne—Zoonosis.

Introduction

HANTAVIRUSES BELONG TO THE FAMILY Bunyaviridae, genus *Hantavirus*. To date 24 hantavirus species have been identified, and new viruses continue to be found (ICTV 2012). The significance of this virus genus for public health interrelates to the human pathogenicity of some hantaviruses. These viruses may cause hemorrhagic fever with renal syndrome (HFRS), which occurs in Eurasia, where the case fatality rate reaches 12%, as well as hantavirus cardiopulmonary syndrome (HCPS), which occurs in both Americas with a fatality rate of approximately 30–35% (Jonsson et al. 2010). However, more and more reports in the literature indicate that

the symptoms of these two diseases, HCPS and HFRS, overlap, and thus it may be time to revise this division (Rasmuson et al. 2011, Clement et al. 2012).

The natural reservoirs of viruses are asymptotically infected rodents that belong to the families Muridae (subfamily Murinae) and Cricetidae (subfamilies Arvicolinae, Sigmodontinae, and Neotominae) (Bartoszcze et al. 1990, Jonsson et al. 2010). Recently, new hantaviruses have also been found in Africa and Asia among insectivores (shrews, moles) and bats (Guo et al. 2011, Kang et al. 2011, Weiss et al. 2012). Infection in humans occurs via the inhalation of aerosolized virus particles from the excreta of infected animals and occasionally through rodent bites. Most hantavirus

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FIG. 1. Location of study areas in Poland. Grey areas: Districts where HFRS cases were registered 2006–2012. Area surrounded by dark black line: Districts where HFRS cases were registered during the current study. Areas with dark grey slanted stripes: Districts where positive rodents were captured. Numbers indicate districts: 1, Stalowowolski; 2, Dębicki; 3, Rzeszowski; 4, Łańcucki; 5, Krośniński; 6, Brzozowski; 7, Przemyski; 8, Sanocki; 9, Leski.

species are closely associated with a specific rodent species (Vaehri et al. 2013). In Europe, most HFRS cases are caused by Puumala virus (PUUV), which is carried by the bank vole (*Myodes glareolus*), and Dobrava–Belgrade virus (DOBV), which is carried by the yellow-necked mouse (*Apodemus flavicollis*) and striped field mouse (*A. agrarius*). Tula virus (TULV), which is slightly or not at all pathogenic for humans, is mainly transmitted by the common vole (*Microtus arvalis*) and other species from the *Microtus* genus (Vapalahti et al. 2003, Vaehri et al. 2013). The course of human disease depends on the hantavirus species. For example, PUUV causes the mildest form of HFRS, called nephropathia epidemica, with a mortality rate of approximately 0.1%, whereas DOBV causes the most severe form of HFRS in Europe with a mortality rate up to 12% (Jonsson et al. 2010). Periodical mast years (seed years) of oak or beech in some countries are important factors of cyclic increase of rodent populations and risk related to hantavirus exposure in following year (Clement et al. 2009).

Laboratory diagnosis of hantavirus infections among humans in Poland is quite rare, and therefore the precise morbidity rate is unknown. Only particular epidemiological sanitary stations in Poland have undertaken laboratory HFRS diagnostics. On the other hand, individual studies of seroprevalence or analysis of acute cases of renal disorders, which are limited to certain areas or populations, have been the subject of scientific and epidemiological projects (Knap et al. 2006).

Since the first serologically confirmed case of hantavirus infection in humans in 2004 in the Subcarpathian region of

Poland (Fig. 1), 47 cases have been reported to date (Knap et al. 2006, Czarkowski et al. 2012). However, hantavirus infections were described much earlier in neighboring countries, and the real scale of infections in Poland may be underdiagnosed (Vaehri et al. 2013). Moreover, detection of hantavirus infections in humans has led to increased interest in the animal reservoir of the viruses in Poland. The natural environment in Polish Subcarpathia is a suitable habitat for wild rodents, which are reservoirs for hantaviruses (Sadkowska-Todys et al. 2007, Knap et al. 2009). Thus, the aim of this study was to assess the relationship between hantavirus infections in humans and the occurrence of the virus among wild rodents. The study was divided into two stages. The first step was to verify and trace the hantavirus disease epidemic with a focus in the human population of the Subcarpathian region. The purpose of the second stage was to investigate the presence of hantaviruses among wild rodents from areas where infections in humans were discovered.

