Genetic analysis of wild-type Dobrava hantavirus in Slovenia: co-existence of two distinct genetic lineages within the same natural focus

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Genetic analysis was performed of wild-type (wt) Dobrava hantavirus (DOB) strains from Slovenia, the country where the virus was first discovered and where it was found to cause haemorrhagic fever with renal syndrome (HFRS), with a fatality rate of 12%. Two hundred and sixty mice of the genus Apodemus, trapped in five natural foci of DOB-associated HFRS during 1990–1996, were screened for the presence of anti-hantavirus antibodies and 49 Apodemus flavicollis and four Apodemus agrarius were found to be positive. RT–PCR was used to recover partial sequences of the wt-DOB medium (M) and small (S) genome segments from nine A. flavicollis and one A. agrarius.

Sequence comparison and phylogenetic analysis of the Slovenian wt-DOB strains revealed close relatedness of all A. flavicollis-derived virus sequences (nucleotide diversity up to 6% for the M segment and 5% for the S segment) and the geographical clustering of genetic variants. In contrast, the strain harboured by A. agrarius showed a high level of genetic diversity from other Slovenian DOB strains (14%) and clustered together on phylogenetic trees with other DOB strains harboured by A. agrarius from Russia, Estonia and Slovakia. These findings suggest that the DOB variants carried by the two species of Apodemus in Europe represent two distinct genetic lineages.

Introduction

Hantaviruses comprise a genus of the family Bunyaviridae. They possess a tripartite, negative-sense RNA genome encoding a viral RNA polymerase, surface glycoproteins G1 and G2 and a nucleocapsid (N) protein (for reviews see Elliott et al., 1991; Schmaljohn & Hjelle, 1997). The number of known distinct hantavirus sero- and genotypes has grown significantly during recent years (for reviews see Plyusnin et al., 1996, 1999b) and currently totals 22 (Elliott et al., 2000). Members of the genus Hantavirus are maintained in nature in persistently infected rodents and, unlike other bunyaviruses, are not transmitted by arthropod vectors. They are thought to be transmitted primarily via infectious aerosols generated by contaminated rodent urine, faeces and saliva (Lee & van der Groen, 1989). One of the main features of hantaviruses is the close association between the virus type and the host species. This results in the circulation of distinct hantaviruses in the Old and New Worlds and in geographical clusters of hantavirus genetic variants (Plyusnin et al., 1996). Many hantaviruses are pathogenic for humans. Hantaan (HTN), Seoul (SEO), Puumala (PUU) and Dobrava (DOB) viruses cause haemorrhagic fever with renal syndrome (HFRS) in Eurasia, whereas Sin Nombre (SN), New York (NY), Black Creek Canal (BCC), Bayou (BAY), Laguna Negra (LN) and Andes viruses are the causative agents of hantavirus pulmonary syndrome and occur endemically in the Americas (Schmaljohn & Hjelle, 1997).

Two pathogenic hantaviruses that cause HFRS have so far been proven to circulate in Europe: PUU and DOB. DOB was isolated originally from the lungs of a yellow-necked mouse (Apodemus flavicollis) trapped in the Dolenjska region of Slovenia, where a number of severe HFRS cases had occurred...
Extensive antigenic and genetic characterization identified DOB as a unique hantavirus type (Avsic-Zupanc et al., 1999). It was shown that DOB, transmitted to humans by A. flavicollis, is the aetiological agent of a severe form of HFRS that occurs in the Balkans (Antoniadis et al., 1996; Lundkvist et al., 1997a; Papa et al., 1998; Avsic-Zupanc et al., 1999). DOB has also been found in striped field mice (Apodemus agrarius) in Estonia (Plyusnin et al., 1997; Nemirov et al., 1999), Russia (Plyusnin et al., 1999) and Slovakia (Sibold et al., 1999). These findings suggest that striped field mice, which are known to harbour HTN in Asia, also carry DOB in Central and Eastern Europe. Human DOB infections have been detected in Russia (Lundkvist et al., 1997a), Estonia (Lundkvist et al., 1998) and Germany (Meisel et al., 1998). Notably, no casualties were associated with any of the DOB-HFRS cases in these countries, where the virus is probably carried by A. agrarius. This is in sharp contrast to the Balkans, where 9–12% fatality rates have been reported for hospitalized DOB-HFRS cases (Papa et al., 1998; Avsic-Zupanc et al., 1999).

