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ORIGINAL ARTICLE

First molecular characterization of Dobrava-Belgrade virus found in *Apodemus flavicollis* in Poland

Marcin Kolodziej[1,A-D], Alicja Melgies[1,A-D], Justyna Joniec-Wiechetek[1,A-D], Aleksander Michalski[1,A,E], Anna Nowakowska[2,C,E], Grzegorz Pitucha[3,B,E], Marcin N Niemcewicz[1,F]

[1] *Biological Threats Identification and Countermeasure Centre, Military Institute of Hygiene and Epidemiology, Pulawy, Poland*
[2] *Provincial Sanitary Epidemiological Station, Rzeszow, Poland*
[3] *Department of Zoology, Faculty of Biology and Agriculture, University of Rzeszow, Poland*
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Abstract

Introduction. Dobrava-Belgrade virus (DOBV) is one of the emerging pathogens which have been reported during the last decades in Europe and have attracted the attention of researchers. The course of infection among humans may have a varied course – from the completely asymptomatic to the more severe forms, such as haemorrhagic fever with renal syndrome (HFRS). DOBV is hosted and carried by rodents like *Apodemus flavicollis* or *A. agrarius*, which occur commonly in Europe.

Objective. To-date, orthohantaviruses have been reported in Poland, both in humans and animals, but detailed country-scale studies have not yet been carried out. The aim of the study was molecular characterization of a strain which was found in *A. flavicollis* in south-eastern Poland.

Materials and method. The phylogenetic analysis of the first Dobrava-Belgrade virus found in *A. flavicollis* in the subcarpathian region of south-eastern Poland, presented in this study, was performed after virus proliferation in cell culture and sequencing of specific PCR products.

Results. Based on genetic sequences of fragments of three segments (S, M and L), the isolated virus was assigned to the Dobrava genotype, taking into consideration the most current classification of the DOBV species.

Conclusions. The Dobrava-Belgrade virus strain isolated from *A. flavicollis* in the subcarpathian region of south-eastern Poland, has been molecularly characterized and assigned to Dobrava genotype, thereby the occurrence of that genotype in Poland has been confirmed by molecular techniques.

Key words

Poland, phylogenetic analysis, Apodemus flavicollis, Dobrava-Belgrade virus, orthohantaviruses

INTRODUCTION

Dobrava-Belgrade virus is one of several species from the genus *Orthohantavirus* reported in Europe, which are pathogenic to humans. Their negative-stranded RNA genome is divided into three segments of different sizes. Three ribonucleocapsids are enclosed in a common envelope. The large (L) segment encodes the viral RNA-dependent RNA polymerase, the medium (M) segment encodes the precursor of Gn and Gc glycoproteins, and the small (S) segment encodes the nucleocapsid protein. In contrast to other arthropod-borne genera of the family *Bunyaviridae*, orthohantaviruses are hosted by small mammals from the orders of Rodentia (mice, rats), Soricomorpha (moles and shrews), and even by Chiroptera (bats), with no apparent symptoms of infection [1, 2]. It is assumed that the particular orthohantavirus species have evolved over many millions of years in strict relation to specific mammal host species, with only a few episodes of cross-species transmission [1]. Therefore, the occurrence of a virus species is connected with the area where its host is present. DOBV can be hosted by mice from the sub-family Murinae: yellow-necked mice (*Apodemus flavicollis*), striped field mice (*A. agrarius*), or Black Sea field mice (*A. ponticus*). Humans are only accidental hosts of DOBV – they usually become infected by inhaling aerosolized dried rodent excreta [3]. Horizontal transmission of DOBV from person-to-person has not been observed. Infection with DOBV can develop even in haemorrhagic fever with renal syndrome (HFRS), the fatality rate of which reaches up to 12% in some regions, although many cases are mild or moderate. [4, 5]. Human infections with other species from the genus *Orthohantavirus*, e.g. Puumala virus (PUUV), have a typically less symptomatic course.