Materials and Methods

Seroepidemiological study

Cases of the renal disorder diseases associated with fever symptoms were identified in hospitals and health care institutions from the Subcarpathian region from September, 2009, to September, 2011. Serodiagnosis of suspected HFRS cases by both immunoblotting and immunofluorescence methods was performed at the laboratory of Voivodeship Sanitary-Epidemiological Station in Rzeszów. Immunoblotting

analysis was used for the detection of anti-hantavirus antibodies in suspected sera (recomLine Bunyavirus IgG/IgM, Mikrogen GmbH, Neuried, Germany) according to the manufacturer's instructions. Strips used in the experiment contained a recombinant nucleocapsid protein of the following viruses: DOBV, Hantaan (HTNV), PUUV, and Seoul (SEOV) as well as the sandfly fever virus. The indirect immunofluorescence test (IIFT) was used to detect anti-hantavirus antibodies in serum (Hantavirus Mosaic 1, IIFT, immunoglobulin G [IgG] and IgM, Euroimmun AG, Lübeck, Germany). The detection of IgG and IgM against HTNV, Sin Nombre, PUUV, DOBV, SEOV, and Saaremaa viruses was performed according to the manufacturer's instructions. All positive cases were analyzed in detail through a survey.

The survey included information regarding patient data, clinical symptoms, and possible exposure circumstances (*e.g.*, living nearby; seasonal working in the garden, fields or forest; cleaning of basements or granaries; camping; staying in forest complexes; mowing; and so on). On the basis of results from the questionnaire, several areas of higher prevalence of suspected and confirmed HFRS cases were identified.

Animal reservoir study

The following species were selected for analysis: yellow-necked mice, striped field mice, bank voles, common voles, and house mice (*Mus musculus*). Permission no. 11/2009 of the National Ethics Committee on Animal Experimentation was issued for the study. Rodents were captured between November, 2009, and October, 2010, using live traps (PPHU Marcinkiewicz, Poland) in districts Sanocki, Przemyski, Krośniceński, and Brzozowski (Fig. 1). Live traps were employed in 12 locations on the basis of the rodents' behavior and the places of residence of currently and retrospectively confirmed human cases. All captured animals were subjected to taxonomy, sex identification, and weighing. After capturing, the animals were transported to the animal laboratory, euthanized, and necropsied. Internal organs were subsequently collected, including lungs, heart, spleen, bladder, and kidneys, and placed in RNAlater Stabilization Reagent (Qiagen NV, Venlo, The Netherlands) and stored at -20°C for further analysis. Serum was prepared from blood samples obtained by retro-orbital sinus access before euthanasia.

Tissues were cut and homogenized using a rotor-stator homogenizer (Stuart[®] SHM1). Total RNA was extracted using the High Pure RNA Tissue Kit (Roche AG, Basel, Switzerland), and cDNA was prepared using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche AG) as described by the manufacturer.

Molecular analysis

Genetic material (cDNA) from five organs of each individual rodent was pooled and 5 μL of cDNA was used as a template for real-time PCR. The presence of DOBV, PUUV, TULV, and HTNV/SEOV genetic material was analyzed by the amplification of a highly conserved region within the S-segment in three capillaries (duplex reaction in the case of DOBV and PUUV, and single for HTNV/SEOV and TULV). Sequences of primers and probes (synthesized by Genomed, Warsaw, Poland) were used as described by Kramski et al. (2007). Thermal profiles were slightly modified (increased annealing time) for the Light Cycler 2.0 chemistry (Light-

Cycler[®] TaqMan[®] Master, Roche AG). In the case of positive or unclear results, the samples were reanalyzed in detail, and then cDNA from each organ was individually tested with another set of probes and primers for DOBV, TULV, and PUUV. The second set was as follows for: DOBV (DOBF, TCCCGTGCAAGCTACTATCTGA; DOBVR, GCGCTCC TTGTCTTTGATTCA; DOBVP, FAM-ACCAAAGGCCCA TCCACCAATCGT-TAMRA), PUUV (PUUVF, TACAA GAGAAGAATGGCAGATGCT; PUUVR, CATTACATC AAGGACATTTCCA; PUUVP, FAM-CTGACCCGACTG GGATTGAACCTGA-TAMRA), and TULV (TULVF, CCA GGTGTTGCACATTCTCTTG; TULVR, GAGGAATAG CTAGCCAGCCAAA; TULVP, FAM-TGAATTATGTGTT CCTGGGCTGCATGG-TAMRA) (Maes et al. 2007).