In Slovenia, hantavirus infection has been demonstrated in multiple rodent species and other mammalian orders by detection of viral antigen and antibodies (Avsic-Zupanc, 1999). Earlier epidemiological surveys indicated that A. flavicollis and bank voles (Clethrionomys glareolus), which are common throughout Central Europe, were most frequently infected with hantaviruses. The purpose of the present study was to estimate the rate of DOB infection in rodents and to evaluate the genetic variability of DOB in Slovenia.

**Methods**

**Trapping of rodents.** Rodents were trapped by a professional mammalogist in five areas of Slovenia (Fig. 1): Dobrava village, Kocevje, Tenetise, Gorjanci and Prekmurje. Animals were live-trapped in July 1990 (Dobrava), June–August 1993 (Kocevje), July 1995 (Tenetise), July and August 1995 (Gorjanci) and September 1996 (Prekmurje). These study sites were selected with regard to data on reported HFRS cases. The traps were placed in fields, at the edges of forests and in forests near the residences of diagnosed HFRS cases. The captured rodents were euthanized with ether, weighed, measured, sexed, identified and then autopsied. Blood samples were obtained by cardiac puncture. Heart, lung, liver, spleen and kidney samples were collected and stored at −70 °C until processed further. The remains of each of the animal were collected for species identification by the Slovenian Museum of Natural History.

**Screening of rodent sera.** Sera collected from rodents were initially screened for the presence of IgG antibodies to hantaviruses by using an enzyme immunoassay (EIA) as described previously, except that the positive control consisted of a mixture of anti-hantavirus monoclonal antibodies (Avsic-Zupanc et al., 1992) and serum from an uninfected laboratory mouse was used as a negative control. In addition, anti-mouse IgG–peroxidase conjugate (A-4416; Sigma) was used. Sera were considered positive when they gave an absorbance four or more times that of the negative control. All positive sera were confirmed by indirect immunofluorescence assay (IFA) by using spot slides containing Vero E6 cells infected with HTN, PUU and DOB. Sera that gave a characteristic hantavirus cytoplasmic fluorescence at a dilution of 1:64 were considered positive.

**RT–PCR.** RNA was extracted from kidney tissue of seropositive rodents. Approximately 100 mg kidney tissue was ground in 1 ml TRIzol Reagent (Gibco BRL) and mixed with 0.2 ml chloroform. After incubation at room temperature for 2–3 min, the mixture was centrifuged at 12,000 g for 15 min at 4 °C. After centrifugation, the RNA was precipitated from the aqueous phase by mixing with 0.5 ml ice-cold 2-propanol and subsequent centrifugation at 12,000 g for 10 min at 4 °C. The RNA pellet was washed with 75% ethanol by vortexing and centrifuged at 8000 g for 5 min at 4 °C. The RNA was briefly air-dried and dissolved in 50 µl nuclease-free water.

RT–PCR was performed initially on all seropositive rodent samples by using the cross-reactive outer primers MOF103 and MOR204, described previously (Chu et al., 1995), which amplify a 490 bp region from the M segment (encoding G1) of a number of different hantaviruses (nt 1190–1680). RT was carried out with the SuperScript pre-amplification system for first-strand cDNA synthesis (Gibco BRL) according to the manufacturer’s instructions. Ten µl first-strand cDNA was then mixed with 4 µl 10 × PCR buffer, 2 µl 25 mM MgCl2, 0.5 µl (100 pmol) each of primers MOF103 and MOR204, 32–7 µl distilled water and 0.3 µl (2.5 U) Taq DNA polymerase. The reaction mixture was then subjected to 35 cycles, each consisting of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C. A second-round PCR (for nt 1309–1599) was carried out with DOB-specific primers DOB G1F (5’ ATGCCAGCGGTGCACCAAA 3’) and DOB G1R (5’ GAGCTATAGTAAGATGCC 3’), which reside within the amplified region of the RT–PCR primers in a nested fashion. For nested PCR, the annealing temperature was increased to 55 °C. The amplified products were analysed by electrophoresis in 2% agarose gels in Tris–acetate buffer. After staining with ethidium bromide, the PCR products were visualized by UV trans-illumination.

