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Dobrava-Belgrade virus: Phylogeny, epidemiology, disease

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ABSTRACT

Dobrava-Belgrade virus (DOBV) is an Old World hantavirus that causes hemorrhagic fever with renal syndrome in humans. With a case fatality rate up to 12%, DOBV infection is the most life-threatening hantavirus disease in Europe. The virus was initially identified in the Balkans, but the discovery of new endemic foci have expanded its recognized geographic range. The recent description of novel genetic variants with different degrees of pathogenicity have complicated its taxonomic analysis. The original rodent host of DOBV is *Apodemus flavicollis*, however additional *Apodemus* species, such *Apodemus agrarius* and *Apodemus ponticus*, have been found to serve as hosts of the various DOBV genotypes. The complex evolution and genetic diversity of the virus are still under investigation. The present review aims to provide an update on the phylogeny of DOBV and the epidemiology of infection in rodents and humans; to describe the clinical characteristics of the disease; to present current knowledge about laboratory diagnosis, treatment and prevention; discuss the current state of the art in antiviral drug and vaccine development.

Contents

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1. Introduction historical perspective

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) cause two clinical syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, and hantavirus pulmonary (or cardiopulmonary) syndrome (HPS) in the Americas, with fatality rates of up to 12% and 60%, respectively (Jonsson et al., 2010). They are transmitted to humans through inhalation of the aerosolized excreta of persistently, but asymptomatically infected rodents, belonging to the *Murinae*, *Arvicolinae* and *Sigmodontinae* subfamilies of the *Muridae* family. Shrews, moles, and bats are also reservoirs of hantaviruses, but of so far undetermined pathogenicity for humans (Schlegel et al., 2012; Song et al., 2007). The two pathogenic hantaviruses in Europe are Puumala virus (PUUV), which has been known for several decades as a cause of mild HFRS in northern Europe, and DOBV, a more recently recognized agent in southeastern Europe, which exhibits a case-fatality rate up to 12%. Since DOBV was first described in 1992, many genetic variants have been detected in new geographic areas, providing insights into the ecology of the reservoir and the etiology of the human disease. These studies have prompted investigation of the genetic determinants implicated in pathogenicity variations.

The existence of a second hantavirus in Europe, in addition to PUUV, was first suspected in the late 1980s, when there was evidence that a virus causing severe HFRS was circulating in the Balkans (Antoniades et al., 1987; Antoniadis et al., 1984,1987; Avsic-Zupanc et al., 1989; Gligic et al., 1989a; Gligic et al., 1988). In 1986, a hantavirus (Porogia virus) was isolated from the urine of a severely ill patient with HFRS in Greece, who presented with acute and persistent pulmonary edema and progressive renal failure, and who gradually improved with daily hemodialysis and respiratory support (Antoniades et al., 1987). Convalescent sera from Greek patients with severe HFRS showed highest titers in plaque-reduction neutralization tests with Porogia virus, rather than with the prototype Hantaan virus (HTNV) (Antoniades et al., 1987). No molecular methods were performed that time, but 10 years later, PCR analysis of a virus stock revealed that it was indistinguishable from the protoype HTNV, most probably due to subsequent contamination. However, Porogia virus was the first murine HTNV-like hantavirus isolated in Europe (Clement et al., 2012).

In 1992, two reports from the former Yugoslavia described the isolation of a previously unrecognized hantavirus circulating in the Balkans. One isolate, named Belgrade virus, was recovered from the blood and urine of patients with severe HFRS (Gligic et al., 1992a), while the other, named Dobrava virus, was recovered from the lungs of *Apodemus flavicollis* (yellow-necked mouse) trapped in Dobrava village in Slovenia (Avsic-Zupanc et al., 1992). A phylogenetic tree based on partial M-segment sequences showed that Dobrava virus was genetically distinct from other hantaviruses, and it was suggested that it represented a new virus in the genus *Hantavirus* (Xiao et al., 1993). As the Belgrade and Dobrava viruses have been shown to be genetically virtually identical, they are considered to be the same virus (Taller et al., 1993), and the International Committee for Taxonomy of Viruses (ICTV) proposed the name Dobrava-Belgrade virus (DOBV) for this species (Fauquet et al., 2005). A few years later, DOBV RNA was detected in blood samples from a Greek and an Albanian patient with severe HFRS (Antoniadis et al., 1996). Identical sequences were obtained from *A. flavicollis* captured in the region where the Greek patient was infected (Papa et al., 2000b).

Molecular technology has contributed largely to the detection and genetic characterization of additional DOBV strains in other parts of Europe and Russia, and additional rodent species have been found to serve as reservoirs, unraveling the epidemiology of the virus. Serological evidence for DOBV infections in humans has been found in European Russia (Lundkvist et al., 1997a), and DOBV RNA was detected in *Apodemus agrarius* (striped field mouse) trapped in 1998 near Kurkino village, in the Tula region of Russia (Plyusnin et al., 1999). The Kurkino strain clusters with other DOBV sequences subsequently obtained from *A. agrarius*, as well as from HFRS patients from Slovakia, Germany, Russia, and other countries (see *Epidemiology*), forming a distinct lineage, provisionally named DOBV-Aa, which currently is not officially recognized by the ICTV (Dzagurova et al., 2009; Klempa et al., 2003a, 2004, 2005, 2008; Sibold et al., 1999, 2001). DOBV-Aa is recognized as a principal pathogen in Central Europe and European Russia, causing several hundred cases of HFRS in Russia and about 30 cases in Germany annually.

In late 1990, a DOBV-like virus was isolated from the western subspecies of *A. agrarius* (striped field mouse) trapped on Saaremaa island in Estonia (Nemirov et al., 1999; Plyusnin et al., 1997). A host-switch event was suggested (Nemirov et al., 2002). The authors initially designated the virus DOBV, but later it was named Saaremaa virus (SAAV) (Sjolander et al., 2002). Phylogenetic analysis has suggested that DOBV (DOBV-Af) and SAAV are distinct, and it was estimated that their split happened 3.0–3.7 million years ago (Sironen et al., 2005). There has been taxonomic controversy as to whether SAAV forms a unique virus species, distinct from DOBV (Dzagurova et al., 2009; Klempa et al., 2003a,b, 2004; Plyusnin et al., 2003). Currently, SAAV is accepted by the ICTV as a distinct virus. Another DOBV variant has also been detected in several *Apodemus ponticus* (Caucasian wood mouse) trapped in the Sochi district in the southern part of European Russia (Tkachenko et al., 2005). Virus isolated from lung tissues of *A. ponticus* was named Sochi virus or DOBV-Ap, a designation currently not officially recognized by the ICTV (Klempa et al., 2008). More recently, a virus was obtained from a fatal HFRS case in the city of Sochi (Dzagurova et al., 2012).

At present, the various genotypes of DOBV are designated as DOBV-Aa and DOBV-Ap, while the one originally associated with *A. flavicollis* is referred as DOBV-Af. Although I have kept these names in the present review, the evolution and diversity of DOBV are complex and are still under investigation, so that it is likely that these designations will be replaced, either by the names of the sites where the viruses were first discovered, or by alphabetic or nu-

meric lineages. Genetic characterization of additional strains from reservoir hosts and (even better) from human cases will facilitate the taxonomic classification and pathogenicity determination for each genotype.

2. Virion structure and genome

The hantavirus virion is spherical, with an average diameter of 80–120 nm, and a surface structure composed of a grid-like pattern due to glycoprotein projections anchored in the lipid bilayer envelope (Battisti et al., 2011; Huiskonen et al., 2010). It contains a single-stranded, negative-sense RNA genome consisting of three segments, the small (S), medium (M) and large (L), all of which are required for infectivity. The 5′and 3′ terminal sequences of each segment are highly conserved and specific for the genus. As the ends are reverse-complementary, they are able to form panhandle structures, playing a role in the proposed prime-and-realign mechanism of replication (Schmaljohn and Nichol, 2007).

The S RNA segment encodes the nucleoprotein (N), which is the major structural component of the virus. The N protein protects and encapsidates the three genomic RNAs, forming three viral ribonucleocapsids; facilitates the translation of viral messenger RNA (mRNA) in cells; and in conjunction with the L protein, plays an important role in transcription and replication of the viral genome. Within the first 118 amino acids of the amino-terminal of DOBV N protein there are the main epitopes for human IgG immune response (Kallio-Kokko et al., 2000). In general, the internally located N protein is relatively conserved among the hantavirus species, inducing a highly cross-reactive humoral immune response, probably by antibody-dependent cytotoxic T lymphocytes (Kruger et al., 2001).