Indirect immunofluorescence

Hantavirus Mosaic 1, IIFT, IgG, and IgM (Euroimmun) were used for the detection of anti-hantavirus antibodies in rodents' sera (1:10 diluted) obtained from molecularly positive individuals. Rabbit anti-mouse IgG + IgM + IgA conjugated to fluorescein isothiocyanate (FITC) (ab8517 Abcam PLC, Cambridge, UK) was used as a secondary antibody (1:50 dilution) instead of the in-package anti-human IgG and IgM antibodies. The remaining procedure was carried out according to the manufacturer's instructions. Depending on the intensity of the signal, fluorescence was graded as: + + +, + +, +, or +/-. The scores +, + +, and + + + were considered as positive.

Results

Seroepidemiological study of suspected human cases

The seroepidemiological study revealed the presence of anti-hantavirus antibodies in 18 patients of 70 suspected cases with symptoms corresponding to the clinical picture of HFRS. The etiologic agent was proposed as PUUV in 11 patients and DOBV in seven patients. The cases were registered in the following districts: Sanocki (11), Brzozowski (3), Leski (3), and Krośniceński (1) (Fig. 1). All suspected cases exhibited acute onset as flu-like symptoms, pain syndrome (*e.g.*, headache, myalgia, back pain, arthralgia), as well as fever (38.5°C and above) and further distinctive symptoms. There were three cases in 2009 (from September), seven cases in 2010, and eight cases to the end of August, 2011. Five out of 18 identified cases were described as convalescent-phase patients (only IgG were detected) in Leski (3) and Sanocki (2) districts. Another 13 were described as acute-phase patients (both IgG and IgM were detected). Results were consistent in both methods used. There were 14 reported cases among men and four cases among women. The range of age in the confirmed cases varied from 15 to 74 years old (average 50 years old). Furthermore, development of acute kidney injury (AKI) International Classification of Diseases, 10th revision (ICD-10), code N17 was observed in 13 cases. The most frequent symptoms noticed among 94% patients were acute influenza-like symptoms, fever (38.5°C and above), pain syndrome (*e.g.*, headache, myalgia, back pain, arthralgia), infiltrates, effusions, and enlarged organs. Other symptoms were proteinuria (83%), thrombocytopenia (72%), and liver damage (55%). Moreover, symptoms that were observed less often included nausea, vomiting (44%), water-electrolyte imbalance (33%), oliguria (28%), hematuria

(22%), cough or pneumonia-like symptoms (16%), and acute myopia (5%). The subsequent survey revealed exposure circumstances among almost all analyzed cases (15 of 18) that were related most often with living and work conditions as well as frequent contact with rodents and their feces. These included habitation near forests, rodent presence in the farmyard (five cases), habitation near fields/meadows, rodent presence in the farmyard (six cases), professional exposure with forest workers, camping cleaners (three cases), frequent camping (one case), and other contact with rodents (four cases). Putative exposure was not determined in three cases.

Animal reservoir investigation

A total of 194 rodents were captured: 64 *A. flavicollis* (31 females, 33 males), 55 *A. agrarius* (26 females, 29 males), 40 *M. glareolus* (19 females, 21 males), 21 *M. musculus* (nine females, 12 males), and 14 *M. arvalis* (nine females, five males). The successful trap location was related to the specific habitats and natural behavior of captured animals (Table 1), which included a food search and acquisition (yellow-necked mice captured in fresh upland forests and intrafield lanes of shrubbery, striped field mice captured in residential areas, common voles captured in the meadow, and bank voles captured in alder forest along stream banks). In some cases, rodents were trapped at the border of inhabited areas, such as a house mouse in a forest or mid-field forestation. However, *Apodemus sylvaticus* and *Microtus agrestis*, which might be involved, were not captured. Habitats selected for trap location were characteristic for *A. flavicollis* and *M. arvalis*, but not for *A. sylvaticus* and *M. agrestis*, the rarely observed in the area of study. Identification was confirmed with craniometric measures (Pucek 1981).