Although orthohantaviruses are reported in most European countries, information about their occurrence in Poland is not well documented due to lack of large-scale studies on animal reservoirs or routine serological tests among human populations in rural and forested areas. There are some serological proofs of human [6, 7] or rodent [8, 3] contact with orthohantaviruses; however, molecular data characterizing viral genetic sequences are limited [8,

Address for correspondence: Marcin Kolodziej, Biological Threats Identification and Countermeasure Center, Military Institute of Hygiene and Epidemiology, Biological Threats Identification and Countermeasure Center, Military Institute of Hygiene and Epide, 2 Lubelska St., 24–100 Pulawy, Poland
e-mail: marcin.kolodziej84@gmail.com

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9, 10]. Michalski *et al.* (2014) surveyed an animal reservoir from an endemic area of orthohantavirus infections in the subcarpathian region of south-eastern Poland and detected 3 different orthohantaviruses (DOBV – *A. flavicollis*, *M. glareolus*, PUUV – *M. glareolus*, *A. agrarius*, TULV – *M. arvalis*) by molecular method [3]. The presented study is a supplement to the research by Michalski *et al.*

Orthohantavirus infections in humans have been monitored officially in Poland since 2007. The number of symptomatic and serologically confirmed cases per year is relatively low in comparison with other European countries [26] and confined to the subcarpathian region. These are as follows: 13 cases in 2007, 2008 – 7, 2009 – 5, 2010 – 6, 2011 – 8, 2012 – 3, 2013 – 8, 2014 – 54, 2015 – 6, 2016 – 8 and in 2017 – 14. No fatal case have been reported [27].

Considering the lack of a comprehensive overview of orthohantaviruses circulation in Poland, new knowledge of *Orthohantavirus* ecology and epidemiology in the country is valuable. The aim of this study was molecular characterization of DOBV isolate found in *A. flavicollis* captured in the subcarpathian region of south-eastern Poland, acquiring partial data on its genomic sequences and phylogenetic analysis.

MATERIALS AND METHOD

Animal studies. Studies on the rodent reservoir of orthohantaviruses in the Subcarpathian Province were described previously by Michalski *et al.* (2014) [3]. Briefly, with the permission of the National Ethics Committee for Animal Experiments (No. 11/2009), 194 rodents were captured in the districts of Sanocki, Przemyski, Krośnieński and Brzozowski in the Subcarpathian Province between November 2009 – October 2010, using live traps (PPHU Marcinkiewicz, Poland). The rodents belonged to the following species: yellow-necked mice *A. flavicollis*, striped field mice *A. agrarius*, bank voles *M. glareolus* (formerly known as *Clethrionomys glareolus*), common voles *M. arvalis*, and house mice *Mus musculus*. After identification, they were euthanized and necropsied in the animal laboratory. The internal organs were stored in RNAlater solution (lungs, heart, spleen, bladder and kidneys) and then homogenized using a rotor-stator homogenizer (SHM1, Stuart). Total RNA was extracted using High Pure RNA Tissue Kit (Roche), cDNA was synthesized using a Transcriptor High Fidelity cDNA Synthesis Kit (Applied Biosystems), and analyzed by real time PCR using primers and probes designed by Kramski *et al.* (2007) [11] and Maes *et al.* (2007) [12]. Sera were obtained from blood samples and analyzed serologically using Hantavirus Mosaic 1 IIFT, IgG and IgM (Euroimmun), combined with secondary antibodies (rabbit anti-mouse IgM/IgG) labelled with FITC (ab8517, Abcam).

