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Central European Dobrava Hantavirus Isolate from a Striped Field Mouse (*Apodemus agrarius*)

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Dobrava virus (DOBV) is a hantavirus that causes hemorrhagic fever with renal syndrome (HFRS) in Europe. It is hosted by at least two rodent species, *Apodemus flavicollis* and *A. agrarius*. According to their natural hosts they form the distinct genetic lineages DOBV-Af and DOBV-Aa, respectively. We have now established a DOBV isolate named Slovakia (SK/Aa) from an *A. agrarius* animal captured in Slovakia. The complete S and M and partial L segment nucleotide sequences of the new isolate were determined. Phylogenetic analyses showed that the SK/Aa isolate clustered together with the other DOBV-Aa sequences amplified from *A. agrarius* before and can be taken as the representative of this genetic lineage. SK/Aa, in comparison with a DOBV-Af isolate, was used for serotyping neutralizing antibodies of HFRS patients in Central Europe. Most patients' sera exhibited a higher endpoint titer when probed with our new isolate, suggesting that DOBV-Aa strains are responsible for most of the DOBV-caused HFRS cases in this region.

Hantaviruses represent a unique genus *Hantavirus* within the *Bunyaviridae* family. They are "emerging viruses" that cause two human zoonoses: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome. The virus genome consists of three segments of negative-stranded RNA; the large (L) segment encodes the viral RNA-dependent RNA polymerase, the medium (M) segment the glycoprotein precursor (GPC), and the small (S) segment of the nucleocapsid (N) protein. In contrast to other genera of the *Bunyaviridae* family, hantaviruses are not transmitted by arthropods. Instead, they are spread by aerosolized rodent excreta and produce a chronic infection with no apparent harm in their natural hosts, rodents of the family *Muridae* (18, 28, 45, 46). In Europe, at least two hantaviruses, Dobrava virus (DOBV) and Puumala virus (PUUV), are known to be pathogenic for humans (for reviews, see references 18 and 37). In addition, a single case of renal syndrome with pulmonary involvement has been very recently associated with infection by Tula virus (14). Currently, DOBV is intensively studied because of its unique properties; different virus lineages exist in different regions of Europe, and these lineages are harbored by different host reservoirs and probably display different levels of virulence in humans.

A viable DOBV strain was isolated from lungs of a yellow-necked mouse (*Apodemus flavicollis*) captured in a natural focus of HFRS in Dobrava village, Slovenia, South-East Europe (5). This virus isolate represents the DOBV prototype strain (named Slovenia or Slo/Af here) from *A. flavicollis*. Moreover, a second DOBV cell culture isolate (Ano-Poroia or AP/Af) has been established from an *A. flavicollis* mouse trapped in Greece (32, 34). Molecular phylogenetic analyses of nucleotide sequences from these cell culture-derived virus isolates, as well as from further South-East European *A. flavicollis*-associated strains, demonstrated their close mutual relationship (2, 4, 34, 35). These strains form a unique *A. flavicollis*-associated DOBV clade, DOBV-Af (15). In addition, DOBV-specific neutralizing antibodies have been detected in HFRS patients from this geographic region (3, 22). A direct proof for DOBV-Af strains as an etiologic agent of HFRS was established by amplification of DOBV-Af nucleotide sequences from HFRS patients in Greece, Albania, and Croatia (1, 25, 33). HFRS cases caused by DOBV infection in South-East Europe were found to be clinically moderate or even severe with a case fatality index of up to 12% (1, 3, 22, 33).

In Central Europe, DOBV RNA has been predominantly detected in a different mouse species, the striped field mouse (*A. agrarius*). Phylogenetic analysis of these viral sequences from *A. agrarius* led to the definition of a second genetic DOBV clade, DOBV-Aa (15, 50, 51). Dozens of HFRS patients with DOBV infection have been diagnosed by typing their neutralizing antibodies, and the clinical parameters of these patients have been well characterized; in contrast to the situation in South-East Europe, HFRS cases caused by DOBV infection in Central Europe appear mostly mild or moderate, and no fatalities have been observed yet. On the other side, pulmonary involvement—sometimes even with life-threatening severity—was observed in a number of cases (26, 27, 48, 49, 51). Direct evidence for a causal role of DOBV-Aa in Central European HFRS cases was provided by amplification of a DOBV-Aa nucleotide sequence from an HFRS patient in Germany (16). However, a virus isolate representing the Central European DOBV-Aa lineage had not yet been described.

In retrospectively studied sera from patients of 1991 and 1992 HFRS outbreaks in the Tula and Ryazan regions in Russia, DOBV-specific antibodies could be detected. There are no data of the clinical characterization of the affected patients

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available, but no fatal cases have been reported (20). The only available viral nucleotide S segment sequences from *A. agrarius* mice trapped in Russia (38) are closely related to the Central European DOBV-Aa sequences (15, 37).