In addition, four partial S segment sequences were recovered. Previously described nested primers (Papa et al., 1998), designed to detect all known hantaviruses associated with rodents of the subfamily Murinae (HTN, DOB, SEO), were used in nested RT–PCR to obtain partial S segment sequences (nt 364–963) from two samples, Slo-1 and Slo-3 (from Dobrava and Kocevje). Two other sequences (Slo-9 and Slo-10, originating from Prekmurje) were recovered by using a different protocol. Briefly, RT and first-round PCR were performed with the DOB-specific
primer DOBS1 (5' CAATTGGTGTAGCCAGGGCAGAAGG 3') and DOBS2 (5' GCCATGGCTGCAAT(G)AACCAGGCAGG 3'), which should yield a 929 bp product (nt 84–1012). Primers DOBS3 (5' CCTTGGATGAGCAAT(A)TACAG 3') and DOBS4 (5' G/C(T)-
CAGATA(A)TAGCTGCA(A/C/GG) 3') were used for the second-round PCR in order to obtain a product that corresponds to nt 354–782 of the S segment. Amplification products of the correct length were separated in agarose gels and purified with the QiAquick kit (Qiagen).

For comparison, the partial M segment sequence (nt 1309–1599) was recovered for the wild-type (wt) DOB strain Kurkino/A44/97 (Pyusyn et al., 1999a) from Russia by using primers DOB G1F and DOB G1R.

- Sequencing. The PCR products were purified from gel slices by using the GeneClean kit (BIO 101) according to the manufacturer's instructions. ABI PRISM Dye Terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (PE Applied Biosystems) and 4 pM of each of the nested primers were used in the sequencing reactions. The products were purified by using Centri-Sep spin columns (Princeton Separations) and sequenced on an ABI 310 Genetic Analyser (PE Applied Biosystems). The chromatograms were analysed and assembled by using the Staden software (MRC Laboratory of Molecular Biology, Cambridge, UK) run on a Linux operating system.

All sequence alignments were done with the SeqApp program, version 1.9A169. The DISTANCES program from the GCC package was used for calculations of distances between nucleotide sequences and amino acid sequences.

- Phylogenetic analysis. The PHYLP program (Felsenstein, 1993) was used to make 500 bootstrap replicates of the sequence data (SEQBOOT). Distance matrices were calculated by using Kimura's two-parameter model (DNADIST) and analysed by the Fitch–Margoliash tree-fitting algorithm (FITC). Alternatively, the DNA pars program was used to find the trees with maximum parsimony. The bootstrap support percentages of particular branching points were calculated from these trees (CONSENSE).

For comparisons, existing sequence data were obtained from sequence databases. The S segment sequence included: HTN strain 76-118 (GenBank accession number M14627); SEO strain SR-11 (M34881); PUU strains Sotkamo (X61035), CG13891 (U22423), Vindeln (Z48586), Umdurania/89/Cg/91 (Z21497) and Cg1820 (M32750); Tula virus (TUL) strains Tula/Moravia/5302v/95 (Z69991) and Tula/76Ma/87 (Z30941); Prospek Hill virus (PH) strain PH-1 (Z40939); Ila Vista virus (IVL) strain MC-SC-B-1 (U31534); SN strain H10 (L35784); NY strain RI-1 (U34988); El Moro Canyon virus (ELMC) strain RM-97 (U11427); BAY strain Louisiana (L36930); LN strain 510B (AF005728); Rio Segundo virus (RIS) strain RMx-Costa-1 (U18100); Khabarovsky virus (KBR) strain MF-43 (U35255); Topografov virus (TOP) strain Topografov/Ls136V (A011646); and DOB prototype strain (L33685) and strain PH-1 (Z55129); SN strain H10 (L25783); NY strain RI-1 (U36801); ELMC strain RM-97 (U26828); BAY strain Louisiana (L39930); LN strain 510B (AF050728); BCC (L39950); Blue River virus strain Indiana (BR-IN) (AF030551); KBR strain MF-43 (A011648); TOP strain Topografov/Ls136V (A011647); and DOB prototype strain (L33685) and strain Saaremaa/160Aa/96 (A009774).

### Results

#### Screening of rodents

A total of 367 rodents were trapped alive at five study sites during a 6-year period. *A. flavicollis* was the most frequently captured rodent (231 specimens), followed by *C. glareolus* (93 specimens), *A. agrarius* (21), field vole (*Microtus agrestis*) (14) and wood mouse (*Apodemus sylvaticus*) (8). For the purpose of this study, only mice of the genus *Apodemus* were studied further. Anti-hantavirus antibodies were detected in 53 rodents (20–4%) of 260 tested. Specific anti-hantavirus antibodies were found in *A. flavicollis* (49) and *A. agrarius* (4) by using ELA and further confirmed by end-point titration using IFA (Table 1). While seropositive *A. flavicollis* were found in all areas studied in a range from 5–7 to 27–2%, seropositive *A. agrarius* (30–8%) were only found in a single locality, Prekmurje. None of the eight captured wood mice (*A. sylvaticus*) was antibody positive.