Virus proliferation. Homogenized lung tissue sample, one of those which showed positive results in real time PCR and immunofluorescence test performed by Michalski *et al.* [3], was centrifuged. Sub-confluent monolayers of Vero-E6 cells (ATCC CRL 1586) in 25 cm^2 flasks were inoculated with 500 µl of the supernatant and incubated for 1.5 h at 37 °C. Thereafter, MEM (Sigma-Aldrich) supplemented with 2% FBS (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich) was added to the flasks, and the cultures maintained under 37 °C, 5% CO_2 conditions for a month, with exchange of medium once a week. Collected media were aliquoted and stored at -70 °C for further analyses. The entire procedure of virus proliferation was performed in a containment level 3 laboratory.

Detection, sequencing and phylogenetic analysis of hantavirus RNA. RNA from frozen supernatants was extracted using QIAamp Viral Mini Kit (Qiagen) and examined for the presence of orthohantavirus specific sequences by one-step RT real time PCR using primers and probes for DOBV designed by Maes *et al.* (2007) [12]. RNA samples which showed positive results were transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or iScript cDNA Synthesis Kit (Quanta). PCRs for fragments of DOBV segments S, M and L were performed using primers designed by Papa *et al.* (1998) (segment S and Gn coding region of segment M) [13] and Klempa *et al.* (2005) (segment L) [14]. Reference strain SK/Aa (EVA) was used as a positive control. Subsequently, the products were separated by agarose gel electrophoresis. After electrophoresis, the products of expected sizes were cut out, purified from agarose gel using Agarose-Out DNA Purification Kit (EURx) and sequenced from both ends using 3730xl DNA Analyzer (Genomed). All sequences obtained in this study were analyzed using BioEdit and submitted to the GenBank database. Sequences for phylogenetic analysis were selected from those which corresponded to the fragments in the current study and represented the DOBV strains detected in various European countries. All sequences containing gaps and missing data in these regions were eliminated. The sequences were aligned by Clustal Omega with default parameters, in the same way that genetic divergences of nucleotide (nt) and amino acid (aa) sequences were determined. The phylogenetic trees were constructed by the MEGA 6 by Maximum-Likelihood method, based on the Tamura three-parameter model, and a discrete Gamma distribution (+G) with five rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). The bootstrap support percentages of particular branching points were calculated from 1,000 pseudoreplicates.

Sequences. The nucleotide sequence data reported in the presented study are available in the GenBank database under Accession Nos.: KU561221, KU561220 and KU256217.

RESULTS

The earliest positive results of one-step real time PCR for DOBV specific sequence were observed for samples of RNA extracted from media after four weeks of culturing cells which had been inoculated with homogenized *A. flavicollis* lung tissue. After cDNA synthesis, nested PCRs for fragments of segments S and M, and PCR for fragment of segment L were performed. Genetic sequence analysis of amplicons purified from agarose gel resulted in sequences of 326 bp (segment S – positions 393–718 of reference sequence NC_005233.1), 204 bp (segment M – positions 1725–1928 of reference sequence NC_005234.1) and 489 bp (segment L – positions 140–628 of reference sequence NC_005235.1) sizes.

The phylogenetic analysis of partial nt sequence of S segment of the newly-isolated strain showed that it was located among other DOBV strains hosted by *A. flavicollis*,