In Estonia, North-East Europe, the situation seems to be more complex. DOBV nucleotide sequences have been detected in *A. agrarius* animals trapped on the Estonian islands Saaremaa and Vormsi (40). Subsequently, a virus isolate (strain Saaremaa/160V, Saa/160V for short) was established in Vero E6 cells (31) and was later proposed as the prototype even of a unique species, Saaremaa (36, 39). Whereas the prevalence of sera reacting with Slo/Af or Saa/160V in focus reduction neutralization assay was found to be 3.4% in the Estonian (10, 24) and ca. 2.7% in the Latvian (23) population, only one DOBV-infected HFRS patient from this Baltic region has been described (11), and no molecular genetic description of the viral genotype(s) responsible for human cases has been proffered. Very recently, hantavirus nucleotide sequences closely related to Saa/160V have been amplified from an *A. agrarius* mouse trapped on the Lolland island in Denmark (29). Phylogenetic analysis of Saa/160V strain suggested an involvement in genetic reassortment processes during their evolution; in particular, its S-segment sequence seems to be related to the DOBV-Af lineage (15, 37), whereas its M segment resembles sequences from DOBV-Aa (15). By comparative use of human serum samples from South-East Europe and Estonia in neutralization tests (neutralizing antibodies are directed against the M-segment-encoded envelope glycoproteins), Brus Sjolander et al. (6) have shown that many but not all sera exhibited an at least fourfold titer difference with favored neutralization of the local virus (Slo/Af and Saa/160V, respectively).

The claimed different severity of DOBV infections in South-East versus Central Europe could potentially be explained by genetic differences between viruses of the DOBV-Af and DOBV-Aa lineages, respectively, and/or by differences in the human genetic background, e.g., haplotypes, determining the susceptibility to virus infections.

For future studies on the biology of the virus and the pathogenesis of the disease, as well as for the development of antivirals and vaccines, the availability of a DOBV-Aa prototype virus is an essential precondition. Here we describe the isolation and first characterization of a virus from an *A. agrarius* animal originating from Central Europe, named Dobrava-Slovakia (SK/Aa for short, where SK stands for Slovakia and Aa indicates for better clarity the natural host). Our data show that this virus isolate can be taken as the first viable representative of the DOBV-Aa lineage.

MATERIALS AND METHODS

Trapping of rodents. In 2001, rodents were trapped alive at three localities in the Eastern Slovakia by using Swedish bridge metal traps as previously described (51). The trapping sites were selected on the basis of their proximity to reported human HFRS cases. Blood for serological screening was taken from *Sinus orbitalis* of anesthetized rodents. Animals were sacrificed and dissected, and lung and liver tissue samples were collected and immediately stored at −70°C.

Screening of rodent samples. Sera collected from rodents were screened for the presence of hantavirus-specific immunoglobulin G (IgG) antibodies by enzyme-linked immunosorbent assay as described by Sibold et al. (50, 51) using recombinant PUUV and DOBV nucleocapsid proteins generated in the yeast *Saccharomyces cerevisiae* (41). Subsequently, lung tissues of seropositive mice were tested by using reverse transcription-PCR (RT-PCR). The RNA from homogenized lung tissues was extracted by using the FastPrep Instrument and corresponding kit (Bio 101 Systems, Carlsbad, CA) according to the manufacturer's instructions. Hantavirus RNA was detected by a nested RT-PCR with DOBV-specific primers as described previously (51).

Virus isolation. DOBV RT-PCR-positive lung and liver samples from two naturally infected, seropositive *A. agrarius* trapped in Rozhanovce, Eastern Slovakia, were used for virus isolation attempts. The samples were processed as 10% tissue suspensions in Dulbecco medium supplemented with 0.2% bovine serum albumin (BSA). The tissues were triturated in a closed mechanical blender FastPrep Instrument (Bio 101 Systems, Carlsbad, CA). Triturated tissues were briefly centrifuged at low speed to remove larger tissue fragments and inoculated (0.4 ml/flask) onto cultures of confluent Vero E6 cells in 25-cm^2 flasks (three for each sample). Virus was then allowed to adsorb at 37°C. The cell culture medium (minimal essential medium plus 10% fetal calf serum, L-glutamate, penicillin, and streptomycin) was changed for the first time after 90 min and then weekly. At 2- to 3-week intervals, cells, detached by trypsin treatment, were passaged into new culture flasks with the addition of the same amount of fresh uninfected cells according to a recently described protocol (31). With the material, several slides were prepared and examined for characteristic hantavirus antigen expression following immunofluorescence assay (IFA) techniques.

Since hantaviruses do not naturally form plaques in Vero E6 cell culture, allowing plaque purification of the virus, an alternative approach based on limiting dilution was used to obtain a homogenous virus stock. Wells of two six-well plates were inoculated, with virus was diluted to 0.1 to 0.5 focus-forming units (FFU)/well. After 10 days of incubation, supernatant samples were collected, and the cells were examined by chemiluminescent focus assay. The supernatant from one of the few positive wells was then used to infect fresh Vero E6 cells and produce the prototype virus stock for further analyses. Assuming that the positive wells were infected with a single virus particle, we obtained a homogenous virus stock equivalent to a plaque-purified virus.