RNA was extracted from the kidney tissue of these 53 seropositive rodents and initially tested by an RT–PCR that amplifies a portion of the G1-encoding region of the M segment of hantaviruses. Twenty-seven of the 53 samples examined produced a band of the expected size (490 bp) by using the outer (genus-specific) primers MOF103 and MOR204. When a second-round nested PCR with DOB-specific inner primers was performed, 23 additional samples scored positive (band size 291 bp).

### Table 1. Results of antibody detection and RT–PCR for *Apodemus* rodents captured in Slovenia

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<th>Species</th>
<th>Antibody</th>
<th>G1</th>
<th>N</th>
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* RT–PCR was carried out with nested genus- and *N* gene-specific primers as indicated.
**Table 2. Comparison of the nucleotide and amino acid sequences of a 277 nt fragment of the M segments of Murinae-borne hantaviruses**

Sequences covering nt 1314–1590 were compared. Sequence divergences are represented as percentage differences for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences. Data for other hantaviruses are from Schmaljohn et al. (1986) (HTN), Arikawa et al. (1990) (SEO) and Xiao et al. (1994) (THAI).

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* Abbreviated strain names used in this paper are given. Full strain names are: Slo-1, Dobrava/AF-1/90; Dob, Dobrava/3970/87 (Avsic-Zupanc et al., 1995); Slo-3, Kocevje/Af-3/93; Slo-4, Kocevje/Af-4/93; Slo-5, Tenetise/Af-5/95; Slo-6, Tenetise/Af-6/95; Slo-7, Gorjanci/Af-7/95; Slo-8, Gorjanci/Af-8/95; Slo-10, Prekmurje/Af-10/96; Slo-9, Prekmurje/Aa-9/96; Rus-53, Kurkino/Aa53/97; and Est-160, Saaremaa/Aa160v (Nemirov et al., 1999).

**Sequence comparison**

From each of the five areas studied, two M segment PCR products (nt 1314–1590) were chosen for sequence analysis. As some striking differences between the two wt strains originating from Prekmurje (Slo-9 and Slo-10) were observed, the S segment sequences were then recovered for these two wt strains as well as for strains from Dobrava (Slo-1) and Kocevje (Slo-3). Corresponding wt strains, sequences of which were not determined, were designated as follows: Dobrava village (Slo-1 and Slo-2), Kocevje (Slo-3 and Slo-4), Tenetise (Slo-5 and Slo-6), Gorjanci (Slo-7 and Slo-8) and Prekmurje (Slo-9 and Slo-10). Two M sequences recovered from A. flavicollis in the Dobrava locality (Slo-1 and Slo-2) were identical to that of the prototype DOB isolate, which originates from a yellow-necked mouse trapped at the same time.

Comparative analysis of the DOB M segment sequences (Table 2) included nine partial sequences recovered from Slovenian A. flavicollis [Dob (prototype), Slo-1 to Slo-8 and Slo-10] and three sequences recovered from A. agrarius: one Slovenian (Slo-9, from Prekmurje), one Russian (Rus-53) and one Estonian (Est-160). All sequences derived from A. flavicollis were closely related to each other (nucleotide diversity 0–5.8%) and showed clear geographical clustering: nucleotide diversity between strains from the same locality did not exceed 0.4%. In contrast, the sequence derived from A. agrarius (Slo-9) was remarkably different from the other Slovenian strains, including the strain Slo-10 from the same locality (nucleotide diversity of 12.6–13.7%).