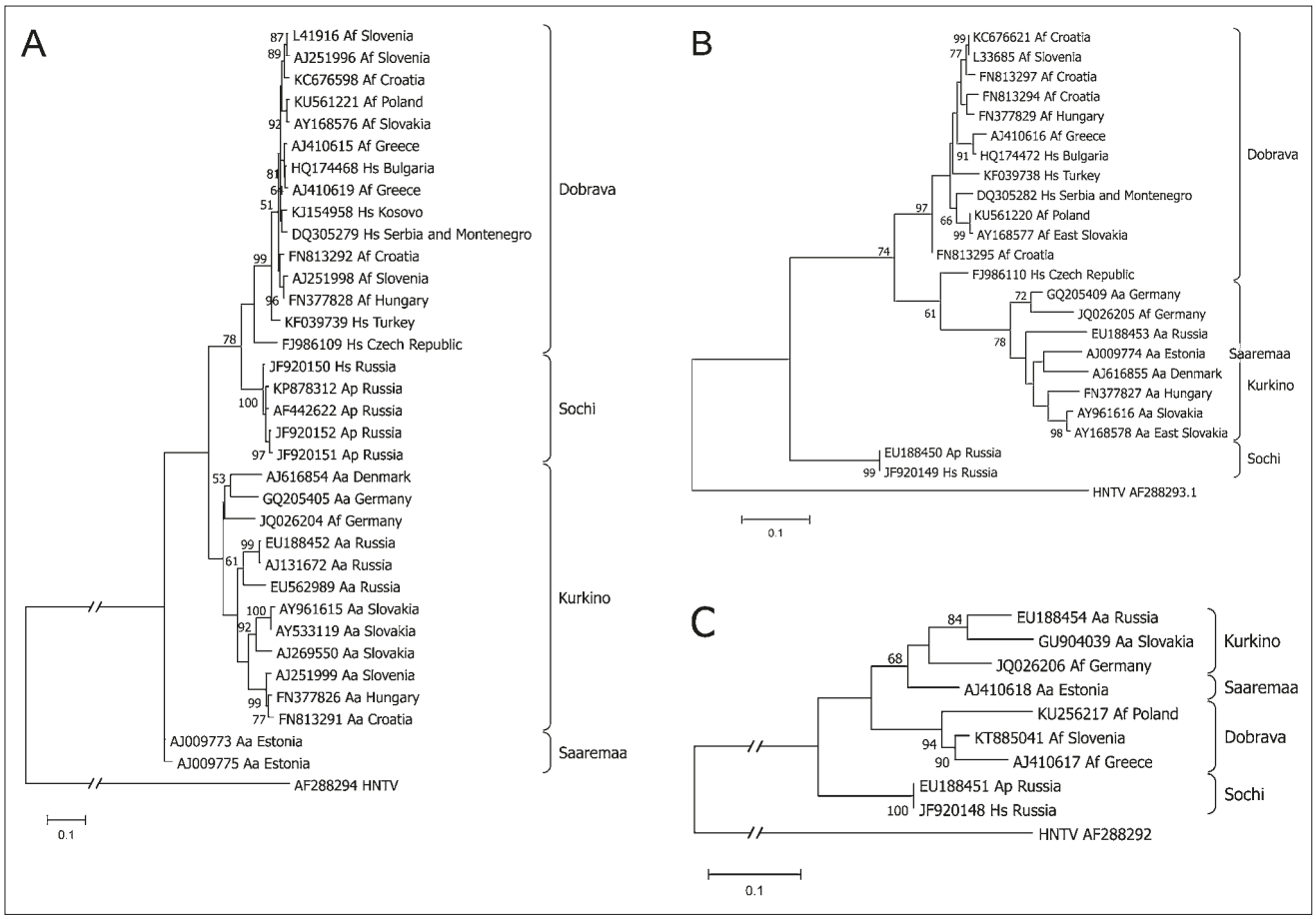

Figure 1. ML phylogenetic trees compiled from: a – 326 nt fragments of the S segment of selected DOBV strains; b – 204 nt fragments of the M segment of selected DOBV strains; c – 489 nt fragments of the L segment of selected DOBV strains deposited in the GenBank database. HNTV as a separate species but carried by Apodemus mice was used as the outgroup species. Sequences in the trees are indicated by GenBank accession number, host and country of origin. The bootstrap values for particular branching are indicated at the nodes, values below 50% are not shown. The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence. DOBV genotypes within the trees were termed according to the subdivision of DOBV suggested by Klempa et al. (2013). Abbreviations: Af – Apodemus flavicollis, Aa – Apodemus agrarius, Ap – Apodemus ponticus, Hs – Homo sapiens, nt – nucleotide.

on Dobrava lineage (Fig. 1a). The aa sequences in this branch demonstrated a high level of homology (Tab. 1). As expected due the geographical location, the Polish isolate showed the highest similarity with the strain from East Slovakia – 98.77% nt identity and 100% aa identity.