Molecular screening and immunological analysis results

The presence of hantavirus genetic material was confirmed in 17 (8.8%) individuals using molecular analysis (Table 2). The animals with positive material were captured in eight of 12 locations of the four districts: Sanocki, Przemyński, Krośniński, and Brzozowski (Table 1, Fig. 1). However, we failed to identify positive rodents in four remaining locations in the following districts: Krośniński (village of Wróblak Szlachecki) and Brzozowski (villages of Przysietnica, Buków, and Haczów). DOBV genetic material was detected in *A. flavicollis* (nine individuals) and *M. glareolus* (one individual). TULV genetic material was isolated from *M. arvalis* (five individuals) and PUUV from *M. glareolus* (one individual) and from *A. agrarius* (one individual). Genetic material was found mostly in the spleen in 88% of positive rodents as well as the lungs or heart in 70% and the kidneys/bladder in 65% of rodents (Table 2). Immunofluorescence assay (IFA) revealed 11 positive samples and one negative sample from a total of 12 specimens that tested positive for DOBV and PUUV by real-time PCR (Table 2). The IFA assay did not include a TULV-specific subfield, and therefore cross-reactivity of TULV PCR-positive samples was only screened and turned out to be negative.

Discussion

A total of 47 cases of hantavirus disease in humans have been confirmed to date in Poland that were more likely as-

sociated with PUUV and DOBV (Nowakowska et al. 2009, Czarkowski et al. 2012). The study, which resulted in 18 new seropositive cases of HFRS, allowed for the continuous analysis of the hantavirus disease epidemic focused in the Subcarpathian region (Knap et al. 2009). No cases from any other part of Poland were registered during this study.

All cases were registered based on rules issued by the state disease surveillance system (National Institute of Public Health, Warsaw, 2011). No fatal hantavirus disease cases occurred during the current study. The analyzed cases were detected in neighboring districts (Sanocki, Leski, Brzozowski, and Krośniński). During the analyzed period, those districts (Fig. 1) were characterized by the higher morbidity rate for HFRS (5.99/100,000) compared to the entire province (0.84/100,000). This observation confirms general trends. The districts Sanocki, Leski, and Brzozowski account for the majority of HFRS cases registered in Poland (Table 1). The increase in the number of cases was noticed in January of 2010 and July of 2011, with three cases monthly. The year 2011 had 11 cases and was the next prominent period characterized by an increased number of infections in Poland (Table 3), followed by the year 2007 with 17 cases (Nowakowska et al. 2009, Heyman et al. 2011, Czarkowski et al. 2012). A similar situation was described in Germany from October, 2011, and Slovenia from January, 2012 (Boone et al. 2012, Kraigher et al. 2012). The observed higher number of infected males was parallel to cases identified in other European countries, where men were more commonly affected due to sex-related differences in the immunological response in early stages of the disease (Klingström et al. 2008), as well as men's higher exposure risk related to more frequent outdoor activities.

Definitive determination of the HFRS etiological agent was not possible with serological methods used in this study due to method limitations (significant sera cross-reactivity). Considering the endemic aspects and careful interpretation of results, only a likely determination was concluded. All analyzed patients were hospitalized due to the severity of the developed symptoms. Therefore, it was difficult to observe differences between the course of the disease caused by PUUV or DOBV. The course of the disease was mild in one case and moderate in 17 cases (see Supplementary Data text; Supplementary Data are available at www.liebertonline.com/vbz/). Hemodialysis was not required, and renal insufficiency was moderate and transient. Respiratory symptoms were observed in 16% of cases. The set of symptoms developed in the early stages of infection led clinicians to perform laboratory HFRS diagnosis. These included influenza-like sudden acute infections with high fever, thrombocytopenia, and confirmed contact with rodents (or residence in endemic areas). However, accurate diagnosis is still difficult at the time of first observation (primary physician care) in Poland. For example, during the flu season, the etiology of HFRS cases with a mild course may be misdiagnosed as influenza-like illnesses. Indeed, underestimation of nephropathia epidemica cases was described in hyperendemic areas (Brummer-Korvenkontio et al. 1999). Consequently, the actual number of infections in Poland might be higher, taking into account the populations and environmental/geographical background (Knap et al. 2006, Heyman et al. 2011, Vaheri et al. 2013). Indeed, some seroprevalence studies concerned with the risk groups revealed the presence of anti-hantavirus antibodies among