The M RNA segment encodes the glycoprotein precursor (GPC), which is further co-translationally cleaved into the envelope glycoproteins Gn and Gc (formerly G1 and G2). The glycoproteins mediate viral attachment and entry and fusion with intercellular organelles. Protective immunity is thought to correlate with neutralizing antibodies to Gn and Gc (Kruger et al., 2001).

The L RNA segment encodes the viral RNA-dependent RNA polymerase (L protein), which acts as a replicase, transcriptase, endonuclease (cleaving cellular mRNAs for the production of capped primers used to initiate transcription), and possibly an RNA helicase. An immune response to the L protein has not been detected.

The S RNA segment of the prototype Slovenian DOBV-Af strain is 1667 nucleotides (nts) in length, with a single ORF encoding a

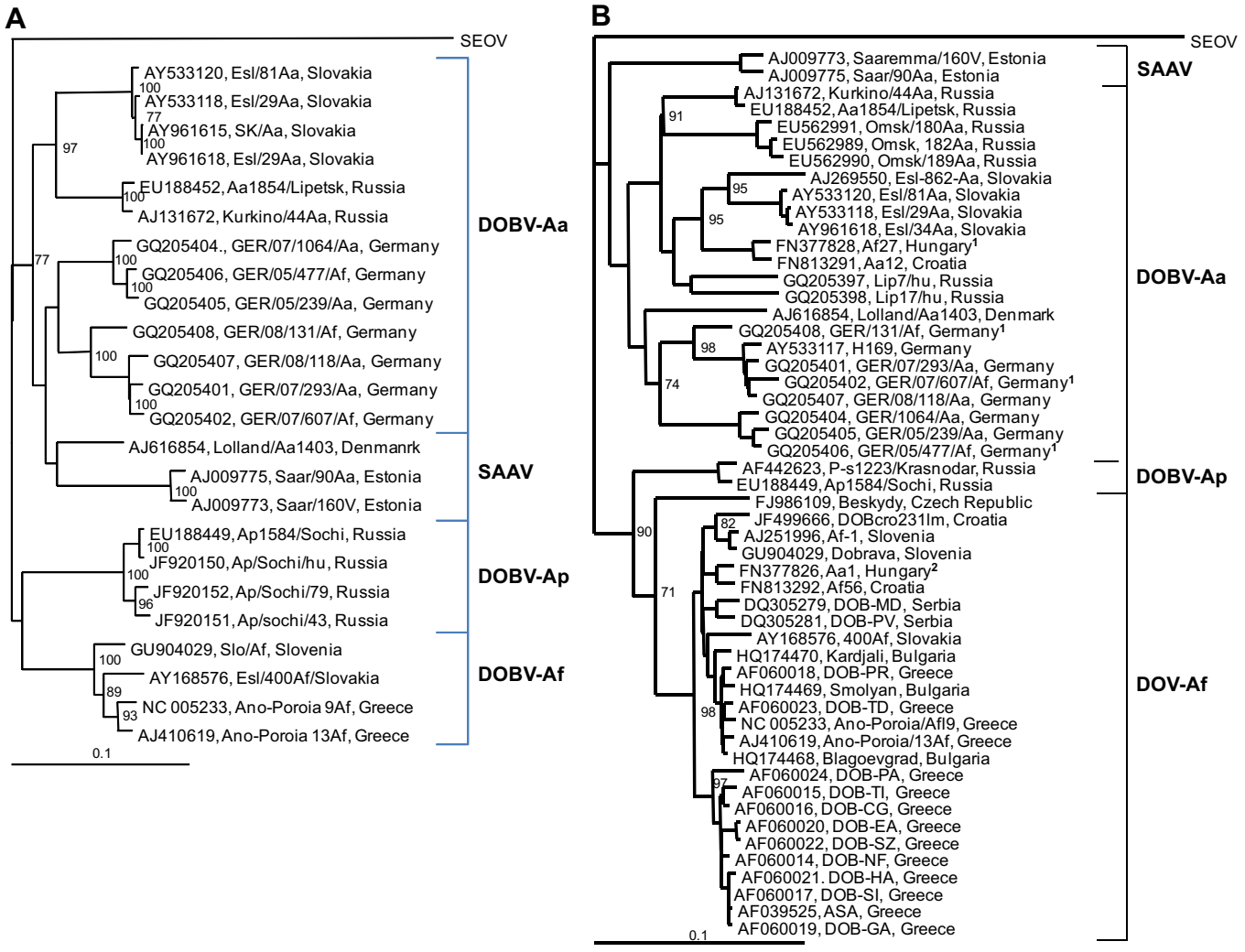

Fig. 1. Neighbor-joining phylogenetic trees of DOBV and SAAV sequences, produced using PHYLIP software, with Seoul virus as the outgroup. (A) Tree based on the complete ORF of the S genome segment. (B) Tree based on a 539-bp fragment of the S RNA segment. Numbers at the nodes indicate percentage bootstrap replicates of 100; values below 60% are not shown. Horizontal distances are proportional to nucleotide differences; the scale bar indicates 10% nucleotide sequence divergence. Sequences are indicated by GenBank accession number, strain name and country.

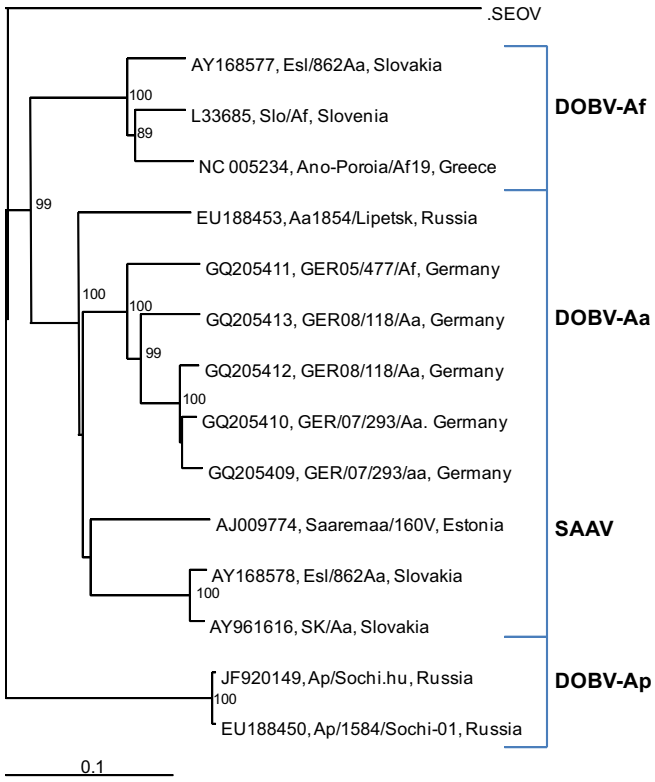

Fig. 2. Neighbor-joining phylogenetic tree based on the complete M genome segment sequence of DOBV and SAAV, produced as described in Fig. 1. Superscripts 1 and 2 indicate spill-over infections.

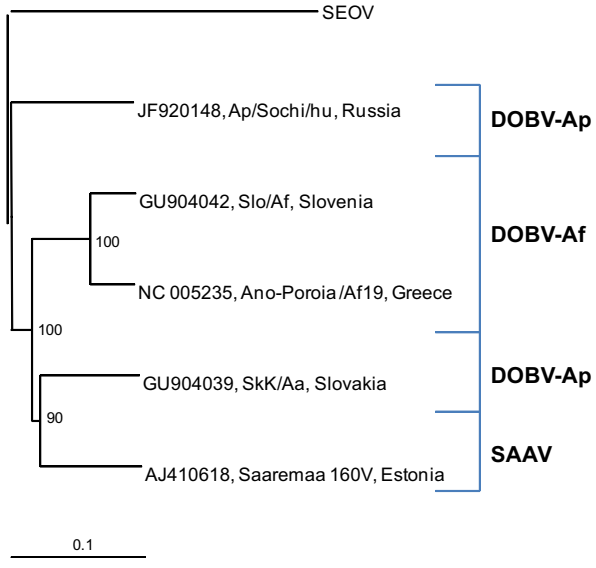

Fig. 3. Neighbor-joining phylogenetic tree based on the complete L genome segment sequence of DOBV and SAAV, produced as described in Fig. 1.

protein of 428 amino acids (aa). The M RNA segment is 3644 nts long, with a coding capacity of 1134 aa in the virus complementary-sense RNA (Avsic-Zupanc et al., 1995). The L segment ORF was determined to be 6453 nt in length, coding for a protein of 2151 aa (Kirsanovs et al., 2010). The N protein, the glycoprotein precursor and the L protein of the Greek DOBV isolate are 429 aa, 1135 aa, and 2151 aa in length (Nemirov et al., 2003). Slight differences are seen in the other genotypes, e.g. the ORF of the glycoprotein precursor of DOBV-Ap (Sochi/Ap) is two amino acids shorter, due to a difference in length of the 3′ noncoding region (Klempa et al., 2008).