The experiments were performed under biosafety level 3 containment conditions in the Institute of Virology, Charité School of Medicine.

Immunofluorescence assay. To prove the success of virus isolation and later to analyze the antigenic properties of the new isolate, a standard IFA on 12-well spot slides with acetone-fixed cells was carried out as described previously (9). During the isolation attempts, an anti-DOBV human convalescent-phase serum was used to detect the virus. For the antigenic characterization of the new isolate, a panel of eight monoclonal antibodies (MAbs) directed against N protein of different hantaviruses was used. The reactivity of the MAbs E5/G6, Eco2, C16D11, C24B4, and B5D9 (55, 56) and 2E2 (a gift from J. Groen) directed against Hantaan virus (HTNV), R31 against Seoul virus (SEOV; Progen Biotechnik, GmbH Heidelberg, Germany), and 5C2/E10 against Andes virus (ANDV; Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany) was analyzed in parallel by using IFA slides with Vero E6 cells infected with DOBV strains SK/Aa and Slo/Af. After 10 days of incubation the infected cells were mixed with the uninfected cells at a ratio of 1:2. A positive reaction was stated to be specific if at least one-third of the cells showed the fluorescence signal. Fluorescein isothiocyanate-conjugated rabbit anti-human and anti-mouse antibodies were used accordingly.

Virus titration. The viral stock, prepared from a cell culture supernatant of infected Vero E6 cells, was titrated by using the chemiluminescence focus assay as described by Heider et al. (13). Briefly, 10-fold serial dilutions of viral stock were inoculated into six-well plates with nearly confluent monolayer of Vero E6 cells. After an adsorption period for 1 h at 37°C, the cells were overlaid with a mixture of 1% agarose and Eagle basal medium. Plates were then incubated for 10 days. Virus-infected cells were detected with anti-DOBV convalescent human serum, followed by peroxidase-labeled goat anti-human IgG and chemiluminescence substrate Super Signal West Dura (Pierce, Rockford, Ill.).

c-FRNT. The chemiluminescence focus reduction neutralization test (c-FRNT) was performed basically according to the protocol described above for the virus titration (13). Human convalescent-phase sera were first diluted serially, mixed with an equal volume containing 30 to 80 FFU of the respective virus, and incubated for 1 h at 37°C prior inoculating the cells. After 10 days of incubation, DOBV-N-specific rabbit antiserum and goat anti-rabbit IgG were used to detect the viral antigen in infected cells. An at least 80% reduction in the number of foci was considered to be the criterion for virus neutralization.

RT-PCR, cloning, and sequencing. Hantaviral RNA was extracted from cell culture supernatant by using the QIAamp Viral RNA minikit (Qiagen, Hilden, Germany). Standard QIAamp viral RNA minispin protocol was performed. Amplification of the entire S segment was undertaken by use of the Robust RT-PCR System, following the application protocol of the manufacturer (Finnzymes, Espoo, Finland). The single genus-specific primer RT-DOB (5′-ttc tgcag TAG TAG TAK RCT CCC TAA ARA G) (heterologous sequences for cloning procedure are in lowercase) was used. For sequencing of the complete M

segment, an RT-PCR amplification of six overlapping M-segment fragments was carried out. The primer sequences were designed from published DOBV entire M-segment sequences and are available upon request. To obtain the partial L-segment sequence, the single primer pair DOBL89F (5′-TCA YTG ACA GCA GTR GAR TG) and DOBL669R (5′-AAC ATK GCY TCY ARA GCA GC) amplifying 580-nucleotide (nt) long L-segment fragment (541 nt when the primer sequences were excluded) was designed from published DOBV entire L-segment sequences.

The amplified products were cloned into pCR2.1-TOPO vector (TA Cloning Kit; Invitrogen, Leek, The Netherlands). At least three recombinant plasmids were sequenced in both directions, and the consensus sequence from the obtained sequences was determined. Dideoxy sequencing was performed on a LICOR sequencer by using the Autoread Kit (Pharmacia-Biotech, Freiburg, Germany) as described by the manufacturer.

Sequence comparison, phylogenetic, and recombination analyses. The obtained overlapping nucleic acid sequences were combined for analysis and edited with the aid of the SEQMAN program from the Lasergene software package (DNASTAR, Madison, Wis.). The sequence data were further analyzed by using the BioEdit software package (12). Sequences were aligned by using CLUSTAL W (53) with default parameters. When aligning the coding sequences, the sequences were first aligned on amino acid level and then reverse translated to nucleotide sequences by using DAMBE software (54). The reliability of the alignment was checked by using DotPlot analysis implemented in BioEdit software package. The alignment was tested for phylogenetic information by Likelihood Mapping analysis (52).