TABLE 1. ENVIRONMENTAL CHARACTERISTICS OF RODENTS' HABITATS

District	Village m.a.s.l. ^a	Habitat	Local vegetation	Captured rodents ^b	Hantavirus detected in animal reservoir	Hantavirus detected among humans (IFA/immunoblot)	Hantaviruses registered during the study against all registered in districts ^c
Sanocki	Lalin 320	Alder forest along stream banks	<i>Alnus glutinosa</i> , <i>Alnus incana</i> , <i>Corylus avellana</i> , <i>Chaerophyllum hirsutum</i>	<i>M. glareolus</i> (2/7) <i>A. flavicollis</i> (0/5) <i>A. agrarius</i> (0/1)	DOBV (<i>M. glareolus</i> ♀, 19 g) PUUV	DOBV, PUUV (nearby)	DOBV (4/12) PUUV (7/14)
	Pisarowce 340	Intra-field lanes of shrubbery	<i>Prunus spinosa</i> , <i>Cerasus avium</i> , <i>Rosa canina</i> , <i>Viburnum opulus</i>	<i>A. flavicollis</i> (1/12) <i>A. agrarius</i> (0/2) <i>M. glareolus</i> (0/3)	DOBV (<i>M. glareolus</i> ♂, 19 g) (<i>A. flavicollis</i> ♀, 33 g)	DOBV, PUUV (nearby)	
	Kostarowce 315	Meadow	<i>Trifolium pratense</i> , <i>Elymus repens</i>	<i>M. arvalis</i> (3/3) <i>M. musculus</i> (0/8)	TULV (<i>M. arvalis</i> ♀, 20 g) (<i>M. arvalis</i> ♂, 20 g) (<i>M. arvalis</i> ♀, 15 g)	DOBV, PUUV (nearby)	
	Zarszyn 295	Meadow	<i>Trifolium pratense</i>	<i>M. arvalis</i> (2/5) <i>A. agrarius</i> (0/6) <i>A. flavicollis</i> (0/1)	TULV (<i>M. arvalis</i> ♂, 13,5 g) (<i>M. arvalis</i> ♀, 16 g)	DOBV, PUUV (nearby)	
Przemyski	Moszczaniec 500	Mixed fir forest	<i>Abies alba</i> , <i>Corylus avellana</i> , <i>Sambucus nigra</i> , <i>Pulmonaria obscura</i>	<i>A. flavicollis</i> (2/7) <i>M. glareolus</i> (0/11)	DOBV (<i>A. flavicollis</i> ♂, 36,5 g) (<i>A. flavicollis</i> ♂, 33,5 g)	PUUV, DOBV (nearby)	
	Bachórzec 240	Oak-hornbeam forest	<i>Fagus sylvatica</i> , <i>Quercus robur</i> , <i>Carpinus betulus</i> , <i>Anemone nemorosa</i>	<i>A. flavicollis</i> (3/10) <i>M. glareolus</i> (0/4)	DOBV (<i>A. flavicollis</i> ♀, 35 g) (<i>A. flavicollis</i> ♀, 37,5 g) (<i>A. flavicollis</i> ♂, 28 g)	DOBV	DOBV (0/2) PUUV (0/0)
Krośniński	Poraj 350	Oak-hornbeam forest	<i>Abies alba</i> , <i>Quercus robur</i> , <i>Carpinus betulus</i> , <i>Galeobdolon luteum</i>	<i>A. flavicollis</i> (3/18) <i>M. glareolus</i> (0/2)	DOBV (<i>A. flavicollis</i> ♂, 44,5 g) (<i>A. flavicollis</i> ♂, 36,5 g) (<i>A. flavicollis</i> ♂, 38,5 g)	DOBV	DOBV (1/2) PUUV (0/0)
	Trzeńców 302	Residential area-buildings, forest	—	<i>A. agrarius</i> (1/38) <i>A. flavicollis</i> (0/4) <i>M. glareolus</i> (0/5) <i>M. musculus</i> (0/1)	PUUV (<i>A. agrarius</i> ♂, 24 g)	DOBV, PUUV (nearby)	DOBV (1/2) PUUV (2/4)

Table includes description of rodent habitats where hantaviruses were found in reservoir.

^aMeters above sea level.^bNumber in parentheses is amount of positive versus all captured in a particular location (village).^cCases registered during the study September, 2009, to August, 2011, in bold against all registered in 2006–2012.