3. Classification and phylogenetics

Hantavirology began in 1978, when Ho Wang Lee and his coworkers isolated Hantaan virus (HTNV), the etiological agent of HFRS in Asia (Korean hemorrhagic fever) from the lungs of *A. agrarius* (Lee et al., 1978). At present, over 20 different hantavirus species are recognized by the ICTV. Most are proven human pathogens, while the rest have been isolated only from rodents or insectivores. HFRS is caused by HTNV, DOBV, Seoul virus (SEOV), and PUUV, while Sin Nombre virus, Andes virus and other related hantaviruses in the Americas cause HPS.

Phylogenetic analysis provides useful data for the classification and molecular epidemiology of the hantaviruses, as well as for their association with human disease and with specific animal hosts. In phylogenetic trees based on the S, M or L RNA segment, DOBV sequences form a distinct clade, differing from all other hantavirus species. It is currently known that at least three DOBV genotypes circulate in many European countries and Russia (DOBV-Af, DOBV-Aa, DOBV-Ap), while SAAV is a DOBV-like virus. The clustering of DOBV genotypes is not the same in phylogenetic trees based on the S, M or L segments. For example, whereas DOBV-Ap and DOBV-Af share a common ancestor in the S-segment tree, DOBV-Ap forms an outgroup from all other DOBV and SAAV sequences (see Figs. 1–4, constructed using the neighbor-joining method). These discrepancies may be the result of genetic reassortment events, or may reflect the limited and unequal number of sequences in the M- and L-segment data sets (Klempa et al., 2008). Because hantaviruses possess a tri-segmented genome, genetic reassortment is a possible event, which can be detected by the presence of discrepancies in phylogenetic trees. Cross-species transmission of DOBV has also been observed. These factors have led to reconsideration of the earlier theory that each hantavirus species coevolved with a specific rodent host, and it is now believed that host-switching and subsequent local adaptation to the new host play an important role in hantavirus evolution (Ramsden et al., 2009).

The S, M and L segments of SAAV differ from DOBV-Af by 12.3%, 17.6% and 14.4% at the nucleotide level, and by 2.8%, 5.9%, and 2.4% in the amino acid sequences of the N, GPC and L protein, respectively (Nemirov et al., 2003). Based on current ICTV criteria, hantaviruses are classified as belonging to different species when their N and GPC amino acid sequences differ by 7% or more, while according to newly proposed criteria, the differences must be even greater: >10% for the N and >12% for the GPC amino acid sequences (Maes et al., 2009). Furthermore, maximum-likelihood and neighbor-joining analysis has revealed that DOBV-Af, DOB-Aa, DOBV-Ap, and SAAV form four well-supported lineages (Maes et al., 2009). Based on these data, a re-evaluation of the taxonomy of DOBV and SAAV is probably needed, especially once additional sequences of the different genotypes have become available. Also, it cannot be assumed that all DOBV-Aa strains are SAAV strains; this has been clarified in a recent article published by all members of a European network on imported viral diseases (ENIVD-CLRN), in which the authors stated that "it should be noted that hantavirus strains associated with *A. agrarius* in Central Europe and Russia have been shown to be phylogenetically distinct from the northeastern European SAAV strains" (Heyman et al., 2011a).

Although DOBV genotypes differ substantially at the nucleotide level, they share a high degree of amino acid sequence similarity, suggesting that most mutations are silent. However, they differ

Incubation 2-4 weeks	Febrile 3-7 days	Hypotensive hours -2 days	Oliguric 3-7 days	Diuretic 1-2 weeks	Convalescence 3-6 weeks
	fever, headache, chills, malaise, myalgia, nausea, abdominal pain, vomiting, diarrhea, facial flushing, conjunctivitis, petechiae	Hypotension, acute renal failure	Oliguria, anuria, microscopic / macroscopic hemorrhagic manifestation, shock	Polyuria, improvement of renal function	Usually without sequlae
	Sudden onset	Thrombocyto-penia	Hemodialysis often needed, High fatality	Good prognostic marker	

Fig. 4. Clinical course of hemorrhagic fever with renal syndrome caused by Dobrava virus, based on articles cited in the text.

in their virulence for humans, prompting further studies to identify the factors associated with these differences.

3.1. The S segment

In the phylogenetic tree based on the S-segment ORF, it is seen that DOBV-Af, DOBV-Aa, DOBV-Ap and SAAV form four distinct, but closely related, genetic clades (Fig. 1A). The nucleotide sequence of the Greek DOBV-Af strain (NC_005233) differs by 3.8% from the Slovenian one, and by 13–16% from the others. Similar clustering is seen in the tree based on a larger number of partial S-segment sequences (Fig. 1B). An additional subclade in the DOBV-Af is seen, containing a sequence obtained from a Czech HFRS patient. It differs by 7–8.5% from the DOBV-Af strains at the nucleotide level; it is not known if it is associated with *A. flavicollis* or other rodent species, as no such sequence has been detected in rodents so far. Whole-genome sequencing of the Czech isolate will identify its precise phylogenetic location.

Even from the first genetic epidemiological studies on DOBV-Af, it was shown that there is a considerable nucleotide variation among the strains, while a strict geographic clustering was observed, which is probably related with the limited acting area of the rodents (Papa et al., 1998). A North-South rather than East-West geographic clustering is seen among DOBV-Af strains, which is more obvious among Greek DOBV sequences: e.g. sequences from northeastern part of Greece (DOB-PR and DOB-TD) are genetically closer to sequences from Bulgaria (North of Greece) rather than sequences from the western Greece (e.g. DOB-GA). This geographic clustering can be explained by the evolution of the virus during rodent migration through the mountain ranges million of years ago.

The nucleotide difference among DOBV-Aa strains is higher than that among DOBV-Af strains (Klempa et al., 2008). Especially sequences from Russia, form 4 clusters: two, containing sequences from HFRS cases observed in Lipetsk during the outbreak in 2006–07, the third, containing sequences from *A. agrarius* trapped in Lipetsk during the outbreak of 2001–02, and from *A. agrarius* trapped in Kurkino in 1998, and the fourth cluster, containing sequences from Omsk (Fig. 1B). The high degree of sequence variability in the small area of Lipetsk suggests the long-term survival and evolution of DOBV-Aa in this region (Dzagurova et al., 2009). So far, DOBV-Aa sequences form two main clades, one containing sequences from Russia, Slovakia, Hungary and Croatia, and the second containing sequences from Germany.

Up to now, the DOBV-Ap lineage contains sequences from *A. ponticus* trapped in Sochi and from HFRS cases observed in Sochi and Krasnodar. Similar to other DOBV lineages, the geographic clustering of DOBV-Ap indicates long-term presence of the virus in the area (Dzagurova et al., 2012).

3.2. The M segment

Greater nucleotide genetic differences between viruses are seen in the M segment than in the S segment. In the phylogenetic tree of the M segment ORF, SAAV clusters together with DOBV-Aa sequences (Fig. 2). The DOBV-Aa strains differ by 11–12% from SAAV at the nucleotide level, and by approximately 4% at the amino acid level. DOBV-Ap differs by approximately 20% from DOBV-Af and DOBV-Aa at nucleotide level, and by 7–9% at amino acid level.

North-south geographic clustering is more evident in this segment: the DOBV-Af Ano-Poroia strain from northeastern Greece differs by 12.7% from strains from northwestern Greece (e.g. DOB-EA) and by only 5.9% from strains from Slovakia which is much further (data not shown). The locations where the Ano-Poroia and DOB-EA strains were isolated are separated by high mountains (the Pindos mountain chain), which stretch in a north-south direction.

