Genetic and antigenic properties of Dobrava virus: a unique member of the *Hantavirus* genus, family *Bunyaviridae*

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We examined the genetic and antigenic properties of Dobrava (DOB) virus, a hantavirus associated with severe haemorrhagic fever with renal syndrome in Europe. Cloning and sequence analyses revealed the DOB M segment to consist of 3644 nucleotides, with a coding capacity of 1134 amino acids in the virus complementary-sense RNA (cRNA). Seven potential asparagine-linked glycosylation sites were identified in the M segment gene product, one in the G2 and six in the G1 coding regions. The S segment is 1667 nucleotides long, and has a single ORF in the cRNA capable of encoding a protein of 428 amino acids. Phylogenetic comparisons of the M and S segments of DOB virus to those of other hantviruses indicated that DOB virus is similar to, but clearly distinct from Hantaan (HTN) and Seoul (SEO) viruses. Certain G2-specific, but not G1-specific monoclonal antibodies to HTN virus reacted to the same titre with DOB and homologous viral antigen. Plaque-reduction neutralization tests indicated that, of the sera tested, only antisera to SEO virus were able to neutralize DOB virus to a titre greater than 1:10; however, this neutralization titre was eightfold lower than that observed with homologous SEO virus. The data reported here confirm that DOB virus is a unique species in the *Hantavirus* genus, family *Bunyaviridae*.

Introduction

The genus *Hantavirus* includes the aetiological agents of haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Among the hantaviruses known to be associated with HFRS which have been propagated in cell culture are Hantaan (HTN), Seoul (SEO) and Puumala (PUU) viruses (Lee & van der Groen, 1989). Hantaviruses associated with HPS include Sin Nombre (SN) and Black Creek Canal (BCC) viruses (Elliott et al., 1994; Schmaljohn et al., 1995; Rollin et al., 1995). [Initial isolates of Sin Nombre virus were Convict Creek 107 virus (CC107) (Schmaljohn et al., 1995) and shortly thereafter, Four Corners virus, briefly renamed Muerto Canyon virus and finally renamed Sin Nombre virus (Elliott et al., 1994), both of which came from *Peromyscus maniculatus* trapped in the southwestern United States.] In addition to these pathogenic hantaviruses there are three isolates not known to cause human disease: Prospect Hill (PH), Thailand (THAI) and Thottapalayam (TPM) viruses. Genetically and serologically distinct hantaviruses are usually isolated from different rodents; thus, most viruses closely related to HTN virus were isolated from *Apodemus agrarius* and those most similar to SEO, PUU and PH viruses were isolated from *Rattus norvegicus*, *Clethrionomys glareolus* and *Microtus pennsylvanicus*, respectively. The primary rodent host of SN virus is *Peromyscus maniculatus* and that of BCC virus is *Sigmodon hispidus* (Childs et al., 1994; Turell et al., 1995; Rollin et al., 1995). The rodent hosts for THAI and TPM viruses, respectively, are *Bundicota indica* and *Suncus murinus* (Carey et al., 1971). Several other hantaviruses have been detected in different rodent hosts and in patient autopsy material, but have not yet been isolated in cell culture (Hjelle et al., 1994; Morzunov et al., 1995; Plyusnin et al., 1994; Torrez-Martinez et al., 1995).

DOB virus was isolated from *Apodemus flavicolus*, the yellow-necked field mouse, in an area of Slovenia where a number of cases of severe HFRS had occurred (Avsic-Zupanc et al., 1992). Another isolate, identical or nearly identical to DOB virus, was obtained from a fatal HFRS case that occurred in the former Yugoslavia (Gligic et al.,...
1992). Partial characterization of DOB virus suggested it to be a distinct virus (Avsic-Zupanc et al., 1992; Xiao et al., 1993). In this report, we extend the antigenic and genetic characterization of DOB virus and provide conclusive evidence that DOB virus is an additional, unique member of the genus *Hantavirus*.

**Methods**

**Propagation of virus and purification of RNA.** DOB virus was propagated in Vero E6 cells (Vero C1008, ATCC CR L 1586) grown in Eagle’s minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS) and gentamicin (50 µg/ml). Total RNA was extracted from infected cells by using Trizol reagent (GIBCO BRL) according to the manufacturer’s directions. Briefly, the infected cells were washed twice with phosphate-buffered saline (PBS), scraped from flasks and transferred to microcentrifuge tubes. Ten volumes of Trizol reagent were added and the samples were mixed. After incubating for 5 min at room temperature, the samples were extracted once with chloroform and RNA was precipitated by adding isopropanol. RNA pellets were washed twice with 70% ethanol, air dried and resuspended in RNase-free water.