To reconstruct maximum-likelihood (ML) phylogenetic trees, we applied quartet puzzling by using the TREE-PUZZLE package (47, 52). As an evolutionary model for the reconstructions, we used the Tamura-Nei model, missing parameters were reconstructed from the datasets. The values at the tree branches are the resulting PUZZLE support values. Resulting evolutionary trees were then visualized by using TreeView v.1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

For recombination analysis, similarity plotting and bootscanning (42) were performed with Stuart Ray's SimPlot 3.2 (19). A window size of 200 nt, a step size of 20 nt, and 500 bootstrap samples per window were used.

Nucleotide sequence accession numbers. The sequences obtained in the present study have been deposited in the GenBank database under the following accession numbers: Dobrava-Slovakia (SK/Aa) complete S-segment sequence, AY961615; SK/Aa complete M segment sequence, AY961616; SK/Aa partial L-segment sequence, AY961617; and DOBV/Esl/34Aa/01 complete S-segment sequence, AY961618. Previously published partial S-segment sequences (16) of the DOBV strains Esl/29Aa/01 (AY533118) and Esl/81Aa/01 (AY533120) have been updated to the complete S-segment sequences.

For the comparisons, S (and, if available, M and L) sequence data were obtained from the GenBank sequence database: DOBV/East Slovakia/856Aa/97 (accession number AJ269549), DOBV/East Slovakia/862Aa/97 (AJ269550 and AY168578), DOBV/Kurkino/44Aa/98 (AJ131672), DOBV/Kurkino/53Aa/98 (AJ131673), Saaremaa/160V (AJ009773, AJ009774, and AJ410618), DOBV/Saaremaa/90Aa/97 (AJ009775), DOBV/Slovenia (L41916, L33685, and AJ009779), DOBV/East Slovakia/400Af/98 (AY168576 and AY168577), DOBV/Ano-Poroia (AJ410615, AJ410616, and AJ410617), Saaremaa/Loll-1403 (AJ616854), HTNV strain 76-118 (M14626, M14627, and NC_005222), HTNV strain LR1 (AF288294 and AF288293), HTNV strain AH09 (AF285264 and AF285265), SEOV strain SR11 (M34881 and M34882), SEOV strain L99 (AF288299, AF035833, and AF288297), SEOV strain Gou3 (AB027522 and AB027521), Thailand virus strain Thai749 (L08756), PUUV strain Vranica/Hällnäs (U14137 and U14136), PUUV strain Sotkamo (NC_005225), Tula virus strain Moravia/5302v/95 (Z69991, Z69993, and NC_005226), Sin Nombre virus strain NM H10 (L25784, L25783, and NC_005217), El Moro Canyon hantavirus strain RM-97 (U11427 and U26828), and ANDV strain Chile-9717869 (NC_003468).

RESULTS

Rodent screening. As a part of a long-term epizootiologic survey, small rodents were trapped in the Rozhanovce, Sebastovce, and Botany localities, Eastern Slovakia, Central Europe, during three consecutive nights in September and October of 2001. A total of 123 rodents was trapped, including *A. agrarius* ($n = 82$), *A. flavicollis* ($n = 25$), and *Clethrionomys glareolus* ($n = 16$). Antibodies reactive to DOBV antigen were found only in *A. agrarius* rodents; 14 of 82 (17.1%) animals were found to be serologically and RT-PCR positive. Tissue samples from two of them originating from the Rozhanovce locality were selected for isolation attempts: DOBV/East Slovakia/34Aa/01 and DOBV/East Slovakia/35Aa/01 (abbreviated Esl/34Aa and Esl/35Aa, respectively). In addition, strains DOBV/East Slovakia/29Aa/01 (Esl/29Aa) and DOBV/East Slovakia/81Aa/01 (Esl/81Aa) detected in the neighboring localities Sebastovce and Botany, respectively, were used for sequencing of complete S-segment nucleotide sequences.

Isolation of the DOBV-SK/Aa. After the third passage at day 47 postinfection, in the flasks of both samples (cells inoculated with homogenized liver tissue from Esl/34Aa or lung tissue of Esl/35Aa), some hantavirus antigen-positive cells were found by IFA with a DOBV-specific human convalescent-phase serum. After the fourth passage (day 59), most cells were found to be antigen positive. One isolate, originating from the field strain DOBV/East Slovakia/34Aa/2001, was then further passaged and designated Dobrava-Slovakia (abbreviated SK/Aa). Whereas the titers of cell culture supernatants of third- and fourth-passage supernatants were extremely low ($<10^3$ FFU/ml), the fifth-passage supernatant had a markedly higher titer (4.5×10^5 FFU/ml). Cell culture supernatant harvested from fifth passage was used to obtain a genetically homogenous virus stock by "limiting dilution" and subsequent virus growth (see Materials and Methods). The titer of this SK/Aa stock was determined to be 5.0×10^5 FFU/ml.

Sequence analysis of DOBV-SK/Aa genomic segments. The complete S-segment sequence of the new isolate DOBV-SK/Aa was determined to be 1,697 nt in length containing a single open reading frame (ORF; nt 36 to 1,325) for the viral N protein which encodes a putative protein of 429 amino acids (aa). In addition, the complete S-segment nucleotide sequences of strains Esl/29Aa and Esl/81Aa were obtained from the lung tissues of *A. agrarius* animals and were determined to have the same length and coding structure.