Genetic analysis of the DOB S segment sequences (Table 3) included (i) seven sequences from A. flavicollis trapped in the Balkan countries, Slovenia [Dob (prototype), Slo-1, Slo-3 and Slo-10], Greece (Gre-9 and Gre-13) and Bosnia (Bos), (ii) two sequences recovered from HFRS patients from Greece (Gre-SZ and Gre-PR) and (iii) seven partial sequences recovered from A. agrarius trapped in Slovenia (Slo-9), Russia (Rus-44 and Rus-53), Slovakia (Slvk-862 and Slvk-872) and Estonia (Est-90 and Est-160). The three new sequences recovered from Slovenian A. flavicollis were closely related to each other and formed a homogeneous group with nucleotide diversity ranging from 0.5 to 4.9%. Sequences originating from Dobrava (Slo-1) and Kocevje (Slo-3) were closer to the prototype Dobrava strain (0.5–1.0%), whereas the sequence from Prekmurje (Slo-10) was more distant from them (nucleotide diversity of 4.4–4.9%). All
A. flavicollis-derived sequences from Slovenia showed high similarity to the sequences recovered from A. flavicollis trapped in neighbouring Bosnia and Greece (nucleotide diversity of 1.9–4.1% and 3.4–3.9%, respectively), as well as to the sequences recovered from Greek HFRS patients (3.9–6.5%). Thus, sequences originating from A. flavicollis and from HFRS cases formed a group that showed genetic diversity of 0.5–6.5% and clear geographical clustering.

Similar to what was observed for the M sequences, the S sequence recovered from the Slovenian A. agrarius (Slo-9) was quite distinct from other Slovenian S sequences. In fact, it was closer to DOB sequences from A. agrarius trapped in Slovakia and Russia (7.3–7.6% and 9.4% nucleotide differences, respectively) than to the A. flavicollis-derived sequence from the same locality (12.7%). Such differences did not fit the pattern of geographical clustering of genetic variants, but rather showed a host dependence. Thus, comparison of the both M and S segments of the A. agrarius-derived Slo-9 strain showed its significant divergence from all strains harbouring by A. flavicollis.

It should be noted that the values of sequence divergence presented in Tables 2 and 3 are calculated for the partial sequences of the M and S segments. When two isolates, Dobrava (prototype) and Saarcoma/160v, are compared, the values calculated for the partial S/N sequences (11.2 and 2.3% nucleotide and amino acid sequence divergence, respectively) are close to the values determined for the complete sequences (12.2 and 3.0%; Nemirov et al., 1999); the same can be seen for the nucleotide sequences of the M segment (20.6% for the partial sequences vs 18.8% for the complete sequences). However, the amino acid sequence divergence of the deduced G1 sequences shown in Table 2 are almost half those determined for the complete ORF of the M segment (3.3% for the partial sequences vs 6.2% for the complete sequences), suggesting that the divergences presented in Table 2 for the G1 amino acid sequences might be underestimates.

### Phylogenetic analysis

The phylogenetic analysis based on partial sequences of the S segment revealed that sequences originating from A. flavicollis, together with those recovered from HFRS cases, constitute a well-supported group (Fig. 2a). It is divided further...
Fig. 2. Phylogenetic trees based on partial sequences of the S segment (nt 377–761) (a) and partial sequences of the M segment (nt 1309–1590) (b). Bootstrap values are expressed as percentages of the total number of replicates (500). DOB strains originating from different rodent hosts and from specimens of HFRS patients are indicated by different shades of grey. Geographical areas where different subtypes were isolated are shown. Only those bootstrap values that exceed 70% (the bootstrap cut-off level; Hillis & Bull, 1993) are shown. Abbreviations for hantaviruses and DOB strains are given in Methods and footnotes of Tables 2 and 3.
into three lineages that include (i) human sequences from north-western Greece, (ii) sequences from *A. flaviocollis* trapped in Slovenia (Dobrava and Kocevje) and Bosnia and (iii) sequences from north-eastern Greece recovered either from *A. flaviocollis* (Gre-9 and Gre-13) or from HFRS patients (Gre-PR and Gre-TD). The sequence originating from *A. flaviocollis* from Prekmurje (Slo-10) is placed within the third lineage, but with low bootstrap support (48%).

The sequences originating from *A. agrarius* form two well-supported distinct lineages, which include variants from (i) Estonia and (ii) Russia and Slovakia. The clustering of these two lineages together, however, has low bootstrap support (40%), probably indicating a complex evolutionary history of DOB variants harboured by the striped field mouse. Most notably, the strain originating from *A. agrarius* from Slovenia (Slo-9) is situated (with 100% bootstrap support) within the second lineage, showing the closest relatedness to the Slovakian variants. Such a placement is not at all contradictory to the data from sequence comparison (Table 3) and supports the view of host-dependence rather than geographical clustering for this DOB variant. A phylogenetic tree obtained by the maximum parsimony method (which represents a character-based approach, distinct from that used by the distance-matrix methods) showed the same clustering (data not shown).