**Reverse transcriptase–polymerase chain reaction (RT–PCR).** First-strand cDNA synthesis was primed by adding random hexamer primers or specific oligonucleotide primers corresponding to DOB sequences (see below), and was performed according to the SuperScript II RNase H-Reverse Transcriptase kit (GIBCO BRL) instructions. PCR was performed in 100 µl reaction mixtures containing 10 mM-Tris–HCl (pH 8.3), 50 mM-KCl, 25 mM-MgCl₂, 400 mM of each dNTP, 2 µM of each primer and 2.5 U of Taq polymerase (Perkin Elmer-Cetus) for 35 cycles each consisting of 1 min at 94 °C (denaturation), 1 min at 45 °C or 55 °C (annealing) and 3 min at 72 °C (elongation) in a DNA thermal cycler (Perkin Elmer-Cetus). For medium (M) segment PCR, initial oligonucleotide primers were designed based on the published partial sequence of the medium genome segment of DOB virus (Xiao et al., 1993). Oligonucleotide primers initially used for PCR amplification of the small (S) segment corresponded to the sequence of the genus-specific consensus primers published previously (Puthavathana et al., 1992). Additional primers were derived by comparing the gene sequences of other hantaviruses and identifying highly conserved regions of their M segments. The oligonucleotide primers corresponding to the consensus terminal nucleotide sequence found on M or S segments of all known hantaviruses were also used. The primer pairs that were used to amplify the M segment cDNA were: 5' TAGTGTAGACTCCGCAA 3' (M-terminal) and 5' CAGTTC-GGGAAACAAATCCTGGG 3' (M1078F); 5' TCAACACTGATTTAGAT 3' (M817F) and 5' CATACTTAGTACATGCACC 3' (terminial). The primer pairs that were used to amplify the S segment cDNA were: 5' TAGTGTAGACTCCGCAA 3' (S5'-terminal) and 5' GCATTGACAAGAACAGGTGAGGGAAACAATCCTGGG 3' (M1078R); 5' TTCAACCACTGATTTAGAT 3' (S1048F) and 5' TAGTAGTATGCTCC 3' (S3'-terminal).

**Cloning and sequence analysis.** The PCR-amplified products were cloned into the pCRII vector by using the TA-Cloning System (Invitrogen). Competent E. coli DH5α (GIBCO BRL) cells were transformed with the recombinant plasmid vectors. Isolation of plasmid DNA, followed by restriction analyses, was carried out and clones with an insert of the appropriate size were selected for nucleotide sequencing. Sequencing was done by the dideoxy chain-termination procedure with a Sequenase version 2.0 DNA sequencing kit (United States Biochemical) according to the manufacturer’s directions. Overlapping cDNA fragments of both genomic segments were sequenced in both directions for at least two clones.

**Computer analysis.** Nucleic acid and amino acid sequence data were analysed by using the MacVector sequence analysis software (Kodak) and the Genetics Computer Group software (University of Wisconsin). Phylogenetic analyses were performed by using the PAUP 3.1.1 program (David L. Swofford, Smithsonian Institute, Washington, DC, USA). Phylogenetic trees were modified by using Canvas (Deneba) or Powerpoint (Microsoft) software.

**Immune-precipitation and ELISA.** General procedures for immune-precipitation were as described previously (Schmaljohn et al., 1983, 1987). In brief, Vero E6 cells infected with DOB virus were radiolabelled with both [35S]methionine and [35S]cysteine to 28 h after infection, lysed in Zwittergent 3-14 (Calbiochem) buffer and reacted with polyclonal rabbit anti-sera to HTN, SEO or DOB viruses, mouse monoclonal antibodies (Arikawa et al., 1989) or with hyperimmune mouse ascitic fluid to HTN virus. Immune precipitates were collected on protein A-Sepharose, washed, and analysed on 12.5% acrylamide–DATD (N,N'-diallyl tartardiamide) gels. Methods for ELISA were as described previously (Chu et al., 1994). Briefly, antigens were prepared from virus-infected Vero E6 cells harvested 10 to 14 days after infection with HTN (76-118), SEO (SR-11), PUU (P160), PH (PH-1) or DOB (3970) viruses. Cells were scraped from flasks, diluted in borate saline (pH 9.0), pelleted by slow-speed centrifugation, and resuspended to 10% (v/v) in borate saline containing 1% Triton X-100 detergent. After sonication, the lysates were clarified by slow-speed centrifugation and infectivity was inactivated by 2 Mrad of γ-ray irradiation. Optimal antigen dilutions were determined with checkerboard titrations against each homologous rat antiserum. Flat-bottom ELISA plates were coated with antigen diluted in PBS containing 0.05% Tween 20 (PBS-T), and 1:200 dilutions of each MAb in PBS-T containing 5% skim milk were added; plates were then incubated for 1 h at 37 °C. After washing as above, peroxidase-labelled goat anti-mouse IgG (Kirkegaard & Perry Laboratories) was added and incubation was continued for 1 h at 37 °C. After washing, ABTS peroxidase substrate (Kirkegaard & Perry) was added and the plates were incubated for 30 min at 37 °C. The endpoint ELISA titre was determined at 414 nm and was expressed as a ratio greater than or equal to the standard deviation of the mean reading obtained with antigen from mock infected cells.