3.3. The L segment

The number of available L-segment sequences is relatively small. In phylogenetic trees based on the complete L-segment ORF, the DOBV-Af sequences cluster together, differing by 15.5–17.5% from DOBV-Aa and DOBV-Ap at the nucleotide level (Fig. 3). The amino acid differences among the three DOBV genotypes and SAAV are <4%.

3.4. Reassortment and recombination

Reassortment and recombination are better elucidated when the analysis of strains is based on whole genome sequences. Klempa et al. (2003b) gave evidence for genetic reassortment between S and M segments of DOBV-Af and DOBV-Aa and indicated homologous recombination events in DOBV evolution. Natural reassortment occurs between closely related viruses, which circulate in the same habitat and are able to infect the same host animal (Kirsanovs et al., 2010). Multiple spill-over events have been observed in both sides: detection of DOBV-Af in *A. agrarius* and detection of DOBV-Aa in *A. flavicollis* (Plyusnina et al., 2011; Scharninghausen et al., 1999; Schlegel et al., 2009). Furthermore, DOBV-Af has been detected also in *A. sylvaticus* and *Mus musculus,* suggesting that spill-over events seem to be far more common than expected (Weidmann et al., 2005). In vitro studies showed that reassortment between strains Slo/Af (DOBV-Af from Slovenia) and SK/Aa

(DOBV-Aa from Slovakia) happens with high frequency only in the M segment, and not in the S or L segment alone, while the in vitro reassortment frequency for DOBV is substantially higher than that for New World hantaviruses (Kirsanovs et al., 2010).

4. Epidemiology

Rodent species of the genus *Apodemus* have been present in Europe for at least 3 million years (Michaux and Pasquier, 1974). *A. flavicollis*, the reservoir host of DOBV-Af, is found in large forest areas, mainly with trees with heavy seeds, such as oak and hazel, while *A. agrarius*, the host of DoBV-Aa and SAAV in Europe, lives in uncultivated land, gullies, ravines and field edges. DOBV infections in the Balkans, where DOBV-Af predominates, display a strong seasonal distribution, with most cases occurring in the summer. People with outdoor activities are most affected; infection may occur during crop harvesting, the cleaning of poorly ventilated cottages, wood cutting or during military field exercises. Farmers, shepherds, forestry workers and military personnel are at greatest risk of infection. The male-to-female ratio is approximately 3:1. Infection is seen primarily among adults, with the age group 35–50 being the most affected (Papa and Antoniadis, 2001). There are also reports of DOBV infection in children (Bogdanovic et al., 1995; Eboriadou et al., 1999; Peco-Antic et al., 1992). Person-to-person spread has not been observed.

The exact incidence of hantavirus infections cannot be precisely estimated in each European country, because the number of recognized cases is only the tip of the iceberg. Especially for PUUV and DOBV-Aa, which usually cause mild disease, the number of infections is underestimated. The situation for SAAV is similar; there currently is no molecular evidence that the virus is able to infect humans and induce disease. Furthermore, since serological methods cannot clearly distinguish between infections caused by the various DOBV genotypes, the only method to gain this information is through PCR and sequencing (see below).

The presence of at least two hantavirus species in the former Yugoslavia was reported in the 1980, when hantaviruses were recovered from *Clethrionomys* (now *Myodes*) *glareolus* and *A. flavicollis* rodents trapped in regions where HFRS cases had been reported (Avsic-Zupanc et al., 1989; Gligic et al., 1989a). Based on the course of the disease and the results of serological testing with a variety of hantavirus antigens (HTNV, PUUV and SEOV), it can be concluded that DOBV was the cause of thousands of cases in the Balkans. In Greece, almost all HFRS cases are caused by DOBV, while from southern and eastern to western and northern Europe, the DOBV/PUUV infection ratio ranges from 50% in Slovenia to less than 1% in Western Europe and Fennoscandia, where PUUV predominates (Heyman et al., 2009).

The following paragraphs summarize existing knowledge of DOBV infections in various European countries, moving from south to north. It should be mentioned that (a) most human cases were detected by serology, thus the exact DOBV genotype cannot be determined; (b) DOBV-Aa and SAAV infections are not serologically distinguishable, because in contrast to their S segments, their M segments are closely related; and (c) based on molecular data, only the presence of a genotype can be confirmed, and the circulation of others cannot be excluded.

4.1. Greece

The first serological evidence for the presence of a HTNV-like virus in Greece was in 1981 (Lee and Antoniadis, 1981). In the following year, the disease was confirmed in two woodcutters in a forested area, who presented with acute renal insufficiency (Antoniadis et al., 1984). Additional cases were later serologically diagnosed (Antoniadis et al., 1987; Papadimitriou and Antoniadis, 1994). In July-August, 1983, an HFRS outbreak was observed in northwestern Greece (eight cases – one fatal) (Siamopoulos et al., 1985). The background of the isolation of Porogia virus in 1986 has been discussed above. Studies on small mammals suggested that *A. flavicollis* might host a hantavirus (Antoniadis et al., 1987; LeDuc et al., 1986).

The first genetic evidence for the association of DOBV with HFRS was shown in two patients from Greece and Albania (Antoniadis et al., 1996), while a retrospective serologic and genetic study revealed that DOBV-Af was the cause of HFRS in all PCR-positive patients (Papa et al., 1998). The most endemic regions are in the northwestern and northeastern parts of Greece, across the Pindos and Rodopi mountain ranges, where approximately 10% of *A. flavicollis* are infected with DOBV-Af (Papa et al., 2000a). A DOBV-Af (strain Ano-Poroia/Af9V/1999) was isolated from the lungs of an *A. flavicollis* trapped in 1999 in Ano Poroia village (the same village where Porogia virus was isolated in 1986 from an HFRS case) in northeastern Greece (Papa et al., 2001); it later became the first DOBV strain with a complete genome sequence (Nemirov et al., 2003). About five hospitalized cases are observed annually in Greece, which are serologically diagnosed as DOBV. The only genotype detected so far in PCR-positive cases is DOBV-Af.

4.2. Countries of the former Yugoslavia

Since 1952, when the first HFRS cases were clinically recognized in the former Yugoslavia, sporadic cases and outbreaks have been reported every year, with case fatality rates exceeding 10% (Gligic et al., 1989b). HFRS outbreaks were observed in 1961 in Serbia (46 soldiers); in 1967 in Bosnia-Herzegovina, Croatia and Montenegro (more than 200 cases); in 1986 in the whole country (mainly in Montenegro and Serbia with 276 suspected cases, 161 of them laboratory-confirmed); in 1989 in Bosnia-Herzegovina, Serbia and Croatia (609 suspected cases, 226 of them laboratory-confirmed). The largest outbreak took place in 1995–96 in Bosnia, Croatia, Serbia and Montenegro, when more than 300 patients, most of them soldiers, were hospitalized in the Tuzla region of northeast Bosnia during the military conflict (Gligic et al., 1992b, 2010; Hukic et al., 1996). Bosnia-Herzegovina, especially the central and northeast part of the country, is considered one of the most endemic regions in Europe (Hukic et al., 2009). Using neutralizing tests it was found that one third of HFRS cases in 1995 were caused by DOBV, and the rest by PUUV (Lundkvist et al., 1997b).

All of Croatia, with the exception of the coastal region and islands, is endemic for HFRS, caused either by PUUV or DOBV (Markotic et al., 2002b). Croatia was highly affected in 1995, with 125 clinically diagnosed patients and two deaths; most cases were severe or moderate (Kuzman et al., 1997). In 2002, there were 401 clinically diagnosed patients; DOBV was the causative agent for approximately 10%, while the rest were caused by PUUV (Kuzman et al., 2003). Recently, DOBV-Af was detected in 5/27 *A. flavicollis* trapped in northern Croatia (Nemeth et al., 2011).

In Montenegro, the incidence of HFRS during 1995–2005 was estimated to be 2.6 per 100,000, with most cases observed in the rural mountainous areas of northeastern municipalities; the sera of 90% of HFRS patients reacted with DOBV (DOBV-Af) or HTNV antigens, while the rest reacted to PUUV or SEOV (Gledovic et al., 2008). Sequences retrieved from HFRS patients during the outbreak in 2002 clustered together with Balkan DOBV-Af (Papa et al., 2006a).