Results of comparison between 204 bp fragment of M segment of the new strain and analogous sequences of other strains were convergent with data obtained for segment S, and confirmed its affiliation with the Dobrava genotype. The strain from East Slovakia was again the one which showed the

Table 1. Percentage differences of nt sequences (above the diagonal) and percentage differences of aa sequences (below the diagonal), based on 326 nt fragments of the S segment of selected DOBV strains

	Strain	Genotype	Locality	1	2	3	4	5	6	7	8	9	10	11	12
1	KU561221	Dobrava	Poland	-	1.23	3.37	3.37	3.37	3.68	10.43	11.04	11.96	13.19	13.50	16.26
2	AY168576	Dobrava	Slovakia	0.00	-	3.37	3.37	3.37	3.68	9.82	11.04	11.66	12.58	13.19	16.26
3	AJ410619	Dobrava	Greece	0.00	0.00	-	3.07	3.68	3.37	8.90	12.88	13.50	13.80	12.58	16.87
4	FN377828	Dobrava	Hungary	0.00	0.00	0.00	-	3.68	2.76	10.12	11.96	12.88	12.88	13.50	16.26
5	L41916	Dobrava	Slovenia	1.85	1.85	1.85	1.85	-	3.37	9.51	11.66	13.50	11.35	14.42	16.26
6	DQ305279	Dobrava	Serbia & Montenegro	0.00	0.00	0.00	0.00	1.85	-	10.12	11.96	12.58	12.88	13.50	15.95
7	AF442622	Sochi	Russia	3.70	3.70	3.70	3.70	3.70	3.70	-	12.58	12.88	13.50	12.88	15.03
8	AJ251999	Kurkino	Slovenia	1.85	1.85	1.85	1.85	3.70	1.85	1.85	-	10.43	13.80	12.58	9.51
9	EU188452	Kurkino	Russia	0.93	0.93	0.93	0.93	2.78	0.93	2.78	0.93	-	12.88	9.51	8.90
10	AJ009773	Saaremaa	Estonia	2.78	2.78	2.78	2.78	2.78	2.78	3.70	2.78	3.70	-	14.42	14.11
11	JQ026204	Kurkino	Germany	1.85	1.85	1.85	1.85	3.70	1.85	1.85	0.00	0.93	2.78	-	11.66
12	AY961615	Kurkino	Slovakia	0.93	0.93	0.93	0.93	2.78	0.93	2.78	0.93	0.00	3.70	0.93	-

nt – nucleotide, aa – amino acid

Table 2. Percentage differences of nt sequences (above the diagonal) and percentage differences of aa sequences (below the diagonal), based on 204 nt fragments of the M segment of selected DOBV strains. nt – nucleotide, aa – amino acid

	Strain	Genotype	Locality	1	2	3	4	5	6	7	8	9	10	11
1	KU561220	Dobrava	Poland	-	0.49	4.41	4.90	5.39	7.35	12.75	14.71	15.20	16.18	16.67
2	AY168577	Dobrava	Slovakia	0.00	-	4.90	5.39	5.88	7.84	12.25	14.22	14.71	16.67	17.16
3	DQ305282	Dobrava	Serbia & Montenegro	0.00	0.00	-	5.88	5.39	8.82	14.71	15.69	14.22	18.14	16.67
4	FN377829	Dobrava	Hungary	0.00	0.00	0.00	-	3.43	4.90	15.69	13.73	14.71	18.14	16.67
5	L33685	Dobrava	Slovenia	0.00	0.00	0.00	0.00	-	6.37	17.16	16.18	16.18	18.14	17.16
6	AJ410616	Dobrava	Greece	0.00	0.00	0.00	0.00	0.00	-	15.20	14.22	15.69	18.14	17.16
7	JQ026205	Kurkino	Germany	2.99	2.99	2.99	2.99	2.99	2.99	-	12.25	12.75	18.14	11.76
8	AJ009774	Saaremaa	Estonia	2.99	2.99	2.99	2.99	2.99	2.99	0.00	-	8.82	22.06	13.73
9	AY961616	Kurkino	Slovakia	2.99	2.99	2.99	2.99	2.99	2.99	0.00	0.00	-	22.55	10.29
10	EU188450	Sochi	Russia	0.00	0.00	0.00	0.00	0.00	0.00	2.99	2.99	2.99	-	18.63
11	EU188453	Kurkino	Russia	2.99	2.99	2.99	2.99	2.99	2.99	0.00	0.00	0.00	2.99	-