**Results**

**Features of the complete M and S segment sequences**

Because DOB-infected cell culture RNAs were used as templates for RT–PCR, at least two independently derived cDNA clones were sequenced for each gene region to minimize errors due to RNA polymerase. cDNA clones representing the complete nucleotide sequences of the M and S segments were determined and deposited in the Genome Sequence Database under accession numbers L33685 and L41916, respectively. The M segment consisted of 3644 base pairs, with a coding capacity in the virus complementary-sense RNA (cRNA) of 1134 amino acids or a polypeptide of approximately 125-5 kDa. A 5' noncoding region of 40 nucleotides
preceded the M segment ORF and 199 non-coding nucleotides followed the stop codon. Like those of other hantaviruses, and viruses in the Bunyaviridae family in general, the predicted M segment gene product of each isolate was cysteine-rich (5.3%). The positions of 27 of 33 cysteines in DOB virus G1, and 27 of 27 residues in G2 were conserved with those of HTN virus, suggesting structural similarities of these proteins (Schmaljohn et al., 1987).

The deduced amino acids comprising the G1 and G2 proteins of DOB virus contained seven potential asparagine-linked glycosylation sites (N-X-S/T), six in G1 and one in G2. The position of the predicted G2 glycosylation site (amino acids 928 to 930) is conserved within the predicted amino acid sequences of all other hantaviruses studied to date (Schmaljohn, 1995). The potential glycosylation sites in DOB virus G1 were interesting, in that in addition to three sites conserved with all other hantaviruses (amino acids 134 to 136; 347 to 349; and 399 to 401), DOB virus had one unique site (amino acids 252 to 254); one site conserved with HTN, SEO and THAI viruses (amino acids 235 to 237); one site conserved only with SEO and THAI viruses (amino acids 562 to 564); and another site adjacent to a unique site in PH virus (amino acids 518 to 520) (Arikawa et al., 1990; Giebel et al., 1989; Parrington et al., 1991; Schmaljohn et al., 1987).

Like those of other hantaviruses, the predicted amino termini of the G1 and G2 proteins of DOB virus followed stretches of hydrophobic amino acids typical of signal sequences. A possible membrane-spanning region (amino acids 627 to 645) may constitute an intergenic region; however, the exact carboxy terminus has not been described for a G1 protein of any hantavirus; therefore, it was not possible to determine if this region remained a part of G1 or was cleaved. The amino acid sequence WAASA is located at amino acids 644 to 648 of the DOB virus M segment ORF. This sequence was previously identified to be conserved among all known hantaviral M segment gene products, and immediately precedes the amino-terminal residue of G2 of HTN and SEO viruses (Schmaljohn et al., 1987; Arikawa et al., 1990; Schmaljohn, 1995). The A residue is likely to be the site of signalase cleavage for this internal signal sequence. Also, like those of the other hantaviruses, the amino acids adjacent to the carboxy terminus of DOB virus G2 were extremely hydrophobic and were followed by a stretch of hydrophilic residues, suggesting the presence of a membrane-anchoring region. The predicted molecular masses of the envelope proteins of DOB virus were 66 to 72 kDa for G1 and 54 kDa for G2. These values were consistent with size estimates obtained by immune-precipitation of radiolabelled viral proteins and resolution by polyacrylamide gel electrophoresis (PAGE) (Fig. 1 a). The G1 and G2 proteins of DOB virus, immune-precipitated with polyclonal antisera to HTN virus were not well-resolved by SDS-PAGE; however, after immune-precipitation with HTN virus G1- or G2-specific MAbs, both proteins were clearly evident (Fig. 1 b).
Fig. 2. Phylogenetic relationship of the complete M (A) and S (B) segments or of the G1 coding region of M (C), and the G2 coding region of M (D) of DOB virus to other hantaviruses. Sequences of hantaviruses used are all available in the Genome Sequence Database. For each analysis, a single most parsimonious tree was derived by using PAUP 3.1.1 software. Bootstrap values resulting from 200 replications are listed below each branch. Horizontal lengths of branches, and the numbers above each branch, are proportional.
Dobrava virus M and S segment sequences