As expected, the sequences of Esl/29Aa and Esl/81Aa were found to be very similar to the new isolate (98.9 to 99.0% nt and 99.7% amino acid identities). When compared to other DOBV sequences, previously determined DOBV-Aa sequences from East Slovakia (Esl/856Aa and Esl/862Aa) showed the highest similarity (93.3% nt and 99.3% amino acid identities). However, the S segment of the new isolate was found to be 7 nt shorter than the sequences of Esl/856Aa and Esl/862Aa strains due to an 8-nt deletion and a 1-nt insertion located in the 3′ noncoding region. The most dissimilar DOBV strain on the nucleotide level, Esl/400Af (84.4% nt identity), is from the same geographical region of Slovakia but belongs to the DOBV-Af lineage (15). On the deduced N protein amino acid sequence level, the Saa/160V isolate showed the highest diversity to SK/Aa, even higher than the virus isolates of DOBV-Af lineage, Slo/Af and AP/Af (Table 1). Cysteine residues were conserved with all Dobrava isolates and the other hantaviruses analyzed here (Table 1).

We wanted to determine whether the nucleotide sequence of the S segment underwent changes during the virus isolation procedure in cell culture. For this purpose, we compared the sequences of the original hantaviral RNA in the rodent tissue (Esl/34Aa) and of the cell culture-adapted isolate SK/Aa. In the complete S segment we found a single nucleotide exchange

TABLE 1. Complete S- and M-segment nucleotide and amino acid sequence identities of DOBV-SK/Aa with other DOBV, HTNV, and SEOV isolates

Segment and isolate	% Identity[a] with virus strain:					
	SK/Aa	Saa/160V	Slo/Af	AP/Af	$HTNV_{76-118}$	$SEOV_{SR11}$
S segment/N protein						
SK/Aa		86.2	85.7	85.2	70.8	68.1
Saa/160V	96.9		88.3	87.3	69.7	66.2
Slo/Af	97.6	97.4		96.6	70.7	67.4
AP/Af	98.1	97.4	99.5		70.5	67.6
$HTNV_{76-118}$	83.6	82.5	82.5	82.9		67.5
$SEOV_{SR11}$	81.1	79.9	79.9	80.4	82.2	
M segment/GPC						
SK/Aa		86.8	81.5	81.5	69.0	69.6
Saa/160V	95.7		81.3	81.5	69.6	69.5
Slo/Af	93.4	94.1		93.4	69.1	69.4
AP/Af	93.5	94.1	98.5		69.7	69.7
$HTNV_{76-118}$	77.0	76.9	77.1	77.3		71.3
$SEOV_{SR11}$	76.7	76.4	77.2	77.1	76.8	

[a] The percent identities for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.

of G (rodent tissue) to A (isolate) at position 162, resulting in an amino acid exchange Ala (GCA) to Thr (ACA) at amino acid position 43 of viral N protein.

The total M-segment nucleotide sequence of DOBV-SK/Aa was found to be 3,643 nt in length. It contains a single ORF (nt 41 to 3445) encoding the putative GPC of 1,135 aa. Table 1 shows the degree of M-segment nucleotide and deduced amino acid sequence identity between the DOBV isolates. The DOBV-Af isolate Slo/Af was found to be the most dissimilar (only 81.5% nt and 93.4% amino acid identities), which could explain the distinct serological behavior of SK/Aa and Slo/Af in neutralization assays (see below). The GPC amino acid sequence of the of DOBV-SK/Aa showed the typical sequence motifs of hantavirus glycoproteins (44).

In addition, a partial L-segment sequence of 541 nt (nucleotide positions 109 to 649 according to the coordinates in the L segment of DOBV AP/Af [GenBank accession number AJ410617]) from the SK/Aa isolate was determined. The comparison with the only available complete DOBV L-segment sequences showed relatively low nucleotide sequence identities of 86.3 and 85.0% to the Saa/160V and AP/Af strains, respectively. In contrast, the percent identity values of the corresponding amino acids (180 aa [amino acid positions 25 to 204]) were very high, reaching 97.7% for both Saa/160V and AP/Af strains. This shows that most of the nucleotide exchanges represented silent mutations.

Phylogenetic analysis. To avoid the phylogenetic analysis being disturbed by conflicting phylogenetic signals due to potential recombination events, both SK/Aa complete S- and M-segment sequences were first screened by preparing similarity plots and bootscanning. No significant recombination signals, which could disturb construction of the phylogenetic trees, were found in these two sequences (data not shown). Significant conflicting phylogenetic signals suggesting putative recombination events have been detected by bootscanning analysis only in the recently published S-segment sequence of Loll-1403 strain from Denmark (AJ616854). Therefore, in the subsequent phylogenetic analysis, datasets with or without this sequence were used to construct the phylogenetic trees.