Table 3. Percentage differences of nt sequences (above the diagonal) and percentage differences of aa sequences (below the diagonal), based on 489 nt fragments of the L segment of selected DOBV strains. nt – nucleotide, aa – amino acid

	Strain	Genotype	Locality	1	2	3	4	5	6	7	8
1	KU256217	Dobrava	Poland	-	9.41	11.45	13.70	14.31	14.93	15.13	15.54
2	KT885041	Dobrava	Slovenia	1.84	-	6.13	13.09	13.09	13.91	13.91	13.70
3	AJ410617	Dobrava	Greece	1.84	1.23	-	13.50	13.91	13.50	15.13	13.70
4	AJ410618	Saaremaa	Estonia	3.07	2.45	2.45	-	14.93	9.82	13.09	11.25
5	EU188451	Kurkino	Germany	3.68	3.07	3.07	3.68	-	15.75	15.95	14.11
6	JQ025206	Kurkino	Russia	3.07	2.45	2.45	2.45	3.68	-	11.86	10.84
7	GU904039	Kurkino	Slovakia	3.07	2.45	2.45	2.45	3.68	2.45	-	9.00
8	EU188454.1	Sochi	Russia	3.07	2.45	2.45	2.45	3.68	2.45	1.23	-

highest identity – 99.51% for nt sequence, with no difference for aa sequence (Fig. 1b; Tab. 2).

In spite of the considerably lower number of available reference sequences of L segment corresponding to fragment obtained in the presented study (located in N-terminal region), phylogenetic analysis confirmed that the Polish strain can be classified as a Dobrava genotype representative (Fig. 1c; Tab. 3).

DISCUSSION

Orthohantaviruses have attracted the attention of scientists since being recognized as the cause of serious infections in humans, which had been reported for several decades in the Far East, Europe, and the Americas. Among orthohantaviruses, PUUV and DOBV are the most significant threat to public health in Europe. Even though they are carried by different rodent species, the habitats of their hosts overlap and cover most of the continent, including Poland. Moreover, the presence of PUUV, DOBV and TULV among local rodents has been confirmed by serological and molecular techniques [8,3]. The current study is a substantial continuation of the research work carried out by Michalski *et al.* (2014) [3], who detected DOBV in *A. flavicollis* in Poland by real time PCR, but without genetic sequence analysis.

Comparison analyses revealed high degree of similarity of the Polish isolate to the strain from eastern Slovakia in nucleotide sequences of S and M segments. Some point mutations have been found (4 point mutations in 326 bp fragment of S segment and 1 mutation in 204 bp fragment of M segment), however, they did not alter the amino acid sequences.

Both strains, the Slovakian and the newly-characterized Polish strain, were isolated from *A. flavicollis* and are located in the same phylogenetic branch with other strains found in this species of *Apodemus* genus. With a reliably high bootstrap support, all the isolates form one cluster called the Dobrava genotype – in compliance with classification proposed recently by Klempa *et al.* (2013) [2]. The cluster noticeably diverges from DOBV strains found in *A. agrarius* (Kurkino and Saaremaa genotypes) or *A. ponticus* (Sochi genotype) (Fig. 1a, b, and c; Tab. 1, 2 and 3). Moreover, this monophyletic group shows minimal amino acid variability in the examined regions (Tab. 1 – 3).