Source: Current study and personal communication.

m.a.s.l., meters above sea level; IFA, immunofluorescence assay; DOBV, Dobrova-Belgrade virus; PUUV, Puumala virus; TULV, Tula virus.

TABLE 2. RESULTS OF IMMUNOLOGICAL AND MOLECULAR SCREENING OF RODENT RESERVOIR

No. of rodent	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Species	bv	ynm	ynm	ynm	ynm	ynm	ynm	ynm	ynm	ynm	cv	cv	cv	cv	cv	bv	sfm
PCR screening result	DOBV	DOBV	DOBV	DOBV	DOBV	DOBV	DOBV	DOBV	DOBV	DOBV	TULV	TULV	TULV	TULV	TULV	PUUV	PUUV
Result of PCR analysis																	
Lungs	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+
Heart	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-
Spleen	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
Kidneys and bladder	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+	-	-
Result of IFA	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+

bv, bank vole; ynm, yellow necked mouse; cv, common vole; sfm, striped field mouse; DOBV, Dobrova-Belgrade virus; TULV, Tula virus; PUUV, Puumala virus; IFA, indirect immunofluorescence assay.

forest workers in Lubelskie Voivodeship, northeastern Poland, as well as among zoologists, which suggested an unrecognized and/or asymptomatic infection (Panasiak et al. 1989, Sadkowska-Todys et al. 2007, Grygorczuk et al. 2008, Knap et al. 2010). Moreover, the underestimation of hantavirus disease morbidity in Poland was not only the result of mild or asymptomatic cases, but was also affected by a limited recognition of the disease among physicians and diagnosticians, a poor description of endemic areas (Gajdusek et al. 1983), and an assumption that it is not a primary problem of public health in Poland (Sadkowska-Todys et al. 2007).

The apparent exposure to rodents was described in subsequent surveys among almost all human cases. These exposure incidents were related to living conditions as well as professional exposure. Therefore, detailed studies of rodent populations in areas of putative exposure confirmed the environmental risk and presence of hantaviruses among rodents. However, the animal reservoir in the Subcarpathian region of Poland has not been studied to date for the occurrence of hantaviruses. The natural reservoir study gave positive results in central Poland in 1998 for TULV found in bank voles and common voles (Song et al. 2004). Recently, Boginia virus (BOGV) was found in the Eurasian water shrew (*Neomys fodiens*) (Gu et al. 2013), and unclassified hantaviruses were found among small mammals (Wójcik-Fatla 2013).

Hantavirus species, which were never detected in Poland in rodents, were identified in our study (DOBV and PUUV). In general, the molecular analysis results correlated with the IFA assay, with the exception of sample 17, in which an inconsistency between the molecular screening and the IFA result was observed, as well as sample 5, which showed negative IFA results. Moreover, the TULV molecularly positive samples did not cross-react in IFA assays. Reduced sera cross-reactivity of TULV-positive rodents was noticed elsewhere (Tegshduuren et al. 2010). DOBV genetic material was detected more frequently in the spleen and less frequently in the kidney, which is in agreement with findings of Korva et al. (2009b). PUUV was detected only in the spleen and lungs. Due to the limited number of samples, it was problematic to make definitive conclusions, but the increased viral load for PUUV in lungs has been noted elsewhere (Korva et al. 2009b).

There was also a geographic correlation between confirmed DOBV in rodents and human cases of DOBV in four villages (Poraj, Lalin, Pisarowce, and Bachórzec). Our findings suggested that human cases of PUUV (Przysietnica) could be connected with animal reservoirs in neighboring villages (Trześniów and Lalin), and human cases of DOBV in Bukowsko and Odrzechowa (district Sanocki) could be related to neighboring animal reservoirs of DOBV in Pisarowce and Moszczanec. Surprisingly, we did not identify any DOBV-positive rodents in six trapping sites (including Zarszyn, Kostarowce, and Trześniów) in the area where prominent aggregation of DOBV human cases (retrospective and current) were noticed. This may be influenced by the unexpected small amount of its carrier *A. flavicollis* in the relevant rodent yield (Table 1), as well as environmental circumstances, because the area of interest is more open-field and agricultural. Instead, TULV-positive *M. arvalis* was captured in Zarszyn and Kostarowce in the appropriate and expected environment. Interestingly, PUUV in Trześniów was detected by PCR in a striped field mouse, which was