In Slovenia, DOBV-Af was originally isolated from *A. flavicollis* in the village of Dobrava in the Dolenjska region, where nearly half of the total HFRS cases have been registered (Avsic-Zupanc et al., 1999). DOBV antigen or antibodies were detected in approximately 20% of *A. flavicollis* in endemic areas (Avsic-Zupanc et al., 1992).

Since DOBV-Aa was detected in reservoir animals in Slovenia (Avsic-Zupanc et al., 2000) and Croatia (Plyusnina et al., 2011), the possibility cannot be excluded that few of the serologically diagnosed cases are caused by DOBV-Aa.

4.3. Albania

The first laboratory-confirmed HFRS case in Albania was reported in 1987, when a 27-year man presented with acute renal failure and shock (Eltari et al., 1987). Antibodies to hantaviruses were detected in 4/6 patients with HFRS-like illness (Gligic et al., 1989b). A study during 2003–2006 among 34 patients with suspected Crimean-Congo hemorrhagic fever (CCHF) showed that 11.7% of the cases were HFRS, while 38.2%, 29.4% and 2.9% were CCHF, leptospirosis and rickettsiosis, respectively (Papa et al., 2008). DOBV-Af sequences obtained from *A. flavicollis* rodents trapped in Albania cluster with those taken from HFRS patients in northwestern Greece (Papa et al., 2006b).

4.4. Bulgaria

Since 1955, when the first HFRS cases were clinically diagnosed in Bulgaria, several severe cases have been observed (Chumakov et al., 1988; Gavrilovskaya et al., 1984). During the past decade, 36 cases have been reported, mainly across the Balkan and Rila-Pirin-Rodopa mountains in southwestern Bulgaria (Papa and Christova, 2011). Bulgarian DOBV-Af sequences cluster with viruses obtained either from *A. flavicollis* collected in Greece (near the border with Bulgaria) or from DOBV-Af patients in central and southern Europe (Papa and Christova, 2011).

4.5. Slovakia

A previous study showed that in eastern Slovakia *A. agrarius* rather than *A. flavicollis* carries DOBV (Sibold et al., 1999). In 2001, DOBV-Af was detected in *A. flavicollis* in eastern Slovakia, suggesting for the first time that the virus circulates not only in the Balkans, but also in central Europe (Sibold et al., 2001). DOBV-Aa has also been isolated from *A. agrarius* in Slovakia, clustering with previously obtained DOBV-Aa sequences. It was suggested that these strains are responsible for most DOBV-caused HFRS cases in this region (Klempa et al., 2005). In the late autumn of 2008, two Czech travelers were infected with DOBV-Af while staying in a mountain hut in northern Slovakia, close to the border with the Czech Republic (Zelena et al., 2011).

4.6. Hungary

A specimen of DOBV isolated from *A. agrarius* in Hungary differed by 12% at the nucleotide level from DOBV-Af (Scharninghausen et al., 1999). More recently, DOBV was detected in *A. flavicollis and A. agrarius* trapped in the Transdanubian region, most closely related to DOBV-Af and DOBV-Aa strains, respectively, from Slovenia (Prekmurje region) (Plyusnina et al., 2009). Recently, DOBV sequences were obtained from *A. flavicollis* trapped in the south Transdanubian region of Hungary, and the authors concluded that the prevalence of DOBV is much higher than previously anticipated (Nemeth et al., 2011).

4.7. Germany

The first evidence for the circulation of DOBV in Germany was reported in 1998, when high levels of DOBV -neutralizing antibodies were detected by focus reduction neutralization test (FRNT) in the serum of a 19-year old man from a rural region in the eastern part of the country who presented with fever and acute renal failure (Meisel et al., 1998). During the following years, based on FRNT results from HFRS patients, it was suggested that DOBV-Aa strains are responsible for most of the HFRS cases caused by DOBV in this region (i.e., most patients' sera exhibited a higher endpoint titer to DOBV-Aa than to DOBV-Af) (Klempa et al., 2004, 2005). *A. agrarius* has been identified as the reservoir host of DOBV-Aa in three federal states of Germany, where multiple natural spillover infections of *A. flavicollis* with DOBV-Aa have been reported (Schlegel et al., 2009; Schutt et al., 2001; Weidmann et al., 2005). A DOBV-Aa strain was isolated from spill-over-infected *A. flavicollis* trapped near the city of Greifsfeld; this strain was used for receptor-binding experiments and for an antiviral response study (Popugaeva et al., 2012).

4.8. Estonia

DOBV-neutralizing antibodies have been detected in an apparently healthy human population in Estonia (Lundkvist et al., 1998), and a DOBV infection was serologically confirmed using FRNT; the patient presented with increased serum urea nitrogen and creatinine levels and significant thrombocytopenia (Golovljova et al., 2000). SAAV was initially detected in *A. agrarius* trapped in 1996 on Saaremaa island in Estonia (Nemirov et al., 1999; Plyusnin et al., 1997). Soon after, it was realized that DOBV (DOBV-Af) and SAAV are distinct hantavirus serotypes (Sjolander et al., 2002). Among 25 HFRS patients in Estonia, 21 were found by FRNT to be infected by PUUV, three by SAAV and one by DOBV-Af (Golovljova et al., 2007).

4.9. Russia

An outbreak of non-fatal HFRS cases in the winter of 1991–92 in two regions of European Russia (Tula and Ryazan) was retrospectively shown to be caused by DOBV (Lundkvist et al., 1997a). DOBV was detected in two *A. agrarius* rodents trapped in January 1998 in Kurkino village in the Tula region (Plyusnin et al., 1999); the S RNA segment and N protein of this strain differed from DOBV-Af by 12% and 4%, respectively, and clustered together with other DOBV-Aa strains from Slovakia, clearly distinct from DOBV-Af and SAAV.

HFRS cases in the European administrative regions of Russia represent 97% of the total cases in the country. Although the majority are caused by PUUV, DOBV-Aa has been the causative agent of some large outbreaks (Dzagurova et al., 2009; Garanina et al., 2009; Klempa et al., 2008). During the winter of 2006–07, 661 cases were registered in a region southeast of Moscow (Central European Russia). Among 422 of the patients, 58 were infected by PUUV and 364 by DOBV, which by molecular and neutralization analyses (FRNT) was shown to belong to the DOBV-Aa lineage (Dzagurova et al., 2009; Klempa et al., 2008). The findings suggest that DOBV-Aa is a common pathogen in eastern Europe, causing large HFRS outbreaks.

As noted above, DOBV was detected in an additional rodent host, *A. ponticus,* that was captured in the Sochi district in the southern part of European Russia (Tkachenko et al., 2005). The virus was later isolated from the lung tissues of *A. ponticus* trapped in the district and named Sochi virus, or DOBV-Ap (Klempa et al., 2008). More recently, an isolate was obtained from a fatal HFRS case in the city of Sochi (Dzagurova et al., 2012).

4.10. Other European countries

DOBV is present in additional European countries. During an HFRS outbreak in Poland in 2007, serologic tests showed DOBV to be the cause of illness in 10 of 17 patients (Nowakowska et al., 2009). In the summer of 2008, DOBV was detected in a 15-year boy who was hospitalized in pediatric intensive care unit in Ostrava, in the southeastern Czech Republic (Papa et al., 2010).

The strain was genetically similar to Greek DOBV-Af strains (Fig. 2). In late autumn of the same year two more Czech people were infected by DOBV-Af during a stay in a mountain hut in northern Slovakia, close to the Czech border (Zelena et al., 2011).

In Lithuania, a seroprevalence study among cancer patients and blood donors based on ELISA and FRNT showed a dominance of DOBV infections ((Sandmann et al., 2005). In neighbouring Latvia, 6 of 14 FRNT-positive sera were specific for SAAV, 3 for SAAV or DOBV and 5 for PUUV (Lundkvist et al., 2002). SAAV was detected in *A. agrarius* trapped in 2000 on the Lolland island in Denmark, and serum samples from a patient on the island with a history of HFRS-like infection showed higher FRNT reactivity to SAAV and DOBV (equal responses) than to other hantaviruses tested (Nemirov et al., 2004).