According to the European long-term research [2], DOBV as a species is divided into four genotypes: Dobrava, occurring in *A. flavicollis*, Kurkino in *A. agrarius*, Sochi in *A. ponticus* and Saaremaa, hosted by *A. agrarius*. However, DOBV genotypes have also been found in non-typical *Apodemus* mice species [3, 15]. This "spill-over" phenomenon seems to take place especially where host species share ecological niches [16]. Dobrava genotype is the most prevalent DOBV in the Balkan peninsula [5], whereas in central and north-eastern Europe (European Russia, Slovakia, Germany), the Kurkino genotype is considered the dominant one [17, 18, 14]. Nonetheless, the Kurkino genotype presence has been proved also in the Hungarian-Croatian borderland [19], Croatia [20] and Slovenia [21] in *A. agrarius*. In central Europe, both genotypes can be found as a consequence of

their hosts cohabitating in the same area [22]. Furthermore, it has been confirmed that both genotypes can be found in the same foci [21, 22]. Michalski *et al.* (2014) [3] detected DOBV molecularly in over 15% of examined *A. flavicollis* from south-eastern Poland, but none of 55 captured *A. agrarius* mice had DOBV. Despite some serological data implying the presence of the Dobrava genotype in Lithuania [23], this study provides the first molecular evidence of the northernmost occurrence of the Dobrava genotype.

The long-term coexistence of two virus genotypes in two different host species in the same geographic region indicates their genetic stability and evolutionary distinctness [22]. On the other hand, transmitting viruses to non-typical hosts ("spill-over" phenomenon) may occur. Moreover, the exchange of segments between different genotypes is possible as it has been observed in laboratory conditions [24]. Furthermore, the Saaremaa genotype is suspected to be an example of naturally occurred reassortment [2]. Based on the analyzed sequences of the Polish strain, such a phenomenon has been excluded in this case.

The results of phylogenetic analyses concerning particular segments of the Polish isolate are convergent – they clearly indicate that the strain belongs to the same genotype Dobrava. Nevertheless, there are some differences in the topology of phylogenetic trees calculated for individual segments, which were also observed in phylogenetic trees constructed by other authors [2, 18]. Those variations can arise from the length of analyzed sequences (not allowing for very fine analysis), the method of analysis applied, the amount of available analogous sequences in the GenBank database, or various mutation frequencies in particular segments.

Apart from differences in nucleotide and amino acid sequences and affinity to host species, the division of DOBV species into genotypes is reflected in their pathogenicity. The cause of such differences in the course of disease, however, remains obscure [2]. The most severe infection course is usually related with Dobrava or Sochi genotypes, whereas Kurkino and Saaremaa are much less virulent [2]. The Kurkino genotype usually causes disease with a mild course and low fatality rate, although some more severe cases [4] or local epidemics with a high number of the afflicted [25] have also been reported.

The Polish isolate belongs undoubtedly to the Dobrava genotype, which has one of the highest fatality rates among DOBV genotypes [4]. In spite of the fact that in Poland fatal cases of HFRS have not yet been reported, the presented study has confirmed the presence of one of the most pathogenic DOBV genotypes, which poses an ineligible environmental and occupational risk for humans.

CONCLUSION

The molecular characterization of DOBV strain found in south-eastern Poland in *A. flavicollis*, which turned out to belong to Dobrava genotype, is a discovery that emphasizes the risk of occurrence of a more severe form of HFRS in Poland. It also reveals the need to conduct further research on orthohantaviruses in Poland, and to increase the awareness of physicians and local populations about the modes of transmission of the viruses and diagnostic methods.

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Ethical approval
The authors did not utilize human participants or animals in the performance of the study.

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