Similar to what was observed for the S segment sequences, all DOB strains originating from *A. flaviocollis* grouped together on the phylogenetic tree based on partial M segment sequences (nt 1314–1590) (Fig. 2b). Also, a geographical clustering can be seen for the strains from different locations in Slovenia: sequences from Dobrava (Dob), Kocevje (Slo-3 and Slo-4) and Gorjanci (Slo-1 and Slo-10), all situated in the south-eastern part of Slovenia, are clustered together, as are two sequences from the Tenetise area (Slo-5 and Slo-6), which is in the central part of the country. At the same time, like on the S-derived tree, the sequence originating from *A. agrarius* from Slovenia (Slo-9) is placed apart from all sequences recovered from *A. flaviocollis*, as are two other sequences originating from *A. agrarius*, from Estonia and Russia. These three are grouped together, albeit with low bootstrap support (42–44%).

Thus, on both the S- and M-derived phylogenetic trees, the *A. agrarius*-derived Slo-9 strain does not group together with strains originating from *A. flaviocollis* of the same geographical origin, but instead groups with other *A. agrarius*-derived strains from Russia, Slovakia and Estonia; i.e. it shows host-dependent rather than geography-dependent clustering.

### Discussion

#### Natural reservoirs for DOB in Slovenia

This report presents the first genetic evidence that DOB is harboured by two distinct species of *Apodemus* mice in Slovenia, *A. flaviocollis* and *A. agrarius*. Altogether, 53 *Apodemus* mice were found to be positive for hantavirus-specific antibodies. The frequency of hantavirus-positive rodents suggested a high occurrence of infection in all natural HFRS foci in Slovenia that were investigated. In most of the positive animals, the antibody titres were higher for HTN and DOB than for PUU, although no significant differences could be observed in some samples. The results of previous studies already indicated the presence of anti-hantavirus antibodies or hantavirus antigen in Slovenian *A. flaviocollis* and *A. sylvaticus* (Avsic-Zupanc et al., 1990). However, it was impossible to identify the virus by conventional serological methods (EIA and IFA). DOB, which was isolated from *A. flaviocollis* in Slovenia (Avsic-Zupanc et al., 1992), was later shown to be the aetiological agent of a severe form of HFRS occurring in this country as well as in the rest of the Balkans (Antoniadis et al., 1996; Lundkvist et al., 1997b; Avsic-Zupanc et al., 1999).

There is little information available in the literature on direct genetic identification of hantaviruses in rodents in southern Europe (Lundkvist et al., 1997b; Å. Lundkvist, personal communication; A. Papa, K. Nemirov, H. Henttonen, A. Antoniades, A. Vaheri, A. Plyusnin and O. Vapalahti, unpublished results). Therefore, one of the purposes of this study was to define DOB infection in *Apodemus* species in Slovenia by using molecular tools.

All but three serologically positive animals (94%) were found to be positive by a DOB-specific nested RT–PCR assay for the partial M segment and sequences were recovered from the two *Apodemus* species. While *A. flaviocollis* mice were abundant in all areas studied, *A. agrarius* mice were trapped in only two areas (Gorjanci and Prekmurje). That is in concordance with the findings of the mammalogists from the Slovenian Museum of Natural History (Krystufek, 1991). According to their studies of rodent populations in Slovenia, *A. flaviocollis* is distributed widely all over the country, while *A. agrarius* inhabits only the north-eastern area along the border with Hungary and Croatia and the sub-Mediterranean lowland.

It should be mentioned that DOB-positive *A. flaviocollis* were found in all five trapping sites, while positive *A. agrarius* were trapped in a single site only (Prekmurje). This probably reflects the limited distribution of the latter species in Slovenia.

#### Has a host-switch resulted in two subtypes of DOB?

Genetic and phylogenetic analyses provide strong evidence that DOB in Slovenia is represented by two distinct genetic lineages. The first lineage is harboured by *A. flaviocollis* and, at the moment, is represented by eight strains from five different locations distributed across the country. The genetic distances calculated for these strains show that they are closely related to each other, with a direct correlation of the similarity to their geographical distribution (in general, the genetic distances between the strains increase with increasing geographical separation). The data on genetic comparisons of the DOB sequences originating from *A. flaviocollis* are in good agreement with their phylogeny: on both the S- and M-derived trees they group together and, within this group, they form small
separate clusters according to their geographical origin. The reason(s) why such a clustering is seen better on the S-derived tree than on the M-derived tree remains unclear. One factor might be that the region of the M segment selected for the analyses is sub-optimal.