4.11. Turkey

HFRS was first recognized in Turkey in 2009, during an outbreak near the Black Sea (Ertek and Buzgan, 2009). The fatality rate was 8% among hospitalized patients, suggesting the cases were caused by DOBV, rather than PUUV (Heyman et al., 2011b). A fatal DOBV infection was observed in the urban region of Istanbul, in the European part of Turkey (Oncul et al., 2011), and DOBV infection was diagnosed serologically in two patients in Giresun province in the eastern Black Sea region (Kaya et al., 2010).

5. Clinical course

The clinical manifestations of HFRS caused by DOBV range from a mild or moderate febrile illness to fulminant hemorrhagic fever and death. The severity depends on the causative genotype. The most severe disease is seen in DOBV-Af infections, with an 8–12% fatality rate among hospitalized patients (Avsic-Zupanc et al., 1999; Papa and Antoniadis, 2001). The illness caused by DOBV-Ap is moderate to severe, with a fatality rate that was first estimated to be 6% (Klempa et al., 2008), but proved after more extensive studies to be >10% (Dzagurova et al., 2012). DOBV-Aa infections are usually mild to moderate in severity, with a fatality rate <1% (Golovljova et al., 2007). However, 27% of 205 patients in a 2006–07 Russian outbreak exhibited severe illness, and one patient died from kidney failure, with generalized subcutaneous hemorrhage (Dzagurova et al., 2009). A severe DOBV-Aa was also reported in Northern Germany in a woman with severe acute respiratory distress syndrome/pulmonary failure complicated by acute renal insufficiency (Schutt et al., 2004). Currently, SAAV infection has only been detected serologically in cases of mild HFRS, and the virus has not been recovered from patients.

Because DOBV-Af has been known for 20 years, the disease it causes has been studied extensively. Like HFRS caused by HTNV, it typically has five phases, which are not always distinct: febrile (3–7 days), hypotensive (a few hours to 2 days), oliguric (3–7 days), diuretic and convalescent (Fig. 4). After an incubation period of 2–4 weeks, patients present with the sudden onset of fever, headache, chills, malaise, myalgia, abdominal pain, nausea, vomiting and diarrhea. In severe cases, facial flushing, conjunctivitis and a petechial rash are present. Acute thrombocytopenia is seen at the end of the febrile phase. Patients often have blurred vision (transient myopia, due to lens thickening).

The disease then progresses to the hypotensive phase, during which kidney function is decreased, leading to oliguria or even anuria. Most of the clinical characteristics are due to damage of the renal medulla, which is particularly susceptible to ischemia; microscopic hematuria may be seen. Renal biopsy shows interstitial edema, distended lumina, epithelial flattening and interstitial inflammatory cell infiltrates (Papadimitriou, 1995), while a characteristic hemorrhagic interstitial nephritis is seen in autopsy (Siamopoulos et al., 1985). Macroscopic manifestations may include epistaxis, petechiae, hematomas of the skin, bleeding from venipuncture sites and gums, conjunctival hemorrhages, hematemesis and melena; severe hemorrhagic complications can include hemothorax and hemopericardium (Avsic-Zupanc et al., 1999). Findings are sometimes compatible with disseminated intravascular coagulation (DIC). Less common symptoms include impaired consciousness and delirium. Shock develops among 28% of hospitalized patients, usually during the oliguric phase, when half of deaths occur. Hemodialysis is often required.

Severe renal insufficiency, pulmonary edema and shock are the most frequent causes of death. Petechial hemorrhages are seen in several organs in autopsy (Avsic-Zupanc et al., 1999). Pneumonic infiltrations, interstitial edema and atelectasia have been recorded in 10–35% of HFRS cases (Antoniades et al., 1987; Antoniadis et al., 1987; Kuzman et al., 1997; Siamopoulos et al., 1985, 1992). Pulmonary insufficiency resembling HPS has been seen not only in a number of DOBV-Af cases, but also in those caused by DOBV-Ap and DOBV-Aa (Dzagurova et al., 2012; Mentel et al., 1999). The appearance of polyuria is a positive prognostic marker, as renal function starts to recover. The average hospitalization time for HFRS patients is 7–12 days, but longer hospitalization of up to 2 months has been reported, associated with the need for many dialysis sessions. Recovery is usually complete, without sequelae, but can take weeks or months.

A limited number of HFRS patients have developed complete or incomplete renal tubular acidosis type I (distal), reduced urine concentrating ability, hypertension or even chronic renal dysfunction (Avsic-Zupanc et al., 1999; Elisaf et al., 1993b; Papadimitriou, 1995; Siamopoulos et al., 1992). A high prevalence (18%) of hypopituitarism after recovery from HFRS has been identified, and magnetic resonance imaging has revealed pituitary atrophy with an empty sella, suggesting that clinicians should be aware of the possible neuroendocrine consequences of HFRS, as unrecognized hypopituitarism might affect significantly the patient's physical and psychological condition (Avsic-Zupanc et al., 1994; Stojanovic et al., 2008). Micronecrosis of the parenchyma of multiple organs, including the adrenal glands, was reported in a fatal case of DOBV-Ap infection, suggesting that hormonal alterations could be caused by direct viral injury (Dzagurova et al., 2012). DOBV-Af infection can range in severity from mild to severe, and it has been estimated that some 5–10% of cases are not detected (Elisaf et al., 1993a). A scale for grading the severity of HFRS was established by Croatian scientists on the basis of clinical and laboratory parameters (Cebalo et al., 2003). According to the scale, severe cases have serum creatinine and urea levels at least 4 times higher than normal; require hemodialysis; and show hemorrhagic manifestations. Standard criteria for mild, moderate and severe cases were also established by Russian scientists (Tkachenko et al., 2005). According to those criteria, 55% of the DOBV-Ap cases in the Sochi region were severe, 39% were moderate and 6% mild. In contrast, 27% of DOBV-Aa cases in the Lipetsk region were severe, 54% moderate and 19% mild (Klempa et al., 2008).

The principal clinical laboratory findings in DOBV infections include proteinuria, increased serum urea nitrogen and creatinine (beginning on day 5–6 of illness and reaching a maximum on day 9–12), thrombocytopenia, leucocytosis, hypoproteinemia, hematuria, proteinuria and increased C-reactive protein. Most patients show a moderate (2-fold) elevation in serum transaminases levels, with alanine aminotransferase being mostly affected (Avsic-Zupanc et al., 1999; Elisaf et al., 1993c). However, serum liver enzyme levels were highly elevated (5- to 10-fold) in Czech HFRS patients, both children and adults (Papa et al., 2010; Zelena et al., 2011).

The differential diagnosis of DOBV infection include other hemorrhagic fevers (mainly Crimean-Congo hemorrhagic fever),

leptospirosis, and rickettsiosis (Papa et al., 2008). Especially in Balkans, these four diseases are endemic, and present similar epidemiological characteristics, making clinical diagnosis difficult. The presence of severe jaundice sometimes facilitates the diagnosis, as it is suggestive of leptospirosis. Dual infection with DOBV and *Leptospira* spp. has been reported (Markotic et al., 2002a).

6. Pathogenesis

The principal pathophysiologic mechanism of HFRS and HPS is a vascular leak syndrome. Pathogenesis is a multifactorial process, which appears to include immune cell-mediated injury, cytokine-mediated damage and enhanced vascular endothelial growth factor (VEGF) responses from endothelial junctions, resulting from highly specific virus–integrin interactions (Koster and Mackow, 2012). Although studies specifically of DOBV pathogenesis are limited, it is generally known that hantaviruses predominantly infect and replicate within vascular endothelial cells, increasing capillary permeability and leading to hypotension, hemoconcentration and vasodilatation (Schonrich et al., 2008). A subsequent decrease of blood platelets and damage of small vessels or capillaries is the main pathological change leading to hemorrhagic manifestations. Studies of Croatian HFRS patients have revealed activation of CD8+ T lymphocytes and increased expression of both early and late T-cell activation antigens, e.g. CD25, CD71 and HLA-DR memory cells during the acute phase of illness (Markotic et al., 1999).

6.1. Virus-cell interactions

Hantaviruses replicate in the cell cytoplasm and mature by budding into the lumen of the cis-Golgi complex, where their surface glycoproteins are trafficked, and exit cells by an aberrant secretary process (Schmaljohn and Nichol, 2007). Microvascular barrier disruption is a key determinant in hantavirus-induced endothelial permeability (reviewed by Steinberg et al., 2012). Following binding to human β_3 integrins, pathogenic hantaviruses block endothelial cell migration and enhance the permeability of endothelial cells in response to VEGF, through the internalization of vascular endothelial (VE)-cadherin, a predominant structural component of adherens junctions (Mackow and Gavrilovskaya, 2009). Phosphorylation of the VEGF (VEGFR2) receptor is increased in infected endothelial cells, leading to increased phosphorylation and internalization of VE-cadherin and microvascular leak (Gorbunova et al., 2011).