Complete coding sequences, allowing unambiguous aligning on amino acid level, were used to construct maximum-likelihood (ML) trees assuming the Tamura-Nei evolutionary model. In the S-segment ML phylogenetic tree (Fig. 1a), the new isolate formed a well-supported monophyletic group with all DOBV-Aa strains except Saaremaa strains, which clustered together with the DOBV-Af strains (see below). As expected, all other Slovakian strains were the closest relatives of SK/Aa. The new strains SK/Aa, Esl/29Aa, and Esl/81Aa formed a sister group to the previously characterized strains Esl/856Aa and Esl/862Aa. *A. flavicollis*-derived strains from Slovakia (Esl/400Af), Greece (AP/Af), and Slovenia (Slo/Af) formed the second cluster sharing the common ancestor with the Saaremaa strains. When the putative recombinant sequence of Loll-1403 was included in the analysis, Loll-1403 clustered with the two Saaremaa strains. Although the overall tree topology remained unchanged, the inclusion of the Loll-1403 sequence changed the statistical support (PUZZLE value of 56) for the clustering of Saaremaa and Loll-1403 strains with the DOBV-Af cluster (data not shown).

In the M-segment ML tree (Fig. 1b), SK/Aa again clustered with the Esl/862Aa strain. However, in contrast to the S-segment analysis, SK/Aa shared a common ancestor also with the Saa/160V strain from Estonia. The strains Esl/400Af, AP/Af, and Slo/Af formed again a strongly supported monophyletic group representing the DOBV-Af lineage.

Phylogenetic analysis of partial L-segment sequences (541 nt) showed results consistent with the S-segment analysis. AP/Af strain clustered with the Saa/160V strain while SK/Aa formed an outgroup to this well-supported cluster (data not shown). When using only 374-nt sequences (nucleotide positions 157 to 530), the available partial L-segment sequence of the original DOBV isolate, Slo/Af, could be included in the alignment. Also in this case, SK/Aa was the most ancestral sequence and Saa/160V formed a well-supported monophyletic group with the AP/Af and Slo/Af strains (Fig. 1c).

Antigenic characterization of the DOBV-SK/Aa isolate. First, antigenic characterization was performed by IFA with a panel of eight MAbs directed against HTNV, SEOV, and