The second DOB lineage in Slovenia is so far represented by a single A. agrarius-derived strain from Prekmurje. This variant seems to occur rather rarely in Slovenia, where its rodent host species is not widely distributed. It is genetically and phylogenetically distinct from all DOB strains harbouring by A. flavirostris (including those from Slovenia, Greece and Bosnia) and instead shows closer relatedness to DOB strains harbouring by A. agrarius from Slovakia, Russia and Estonia. Most notably, both DOB lineages were found in co-circulation within the same location, suggesting that they are not mutually exclusive, i.e. they are sympatric.

These observations, taken together with other currently available data, raise several important questions. While the finding of distinct DOB genetic lineages in A. flavirostris and A. agrarius supports the hypothesis of hantavirus–host co-speciation and co-evolution (Plyusnin et al., 1996; Nichol, 1999; Plyusnin & Morzunov, 2000), the large genetic distance between HTN and the DOB variant found in A. agrarius is inconsistent with this hypothesis. In fact, the phylogenetic relationships of HTN and the DOB lineage in A. agrarius seem not to mirror those of their rodent hosts. Even taking into consideration the fact that A. agrarius from Europe (the host for one of the DOB variants) and from the Far East (the host for HTN) belong to two distinct subspecies, A. agrarius agrarius and A. agrarius mantchuricus (Chernukha et al., 1986), one would expect the two hantaviruses to be monophyletic, but the analysis shows them not to be (Fig. 2). Such a discrepancy might be explained by a host-switch event occurring during evolution of these viruses, similar to that reported recently for the hantaviruses TOP and KBR (Vapalahti et al., 1999). If that was the case for the DOB variants harboured by A. flavirostris and A. agrarius, the first could have acted as the ‘donor’ and the second as the ‘recipient’ for a host-switching hantavirus. To date, several other examples of host-switch events in hantaviruses are known (for reviews see Nichol, 1999; Plyusnin & Morzunov, 2000) and all of them are considered exceptions from the general flow of virus–host co-speciation and co-evolution.

The question of whether the two DOB variants represent distinct subtypes or even distinct hantavirus types (species) remains to be answered. Nevertheless, separation of the ‘classic’ DOB, with the Slovenian A. flavirostris-derived Dobrava isolate (Avsic-Zupanc et al., 1995) as the prototype, from Saaremaa virus (SAA), with an Estonian A. agrarius-derived isolate (Nemirov et al., 1999) as the prototype, does not seem totally illogical. Indeed, DOB and SAA, besides occupying distinct ecological niches (i.e. primary rodent reservoirs), show at least 4-fold differences in titres in neutralization tests (Nemirov et al., 1999; Å. Lundkvist, personal communication) and up to 6·2% diversity of amino acid sequences for the complete glycoprotein precursor; i.e. they fulfill two of the criteria currently accepted to define distinct hantavirus species (Elliott et al., 2000) and are very close to fulfilling the third (7% diversity), at least for the G1/G2 sequences. To clarify the issue, comparative serology of the DOB and SAA isolates should be studied in greater detail. Also, phylogenetic studies of the natural hosts of DOB and SAA from different areas of Europe will be needed, similar to those performed for Peromyscus mice, the hosts for SN and related hantaviruses in North America (Morzunov et al., 1998).

Finally, it is worth mentioning that DOB and SAA seem to possess different pathogenicity for humans. Although there is no direct evidence to date for this conclusion, none of the existing data contradict such a statement. The most severe HFRS cases (fatality rate among hospitalized patients of 9–12%) have been reported from the Balkans, where the ‘classic’ DOB is dominant (Antoniadis et al., 1996; Papa et al., 1998; Avsic-Zupanc et al., 1999). In contrast, in other parts of Europe, where one might expect SAA to dominate, together with its host rodent species, no fatality associated with DOB or DOB-like virus types has been registered (Lundkvist et al., 1997a, b, 1998; Meisel et al., 1998). The most prominent example here is the large DOB-associated outbreak in central Russia in 1991–1992, when 130 HFRS patients were hospitalized and no fatal cases occurred (Lundkvist et al., 1997a).

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