The S segment of DOB virus consisted of 1667 nucleotides, and had an ORF in the cRNA with a coding capacity of 428 amino acids, or a polypeptide of approximately 48 kDa. This ORF began at nucleotide 36 with respect to the 5′ terminus of the S segment cRNA, and terminated at nucleotide 1322. Consequently, the 3′ non-coding region (345 nucleotides) was similar in length to that observed with HTN (370 nucleotides), SEO 437 nucleotides), PUU (486 nucleotides) and PH (331 nucleotides) viruses but was much shorter than that reported for SN virus (730 nucleotides) (Spiropoulou et al., 1994; Schmaljohn, 1995). The size of the S segment gene product, the nucleocapsid protein (N), agreed with the migration of radiolabelled N in SDS-containing polyacrylamide gels (Fig. 1). No other significant ORFs were encoded in any of the six possible S segment reading frames.

Phylogenetic analyses

Maximum parsimony analysis (PAUP 3.1.1) was used to study the phylogenetic relationship of DOB virus and other hantaviruses. Both the M and the S segments of DOB virus localized near HTN and SEO viruses. Interestingly, phylogenetic trees constructed with nucleotide sequences encoding N or G1 placed DOB virus nearer to SEO virus, and sequences of the complete M segment or of the G2 coding region placed DOB virus closer to HTN virus (Fig. 2). To examine the possible differing evolution of the G1 and G2 coding regions in relationship to those of HTN and SEO viruses, we performed a dot-matrix comparison of the predicted amino acids for DOB and HTN or SEO viruses. For this analysis, a similarity table (Pam 250 scoring matrix) was used, in which values were assigned to similar amino acids. From these comparisons, it was clear that there were no specific HTN- or SEO-like regions within DOB virus G1 or G2 (Fig. 3).

ELISA and plaque-reduction neutralization test (PRNT)

Previous studies, in which HTN virus G1- or G2-specific MAbs were reacted with DOB virus antigen in an immunofluorescent antibody test, suggested that some epitopes on DOB virus G1 are not conserved with HTN virus (van der Groen, 1990). Similarly, by using a different panel of HTN-specific MAbs (Arikawa et al., 1989), we found that certain HTN virus G2- but not G1-specific MAbs reacted with DOB virus antigen to as high a titre as homologous antigen in an ELISA (Fig. 4). Because PRNT is the most sensitive serological means for differentiating hantaviruses (Chu et al., 1994; Schmaljohn et al., 1985), we used polyclonal antisera to representative hantaviruses in a PRNT with DOB virus. We found that sera to SEO virus were able to neutralize DOB virus to a titre of 1:40, as compared to 1:360 for homologous SEO virus neutralization. None of the other sera tested neutralized DOB virus at a titre greater than to nucleotide sequence differences. Accession numbers for the M segment viral sequences used are: HTN, M14627; THAI, L08756; SEO, M34882; PUU (Sotkamo), X61034; PUU (P360), L08755; PH, X55129; DOB, L33685; SN, L33474; BCC, L39950; ELMC, U26828; BAY, L36931. Accession numbers for the S segment viral sequences used are: HTN, M14626; SEO, M34881; PUU (Sotkamo), X61035; PUU (P360), L11347; PH, X55128; DOB, L41916; SN, L33683; BCC, L39949; ELMC, U14227; TUL, Z30941; BAY, L36929; RIOS, U18100.
Fig. 4. ELISA. Infected cell lysates were prepared as described in Methods and were reacted with four MAbS to HTN virus G1 and ten MAbS to HTN virus G2. The designations a-f refer to competitive inhibition groups (Arikawa et al., 1989). The endpoint ELISA titre was determined at 415 nm and was expressed as the reciprocal of the maximum dilution of antigen giving a ratio > 3 times that obtained with antigen prepared from mock-infected cells.

Table 1. Plaque-reduction neutralization test (PRNT)

<table>
<thead>
<tr>
<th>Serum†</th>
<th>Homologous</th>
<th>DOB</th>
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<tbody>
<tr>
<td>HTN</td>
<td>320</td>
<td>10</td>
</tr>
<tr>
<td>SEO</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td>PUU</td>
<td>160</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>PH</td>
<td>160</td>
<td>10</td>
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<tr>
<td>TPM</td>
<td>640</td>
<td>&lt; 10</td>
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</table>

* Titres are expressed as the reciprocal of the highest dilution causing an 80% reduction in plaques.
† TPM, hyperimmune mouse ascitic fluid; all the others are rabbit sera.