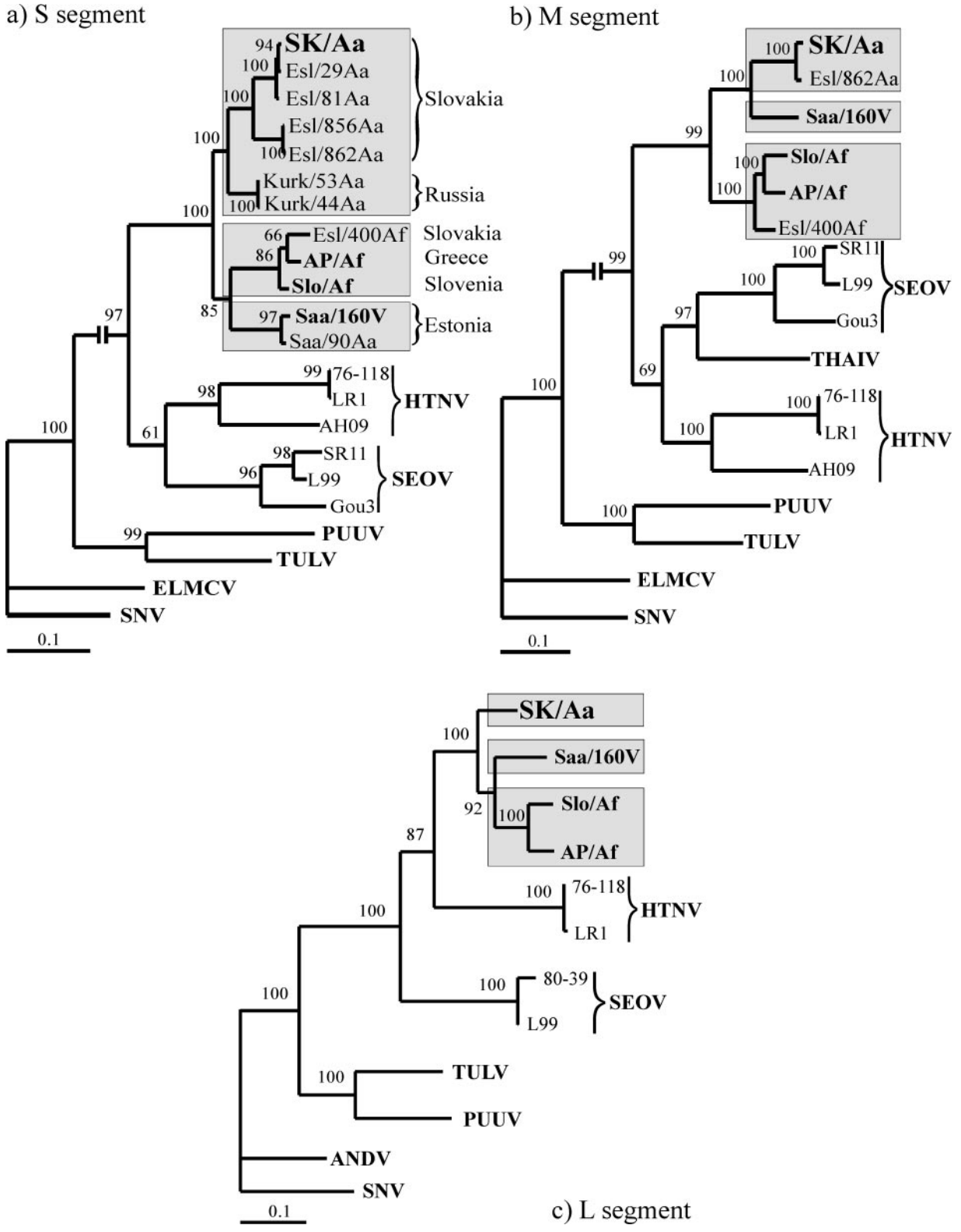

FIG. 1. ML phylogenetic trees of DOBV strains based on complete S-segment (a) and complete M-segment (b) ORF nucleotide sequences corresponding to S-segment nucleotide sequence positions 36 to 1,325 and M-segment nucleotide sequence positions 41 to 3,445, respectively, in SK/Aa, and partial L-segment sequences (374 nt, positions 157 to 530) (c). Names of DOBV isolates are in boldface. Different DOBV clades are indicated by gray boxes. For abbreviations and accession numbers, see the Materials and Methods. The trees were computed with the TREE-PUZZLE package. The values at the tree branches are the PUZZLE support values.

TABLE 2. Comparison of DOBV isolates SK/Aa and Slo/Af with respect to their neutralization by convalescent sera from Central European HFRS patients

Serum no.[a]	c-FRNT (endpoint titer)[b]	
	SK/Aa	Slo/Af
1	10,240	640
2	10,240	2,560
3	2,560	640
4	640	160
5	640	160
6	2,560	2,560
7	640	640
8	2,560	10,240
9	640	2,560

[a] Serum samples of HFRS patients that were clinically characterized elsewhere (sera 1 to 6 [51], serum 7 [16], sera 8 and 9 [unpublished data]). Sera 1 to 7 were obtained from patients in Germany, and sera 8 and 9 were obtained from patients in Slovakia.

[b] That is, reciprocal endpoint titers given as determined by c-FRNT.

ANDV N protein. The obtained reaction pattern for the SK/Aa and Slo/Af isolates was identical; both isolates reacted with Eco2, E5/G6, C16D11, and 2E2 MAbs (all raised against HTNV), but both did not react with B5D9, C24B4 (both raised against HTNV), R31 (against SEOV), and 5C2/E10 (against ANDV). In the cases where the respective epitope locations in the N protein are known, a comparison of the SK/Aa and Slo/Af sequences at these positions showed no or only a few amino acid exchanges, which could all be considered as conservative according to the method of Dayhoff et al. (8).

Nine convalescent-phase sera of HFRS patients sampled at different time points and different places in Germany (sera 1 to 7) and Slovakia (sera 8 and 9) were used to compare the neutralizing antibody titers against the new isolate SK/Aa as the representative of the DOBV-Aa lineage and the prototype strain Slo/Af from the DOBV-Af lineage (Table 2). All sera from German HFRS patients had been previously serotyped as anti-DOBV specific by FRNT (16, 51).

Four patient sera showed 4-fold higher and one serum showed 16-fold higher reciprocal titers to SK/Aa than to Slo/Af. In contrast, two sera exhibited a fourfold-higher reciprocal titer to strain Slo/Af. Two sera reacted at equal endpoint titers with both viruses (Table 2).

DISCUSSION

DOBV-Aa is an important HFRS pathogen in Central Europe; by serotyping and direct molecular proof, dozens of patients with renal failure have been found to be infected with strains of this virus lineage (16, 51). However, all current knowledge about the genetics and molecular phylogeny of DOBV-Aa strains was generated on the basis of nucleic acid isolation from *A. agrarius*- or human-derived specimens and subsequent PCR amplification and nucleotide sequence analysis. Here we describe the isolation of an indigenous DOBV-Aa virus strain that can be taken as the representative of the DOBV-Aa lineage within the DOBV species.

Our sequence and phylogenetic analysis showed that the DOBV-Aa virus isolate, named Slovakia or SK/Aa in brief, is closely related to the other Central European DOBV-Aa strains. Based on the S- and L-segment phylogenetic trees of DOBV strains (Fig. 1a and c), SK/Aa is not only the exclusive DOBV isolate from Central Europe but is the only viable virus strain within the whole DOBV-Aa genetic lineage.