TABLE 3. RELATIONSHIP OF MAST YEARS^a OF BEECH TREES IN THE AREA OF STUDY WITH NUMBER OF HFRS CASES

Forest inspectorate	Villages (district)	2006	2007	2008	2009	2010
Brzozów	Lalin, Pisarowce Trześniów	++++	+	++	++	++
	Kostarowce, Zarszyn (Brzozowski)					
Kańczuga	Bachórzec (Przemyski)	+++	+	+++	+	++
Dukla	Poraj (Krośniński)	++++	+++	+++	++	++
Rymanów	Moszczaniec (Sanocki)	++++	++	++++	+	+++
Number of HFRS cases in Subcarpathia in subsequent year ^b		17 (2007)	3 (2008)	6 (2009)	7 (2010)	11 (2011)

^aInformation kindly provided by the Regional Management of State Forests. Crop of beech trees was estimated in four levels scale. Mast years in bold.

^bData from current study, Heyman et al. (2011), and Czarkowski et al. (2012).
HFRS, hemorrhagic fever with renal syndrome.

captured close to a residence (Tables 1 and 2), although it is usually associated with bank voles. We detected DOBV in one bank vole by PCR, although typically DOBV is associated with yellow-necked, striped field, or Black Sea field mice (Klempa et al. 2013). This finding may indicate “spillover” infection of hantaviruses circulating in the same geographic areas. This phenomenon was also described by other researchers: DOBV genetic material was detected in wild-trapped *A. sylvaticus*, *M. musculus* (Weidmann et al. 2005), *M. glareolus*, *M. arvalis*, and *Sorex araneus* (Garanina et al. 2009). The results might be explained by biotope sharing, asymmetric population occurrence, and the cohabitation of various rodent species, which may enhance the “spillover” phenomenon and the correlation between atypical rodents species and hantavirus species (Weidmann et al. 2005, Korva et al. 2009a, Jonsson et al. 2010).

The Subcarpathian region is characterized by a relatively low degree of urbanization with built-up areas accounting for only 4%. The region is dominated by agricultural areas that cover 55% of the land. Forest land, wooded areas, and shrub land represent 39% of the total area (Śtupczyńska et al. 2009). Forests in this area belong geobotanically to European–Mediterranean montane mixed forests. In addition, it is the only region in the territory of Poland with a predominance of deciduous trees, including beech (*Fagus sylvatica*), oak (*Quercus robur*), maple (*Acer pseudoplatanus*), and ash (*Fraxinus excelsior*) (Śtupczyńska et al. 2009); the nuts of these trees are the main source of food for some rodents. The climate and the periodic abundance of food (phenomenon of heavy seed years) influence the occurrence of the following “mast years” and subsequent “mouse years,” which could be related to the periodical increase of HFRS cases among the human population, as was described for nephropathia epidemica by Clement et al. (2009). There are a very limited number of registered HFRS cases in Poland to draw any strong conclusions about the influence of “mast years” on nephropathia epidemica increase in following year. However, the prominent mast year in 2006 in Subcarpathia was speculated to be an important factor for the increased number of HFRS cases in 2007 in Poland (Knap et al. 2009). During the course of our study, we did not observe such a relation in 2009 after the mast year of 2008. The increase of HFRS cases in 2011 may be related to the seed year of 2010 (Table 3). Climate change may have a vital role in spreading the hantaviruses in the environment via various ecological mechanisms, thus influencing their hosts. A warming climate in

Poland may lead to a shortened “mast years” interval and consequent increased frequency of “mouse years” as well as an increased risk of outbreak among the human population (Clement et al. 2009, Klempa et al. 2009).

Conclusions

The endemic aggregation of HFRS cases in Poland in Subcarpathia was investigated in this study. A detailed analysis of animal reservoirs was performed in regard to registered human cases of hantavirus disease. Furthermore, the study included molecular detection of DOBV and PUUV among rodents for the first time in Poland. Our findings described geographical correlations of viruses found among animal reservoirs and those detected in human cases.

Molecular analyses need to be continued and extended to phylogenetic studies of isolates. Moreover, the monitoring of other parts of Poland (Western Carpathia or Sudeten) would be beneficial for comparing populations of hantaviruses in other regions. Global climate change triggers the need to monitor rodents and the risk for the human population, especially in Poland, where the threat it seems remains underestimated.

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No competing financial interests exist.

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