Discussion

HFRS may have existed in the Balkans for decades. Since 1954, 92 serologically confirmed, sporadic cases have been documented in Slovenia. These include both severe and mild cases, with an overall mortality rate of 3.5% (T. Avsic-Zupanc, unpublished information), which is similar to the 3 to 7% reported for Korean haemorrhagic fever (Lee, 1982). Serological and molecular epidemiological studies of small mammals trapped in fields and forests surrounding villages in Slovenia where HFRS cases were reported found simultaneous circulation of at least three different hantaviruses: HTN virus, detected in humans, Apodemus flavicolis, Microtus agrestis (field vole) and Glis glis (dormouse); PUU virus, detected in Clethrionomys glareolus; and DOB virus, detected in Apodemus flavicolis (Avsic-Zupanc et al., 1990, 1994; Avsic-Zupanc & Poljak, 1994).

To gain a better understanding of the genetic properties of DOB virus, we determined the complete M and S segment nucleotide sequences. The sizes of the M and S segments and the predicted molecular masses of the G1, G2 and N proteins were all consistent with the apparent sizes that were estimated for those molecules by gel electrophoresis and features of the deduced amino acid sequences of the G1 and G2 proteins were similar to those reported previously for other hantaviruses. Although the predicted G1 protein of DOB virus has more potential N-linked glycosylation sites than does any other hantavirus studied to date, the migration of G1 on polyacrylamide gels did not suggest an unusually high degree of glycosylation; and in fact, DOB virus G1 was difficult to resolve from G2, except with specific MAbS. Although we did not perform studies to determine how many of the glycosylation sites were actually used, our results suggest that few of the potential sites in G1 are utilized.

The S segment of DOB virus apparently encoded only N. The S segment of HTN virus has a second ORF, in the same reading frame as N, which initiates four bases after the stop codon for N and has a coding capacity of
about 6 kb (Schmaljohn et al., 1986). The S segments of PUU, PH and SN viruses each have an ORF overlapping that of S which could potentially encode polyptides of 7-6 to 11 kDa (Parrington & Kang, 1990; Spiropoulou et al., 1994; Stohwasser et al., 1990). To date no additional S segment-derived proteins have been identified for any of these viruses. Our sequencing results with DOB virus, like those reported earlier for SEO virus (Arikawa et al., 1990) further indicate that an NSs protein is not generally required by hantaviruses.

Phylogenetic analyses of hantaviruses always result in trees that show two distinct ancestral branches, one of which leads to the HTN, SEO and THAI virus clades and the other to the PUU, PH and SN virus clades (reviewed in Schmaljohn, 1995). Comparison of the M and the S segment sequences of DOB virus with those of other viruses in the genus clearly demonstrated DOB virus to be unique, but to be of the same ancestral lineage as HTN, SEO and THAI viruses. Although the M and G2 trees place DOB virus closer to HTN than to SEO virus, and the S and G1 trees place DOB virus closer to SEO and THAI viruses than to HTN virus, the bootstrap reliabilities are much better for the M and the G2 trees. This finding is consistent with an earlier tree generated from a small region of the G2 gene of DOB and numerous other hantaviruses (Xiao et al., 1994).

Our analyses revealed no evidence that DOB virus descended directly from either HTN or SEO viruses, but rather that DOB, HTN and SEO viruses appear to have diverged at similar evolutionary rates from a theoretical ancestor. Although it might be expected that DOB virus would be more similar to HTN virus than to SEO virus because both HTN and DOB viruses were isolated from Apodemus, while SEO virus was isolated from Rattus, the difference in the species of Apodemus (i.e. agrarius for HTN virus and flavicolis for DOB virus) was apparently sufficient to result in as equally a divergent evolutionary path as is observed with viruses in different genera of rodents.

Both our phylogenetic analyses and antigenic studies confirm earlier reports that the G1 proteins of hantaviruses are more type-specific than are the G2 proteins (Chu et al., 1994). Our neutralization studies indicated that DOB virus was readily distinguishable from HTN, SEO, PUU and PH viruses, but that some neutralization was possible with antisera to SEO virus.

In conclusion, our results all indicate that DOB virus is a unique species in the Hantavirus genus, family Bunyaviridae.

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References


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