Identification of the partial L-segment sequence of the new isolate SK/Aa allowed new insights into the recently discussed topic of reassortment during DOBV evolution (15, 17, 39). The ML analysis showed that also in L segment, the Saa/160V strain (although found in *A. agrarius* rodents) is more related to DOBV-Af strains and only its M segment resembles that of DOBV-Aa strains. This suggests that the M segment encoding the viral glycoproteins is crucial for the hantavirus host specificity.

Further studies on the biological consequences of reassortment in hantaviruses are urgently needed. The availability of DOBV-Af (Slo/Af) and DOBV-Aa (SK/Aa) isolates, as well as the apparently reassorted Saa/160V, is an important step forward to study the consequences of reassortment processes for *Murinae*-associated hantaviruses.

Adaptation of a field virus to Vero E6 cells cell culture may be accompanied by the accumulation of mutations in the viral genome. However, when we compared the original sequence from the *A. agrarius* specimen used for virus isolation (Esl/34Aa) with the nucleotide sequence of the complete S segments of the virus isolate SK/Aa, we found no differences in the noncoding regions, and only one putative amino acid exchange in the N protein occurred. This exchange of Ala to Thr at amino acid position 43 can be considered conservative according to the criteria of Dayhoff and coworkers (8). At this position, both amino acid residues can be also alternatively found in the N protein sequences of other hantavirus species, no matter whether the respective strains were cell-adapted or "wild" strains (data not shown). This let us conclude that our virus isolate, at least in the S segment, represents the natural genetic make-up of the "wild" virus.

Similar analyses were recently undertaken with PUUV strain Kazan (21, 30); the only amino acid substitution was found in the L protein, Ser versus Phe at position 2053. In the entire S-segment sequences, single mutations in both 5′ and 3′ noncoding regions were observed that correlated with a different infectivity of the viruses to bank voles, but no differences could be found in the S-segment coding region and in the entire M segment (21). In other studies, the complete S-segment sequence recovered directly from the lung tissue sample of *A. flavicollis* was found to be identical to that of the subsequent Vero E6 cell culture isolate Dobrava/Ano-Poroia (32). Chizhikov et al. (7) compared the nontranslated regions of the S and M segments of Sin Nombre virus RNAs (strain NMR11) amplified from (i) the kidney tissue from the original trapped *P. maniculatus*, (ii) the lung tissue of the first- and second-passage experimentally virus-infected *P. maniculatus*, and (iii) SN virus harvested from the fifth passage in Vero E6 cells; these researchers found no nucleotide sequence differences among these samples, suggesting that no genetic selection or adaptation is taking place during growth of the virus in the experimentally infected *P. maniculatus* or during the five passages in Vero E6 cells.

Since the amplification of hantaviral nucleic acid from patient material is difficult and rarely successful, FRNT is the only useful method for fine serotyping of human hantavirus

infections in HFRS diagnostics (18). The validity of the approach to serotype neutralizing antibodies in the patient's serum by FRNT mainly depends on the availability of that virus in the assay, which is closely related to and therefore representative for the naturally infecting virus strain. Accordingly, the SK/Aa strain is the virus of choice for fine typing sera from Central European HFRS patients by FRNT.

Here we have investigated convalescent-phase sera from nine HFRS patients from Germany and Slovakia originally diagnosed as DOBV positive on the basis of the ability of their sera to neutralize the Slo/Af prototype strain (16, 51). From these results it is reasonable to conclude that five of nine patients were infected by DOBV strains best represented by our SK/Aa isolate.

Moreover, two of nine sera neutralized the Slo/Af strain significantly better than the SK/Aa strain. Interestingly, both sera were taken from patients from Slovakia, a country in Central Europe where DOBV-Aa and DOBV-Af were demonstrated to exist sympatrically (51). Our data might provide a first hint that in this geographical region not only DOBV-Aa but also DOBV-Af strains could be etiological agents of DOBV-associated HFRS cases.

In the remaining two of nine cases, members of a third DOBV lineage with equal neutralization titers to either DOBV-Aa or DOBV-Af may have infected the patients. It is interesting that not only in the present study (using sera of HFRS patients) but also in seroprevalence studies in Estonia (6, 10), Latvia (23), and most of all in Lithuania (43) a noticeable number of sera reacted equally well with Slo/Af on the one hand and SK/Aa (or Saa/160V) on the other.

Recently, several authors suggested that DOBV-Aa and DOBV-Af strains exhibit different levels of virulence in humans. This idea is mainly based on the fact that most severe DOBV-associated HFRS cases have been reported from the Balkans, where DOBV-Af is believed to be dominant (3, 33). On the other side, usually mild or moderate DOBV-associated clinical courses were described for Central Europe, where DOBV-Aa is supposed to prevail (51). However, the clinical course of the two HFRS cases in Slovakia described here, which could have been caused by DOBV-Af-like virus strains, was rather mild, resembling the situation of clinical DOBV-Aa infections in Central Europe.

In summary, the first Central European DOBV strain has been isolated from an *A. agrarius* rodent. The availability of this virus strain will allow additional studies to examine the antigenic properties of DOBV lineages, their different pathogenic potentials, and the role of genetic reassortment and host-dependent virus evolution.

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