In addition to β_3 integrins, decay-accelerating factor (DAF)/CD55, an inhibitory regulator protein of the complement system, is essential for virus entry. HTNV and PUUV enter polarized epithelial cells after binding first to DAF/CD55, which is abundantly expressed on the apical surface of epithelial cells (Krautkramer and Zeier, 2008). A recent study of a strain of DOBV-Aa isolated from spill-over infected *A. flavicollis* trapped in Germany showed that the virus used the same receptors as the highly pathogenic HTNV, and that it modulated the first-line antiviral response similarly to HTNV (Popugaeva et al., 2012).

CD8+ T-cell responses also contribute to the pathogenesis of hantaviral diseases, and there appears to be a delicate balance between protection and injury (Terajima and Ennis, 2011). A patient's inherited HLA phenotype may play a role in the severity of illness. Thus, PUUV patients with an extended haplotype containing the HLA-B8, -DR3, and -DQ2 alleles developed a severe form of HFRS (Mustonen et al., 1996). In Slovenia, where both DOBV-Af and PUUV infections are seen, but with DOBV cases being more severe, it was found that DOBV-infected patients had a significantly higher frequency of HLA-B*35 (Korva et al., 2011). In the case of HLA class II genes, the biggest difference between groups of PUUV- and DOBV-infected patients was in HLA-DRB1*13, as this genotype was more frequent in PUUV infections, especially in severe cases. In contrast, HLA-B*07 may reduce the severity of PUUV HFRS cases in the Slovenian population. This suggests that the various hantaviruses are presented differently by the same HLA molecules, lead to milder or more severe form of the disease (Korva et al., 2011).

6.2. Virus and the whole organism

Because hantaviruses are not cytopathic for endothelial cells, illness appears to result from immunopathological mechanisms involving innate and adaptive immune responses (Schonrich et al., 2008). Inhibition of the antiviral effects of interferons (IFNs) in HFRS patients has been reported (Stoltz et al., 2007). Monocytes and macrophages are thought to play a significant role in the spread of virus from the primary site of infection, while mediators released by activated macrophages are important in pathogenesis (Kanerva et al., 1998). Increased levels of the proinflammatory cytokines IL-1, IL-2 and TNF-α, along with a marked increase in CD8+ lymphocytes, is seen in both HFRS and HPS (Maes et al., 2004). The DOBV N protein has been shown to interact with various importin-α subunits, inhibiting the activation of NF-κB by TNF-α and translocation of the NF-κB p65 subunit (Taylor et al., 2009).

In a recent study in Slovenia, severely ill DOBV-Af patients had significantly higher serum levels of TNF-α and IL-10 than those with milder disease, but titers were not dependent on the day of illness (Saksida et al., 2011). In contrast, preliminary results among Greek patients with DOBV-Af infection showed that the levels of most cytokines were time-dependent, e.g. the highest level of IL-10 was seen after the first week of illness (Tsergouli et al., 2010). In vitro studies showed that the Slovenian DOBV-Af and Slovakian DOBV-Aa strains induced different innate immune responses, and that the differences were determined by the viral S and L segments (Kirsanovs et al., 2010).

7. Animal models

Research on the pathogenesis of HFRS has been hampered by the lack of suitable animal models. In their rodent reservoirs, hantaviruses cause a persistent infection with no apparent pathology, potentially limiting the role of rodents as models of human disease. Humans and rodents clearly differ in immune responses and the outcome of infection. It has been suggested that the reservoir species are characterized by reduced proinflammatory and antiviral responses and elevated regulatory responses at sites of virus replication (Easterbrook and Klein, 2008). Sex steroids, glucocorticoids and genetic factors may also alter host susceptibility, contributing to viral persistence. Experimental studies of DOBV, SAAV, and PUUV infection of their natural reservoirs (*A. flavicollis*, *A. agrarious*, and *M. glareolus*, respectively) found that hantavirus RNA was present in all internal organs and blood samples tested (Korva et al., 2009). DOBV showed a considerably higher viral load in *A. flavicollis*.

Soon after HTNV was discovered, it was adapted to laboratory rats, and infection was confirmed by the detection of specific antigen in lung tissues (Lee et al., 1981). Since that time, various disease models have been developed in laboratory rodents (suckling and adult mice and Syrian hamsters) and nonhuman primates (Araki et al., 2004; Hammerbeck and Hooper, 2011; Hooper et al., 2001b; Klingstrom et al., 2005; Martinez and Padula, 2012; Safronetz et al., 2011; Schonrich et al., 2008; Seto et al., 2012; Sironen et al., 2008). In contrast to SAAV, DOBV-Af is lethal for suckling mice (Klingstrom et al., 2006). High levels of replicating virus and elevated levels of nitric oxide production are detected, with

higher ratio of IgG2a/IgG1-titers to the viral N protein in DOBV- than in SAAV-infected mice.

8. Laboratory diagnosis

Depending on the time elapsed since the onset of illness, the diagnosis of acute DOBV infection relies on molecular or serologic assays. As in most viral infections, serologic testing of paired samples and the application of more than one method are the most reliable approaches. Isolation of DOBV is a tedious and rarely successful process, which typically requires several blind cell culture passages under BSL-3 containment. It is not used for routine diagnostic purposes.

8.1. Acute phase

DOBV RNA can be detected in blood, serum, urine or biopsy and autopsy tissues in the early phase of the disease. In DOBV-Af cases, virus is detectable up to 15–18 days after onset of the illness (Papa et al., 1998; Saksida et al., 2008). Viral sequences were recovered from 5 of 10 patients with DOBV-Ap infection sampled at days 1–9 days of illness (Dzagurova et al., 2009). A nested RT–PCR protocol for hantaviruses associated with *Murinae* rodents showed that all PCR-positive Greek HFRS patients were infected with DOBV-Af (Papa et al., 1998). Additional protocols amplifying fragments of the S, M, or L segment of DOBV have been described (Klempa et al., 2006; Sibold et al., 2001). Similar techniques applied to tissue samples from small mammals have enabled the association of hantaviruses with specific hosts (Vaheri et al., 2008).

Various real-time RT–PCR protocols with high levels of sensitivity, specificity and reproducibility have been reported (Aitichou et al., 2005; Jakab et al., 2007; Korva et al., 2009; Kramski et al., 2007; Saksida et al., 2008; Weidmann et al., 2005). On average, patients with severe disease had higher viral load than those with milder symptoms (6.15 vs. 4.67 log (10) copies/mL; $p = 0.053$) (Saksida et al., 2008). Technological advances now allow researchers to sequence entire viral genomes, making it possible to identify recombination or reassortment events (Kirsanovs et al., 2010).

IgM and low-avidity IgG antibodies to DOBV can be detected through immunofluorescence assays (IFA) or enzyme immunoassays (EIA). Low-avidity IgG antibodies and granular fluorescence in IFA, caused by anti-N protein antibodies in acute-phase serum, can be used to differentiate past from current infection (Vaheri et al., 2008). Because the two viruses cross-react antigenically, ELISAs for the detection of anti-HTNV IgM and IgG are often used to detect antibodies against DOBV. However, for more specific results it is advisable to use homologous antigen, which gives higher endpoint titers than heterologous antigen (Meisel et al., 2006). Anti-DOBV IgM is best detected by IgM-capture enzyme assays using native virus antigen or recombinant N protein (Araki et al., 2001; Elgh et al., 1997; Heyman et al., 2009; Kallio-Kokko et al., 2000; Sjolander and Lundkvist, 1999). It should be noted that IgM antibodies can be detected up to 2 years after illness, and IgG antibodies may persist life-long. The time-course of IgA expression resembles that of IgM. A rapid immunochromatographic test for the detection of anti-DOBV IgM, or in combination to detect all Eurasian pathogenic hantaviruses, was developed in Finland (Hujakka et al., 2003). Immunohistological techniques are also used to detect DOBV in tissue samples (Heyman et al., 2009).

8.2. Epidemiological monitoring

Epidemiologic studies rely principally on IFA and ELISA to detect high-avidity IgG antibodies to DOBV. Because DOBV, SAAV and HTNV cross-react antigenically, neutralization testing is the method of choice for hantavirus serotyping. The best results are obtained using late-convalescent serum samples, e.g. those taken a month after the onset of illness. Using FRNT, it was found that the majority of serum samples from Estonian patients had at least 4-fold higher titers to SAAV than to DOBV, while sera from the Balkan region reacted more strongly with DOBV, indicating that DOBV and SAAV define unique serotypes (Sjolander et al., 2002). During the 2006–07 outbreak in Russia, it was demonstrated by FRNT that DOBV-Aa was the responsible DOBV genotype, suggesting that it is responsible for large HFRS outbreaks in eastern Europe (Dzagurova et al., 2009; Klempa et al., 2008). DOBV-Ap isolates were neutralized by serum from DOBV-Ap infected patients (Dzagurova et al., 2009).

9. Treatment

There are currently no FDA-approved drugs for the prevention or treatment of HFRS (Maes et al., 2004). Treatment of DOBV patients is symptomatic, and consists of management of electrolytes, hydration, blood pressure, oxygenation support and dialysis, if required (Sargianou et al., 2012). Vasoactive agents should be used in cases of shock, after correcting the volume deficit. Blood transfusion and H_2-receptor antagonists are indicated in cases of gastrointestinal bleeding.

9.1. Passive immunization

Studies in mice have demonstrated that monoclonal antibodies (mab) could be effective candidates for the treatment of HFRS caused by HTNV, and a murine anti-HTNV MAb was shown to be well tolerated and slowly cleared (Xu et al., 2009). The development of effective therapeutic antibodies, e.g. neutralizing mabs against DOBV, might be a life-saving treatment for HFRS in Europe.

9.2. Antiviral drugs

To my knowledge, there are no reports of the specific treatment of DOBV infection. The following are a few data concerning the use of ribavirin in other hantaviral diseases and some findings from recent studies of various compounds. Ribavirin, a nucleoside analog with broad-spectrum antiviral activity, was used in patients with HFRS caused by HTNV, and proved to be effective when initiated during the first 4 days of illness (Huggins et al., 1991; Rusnak et al., 2009). Ribavirin's activity against HTNV correlates with the production of ribavirin triphosphate and an increase in viral mutation frequency (Severson et al., 2003; Sun et al., 2007).

Because the use of ribavirin often results in hemolytic anemia, teratogenic effects and reproductive toxicity, attempts have been made to find compounds with increased selectivity and activity. Studies of 1-β-D-ribofuranosyl-3-ethyl-[1,2,4]triazole (ETAR) have shown that it has antiviral activity against HTNV and Andes virus, by a different mechanism of action than ribavirin, as it acts by inhibition of inosine monophosphate dehydrogenase with reduction of GTP pools, combined with complementary activity possibly affecting the L protein (Chung et al., 2008). Postexposure prophylaxis with ribavirin was given to two laboratory workers who were exposed to New World hantaviruses (Rusnak, 2011). However, the decision to administer it after a laboratory exposure must consider the risk of developing disease, the morbidity and mortality of the disease, and the potential benefit from ribavirin, versus its adverse effects.

Other compounds with known anti-hantavirus activity include cyclic peptides which bind $a_v\beta_3$ integrin as a virus receptor, cyclic peptides presented on nanoparticles and peptidomimetic compounds similar to a cyclic peptide, which were found potent in

the nanomolar range (Hall et al., 2010). In addition, compounds that target the interactions of VEGF, VE-cadherin and αvβ3 integrins could be a potential approach for therapeutic interventions. Specifically, an antibody that binds VEGFR2 is able to block the internalization of VE-cadherin in infected cells (Shrivastava-Ranjan et al., 2010). Because hantaviruses increase endothelial permeability without causing endothelial cell lysis, barrier-stabilizing molecules such as sphingosine-1-phosphate and angiopoietin-1, which inhibit microvascular leak, are considered to have therapeutic potential (Gavrilovskaya et al., 2008). A recently developed high-throughput screening assay for small-molecule inhibitors of virus-cell interactions in a BSL-2 environment may prove helpful (Buranda et al., 2010).

10. Prevention

10.1. Rodent control measures

Because DOBV is carried by rodents, preventing exposure is the most important task. People are recommended to minimize contact with rodents during outdoor activities, and to maintain a clean house, keeping food and water in protected areas, so as not to attract rodents.

10.2. Vaccines

Despite efforts to develop vaccines for hantavirus infections, there is currently no WHO-approved vaccine with widespread acceptance. Mouse-brain and cell-culture-derived inactivated vaccines (HTNV, SEOV, or SEOV/HTNV) have been used for many years in Korea and China [reviewed in (Schmaljohn, 2009)]. The main disadvantage of mouse-brain preparations is concern about the possibility of autoimmune encephalitis, due to the presence of even small quantities of myelin basic protein. The following are a few approaches for the design of new vaccines, especially those targeting DOBV. The immunogenicity and protective efficacy of a recombinant DOBV N protein, given to C57BL/6 mice with alum or Freund's adjuvant, indicated that the strong Th2-type immune response did not result in protection (Klingstrom et al., 2004). In Russia, a cell-culture-derived, inactivated bivalent PUUV/DOBV-Aa vaccine has passed pre-clinical trials (Kruger et al., 2011).

Molecular approaches have also been tried, including chimeric viruses, virus-like particles (VLPs), recombinant proteins, and DNA vaccines (Kruger et al., 2011). When BALB/c and C57BL/6 mice were immunized with a DOBV recombinant N protein expressed in *Saccharomyces cerevisiae*, they produced high titers of N-specific antibodies with a mixed Th1/Th2 immune response, that was cross-reactive with the N proteins of PUUV and HTNV (Geldmacher et al., 2004). Also, chimeric hepatitis B virus core particles carrying the amino-terminal 120 amino acids of the N protein of DOBV, HTNV, or PUUV are highly immunogenic in mice, even without adjuvant (Geldmacher et al., 2005).

The first immunogenicity data for hantavirus DNA vaccines in nonhuman primates were reported in 2001, and it was suggested that a vaccine based on the M genome segment could protect humans against the most severe forms of HFRS, as cross-protection among HTNV, SEOV, and DOBV (but not PUUV) was observed (Hooper et al., 2001a). A rapid, reliable and sensitive flow-cytometric assay was recently developed to measure the potency and stability of DNA vaccines, for delivery either by particle-mediated epidermal delivery (PMED) or electroporation (Badger et al., 2011). Because PUUV also circulates in many DOBV-endemic areas, a comprehensive vaccine for the prevention of HFRS in Europe will require two components (Schmaljohn, 2009).

11. Concluding remarks

A great deal of knowledge of the epidemiology, genetic variability and pathogenicity of DOBV has been gained in recent years. Further studies of viral evolution and its relationship to rodent migration routes will be enhanced by the collection of additional viral sequences from endemic areas. Future environmental and demographic changes may affect the geographic distribution and dynamics of rodent populations, which in turn may influence the epidemiology of hantaviruses, including DOBV. Additional DOBV strains, genotypes or/and reassortants will probably be detected in the near future, associated with *Apodemus* or other rodent or small mammal hosts. Novel diagnostic tools will be needed, capable of detecting and differentiating known and novel DOBV variants, and further modifications to the taxonomy and terminology of hantaviruses may be necessary.

The relative pathogenicity of the three DOBV genotypes and SAAV remains to be elucidated. Systematic epidemiologic and genetic studies will be needed to conclusively address the hypothesis that *A. agrarius*-associated strains are less pathogenic than DOBV-Af and DOBV-Ap. Characterization of pathogenic determinants could be helpful in designing vaccines and developing new therapeutic interventions. Further insights into the pathogenesis of DOBV infection will be gained when a suitable animal model becomes available. Animal studies will also show whether previous DOBV infection protects against other hantaviruses, and vice versa. Although great progress has been made in elucidating the pathophysiology of HFRS, much remains to be learned about the mechanisms of DOBV infection, including identification of pathogen receptors that sense the virus, determination of the components that interfere with viral entry and replication, and characterization of mechanisms responsible for the nature and intensity of the so-called "cytokine storm". Such knowledge will form the basis for drug design and vaccine development, which may be directed to DOBV, to the broader spectrum of hantaviruses, or even to other viruses causing hemorrhagic fever.

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