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Serological divergence of Dobrava and Saaremaa hantaviruses: evidence for two distinct serotypes

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SUMMARY

In order to investigate the serological relationship of Dobrava hantavirus (DOBV, originating from Slovenia) and the Dobrava-like Saaremaa virus (SAAV, recently discovered in Estonia) we analysed 37 human serum samples, 24 from Estonia and 13 from the Balkans, by focus reduction neutralization test (FRNT). Most of the Estonian sera (19), including all sera from Saaremaa island (12), reacted with higher FRNT end-point titres to the local SAAV; the majority of them (15 and 11, respectively), with at least fourfold or higher titres to SAAV than to DOBV. In contrast, out of the 13 sera collected in Slovenia, Bosnia-Herzegovina and Greece, only one reacted more strongly with SAAV (with a twofold higher titre), while 10 of these sera reacted more strongly with the local DOBV (9/10 with fourfold or higher titres). These results indicate that DOBV and SAAV define unique hantavirus serotypes.

INTRODUCTION

Dobrava hantavirus (DOBV) belongs to the *Bunyaviridae* family and causes haemorrhagic fever with renal syndrome (HFRS) in Europe [1–4]. HFRS is a complex of human diseases, which occur in Eurasia and are mainly characterized by fever and renal dysfunction, sometimes with haemorrhagic manifestations. Hantaviruses are rodent-borne enveloped viruses with negative-sense single-stranded RNA genomes, usually with a close association between the virus type and the host species [5]. The genome is tripartite and encodes the nucleocapsid protein (S segment), two envelope glycoproteins, G1 and G2 (M segment), and the viral RNA-polymerase (L segment) [6]. Humans are thought to be infected by inhalation of aerosolized rodent excreta. The serological cross-reactivity among the different hantavirus serotypes is pronounced in assays such as IFA, ELISA or immunoblotting, and to date only the neutralization test is suitable for serotyping.

DOBV was first isolated from the yellow-necked field mouse (*Apodemus flavicollis*) [2]. DOBV or DOB-like viruses have been shown to circulate in large areas of central and eastern Europe and cause outbreaks or sporadic cases of HFRS [1, 3, 4, 7–9]. A DOB-like virus was recently identified and subsequently isolated from the striped field mouse (*Apodemus agrarius*) trapped on Saaremaa island, Estonia [10, 11]. Genetic analysis revealed up to 19% diversity between the M segment sequences of the prototype DOBV from Slovenia and the Saaremaa isolate (SAAV), which reflected a more than 6% diversity of the deduced amino acid sequences of the G1/G2 protein. The virus was initially regarded as an *A. agrarius*-carried variant of DOBV, but with accumulating genetic data, its re-classification as a subtype of DOBV or as a unique hantavirus has been discussed [5, 12].

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Later, SAAV-like strains have been identified in *A. agrarius* in central Russia [13] and Slovakia [9]. Most recently, analysis of *A. flavicollis* and *A. agrarius* from the same locality in Slovenia, revealed significant genetic and phylogenetic differences between the hantavirus sequences recovered from the two rodent species [12]. A similar picture was also observed in Slovakia [14]. These findings have proven that both types of the virus (DOBV in *A. flavicollis* and SAAV in *A. agrarius*) can co-circulate within the same area without mixing, which is in line with the view of close association between hantavirus types and host species [5].

The purpose of this study was to investigate if DOBV and SAAV represent distinct hantavirus serotypes.

METHODS

Patient sera

A total of 37 sera from apparently healthy blood donors or from patients who had recently had HFRS (serum samples drawn at least 1 month after onset of disease) were collected from different localities in Europe: 24 sera from Estonia (Golovljova and Lundkvist, unpublished), 4 from Slovenia [3], 8 from Bosnia-Herzegovina [4], and 1 from Greece (kindly provided by Dr Anna Papa). All sera were previously determined as DOBV-like infections by focus reduction neutralization assay (FRNT) including all hantaviruses known to cause HFRS i.e. DOBV, DOBV-like strain Saaremaa, Puumala (PUUV), Hantaan (HTNV) and Seoul (SEOV) viruses.

Focus-reduction neutralization test (FRNT)

For FRNT analyses, DOBV strain Slovenia [2], DOBV-like strain Saaremaa [11], HTNV strain 76-118, SEOV strain 80-39, and PUUV strain Sotkamo were used.

Focus-reduction neutralization test (FRNT) was performed as described earlier [4]. Briefly, sera were serially diluted and mixed with an equal volume of diluted virus, containing 30–70 focus forming units (FFU)/100 μl. The mixture was incubated at 37 °C for 1 h and subsequently inoculated into wells of six-well tissue culture plates containing confluent Vero E6 cell monolayers. The wells were overlaid with a mixture of agarose and tissue culture medium and incubated for 7–13 days. The agarose was removed from the wells and the cells were fixed. Rabbit anti-hantavirus sera, followed by peroxidase labeled goat antibodies to rabbit IgG, were added to indicate virus-infected cells. TMB was used as substrate and foci were enumerated. An 80% reduction of the number of foci, as compared to the virus control, was used as the criterion for virus neutralization titres.

RESULTS

Serological relationship between DOBV and SAAV

In total, 37 sera, 24 from Estonia and 13 from the Balkans, were examined for neutralizing antibodies to SAAV and DOBV. Most of the Estonian sera (19/24, 79%) showed twofold or higher end-point titres to SAAV, while only 2/24 (8%) reacted with a higher titre to DOBV (Fig. 1). In contrast, of the 13 sera collected in the Balkans, 10/13 (77%) reacted with twofold or higher titres to DOBV, while only one reacted with a twofold higher titre to SAAV. The majority of the samples (15 and 9, respectively) reacted with at least fourfold higher titres to the local virus. Thus, altogether 24 sera reacted with fourfold or higher titres to one of the two viruses. This result clearly identified DOBV and SAAV as two distinct hantavirus serotypes.

Five serum samples, three from Estonia and two from the Balkans, were not separable by FRNT as some had equally low end-point titres (80), while others had high but equal titres (10240) to DOBV and SAAV.

Epidemiology in Estonia

When the FRNT results of the Estonian sera were classified according to their geographical origin, an interesting pattern was observed. Of a total of 24 sera from Estonia, 12 were collected on the Saaremaa island, which is located to the west of mainland Estonia, while the other 12 originated either from the neighbouring island Vormsi or from mainland Estonia. All but 1 of the 12 sera collected on Saaremaa island reacted with at least fourfold higher titres to SAAV as compared to the titres to DOBV (Table 1). Moreover, 9 of these sera reacted with at least 8-fold higher titres, 3 sera showed at least 16-fold higher titres and 1 of them had as much as 32-fold higher titres to SAAV. In contrast, none of the 12 sera originating from mainland Estonia or Vormsi island showed more than eightfold higher titres to SAAV.

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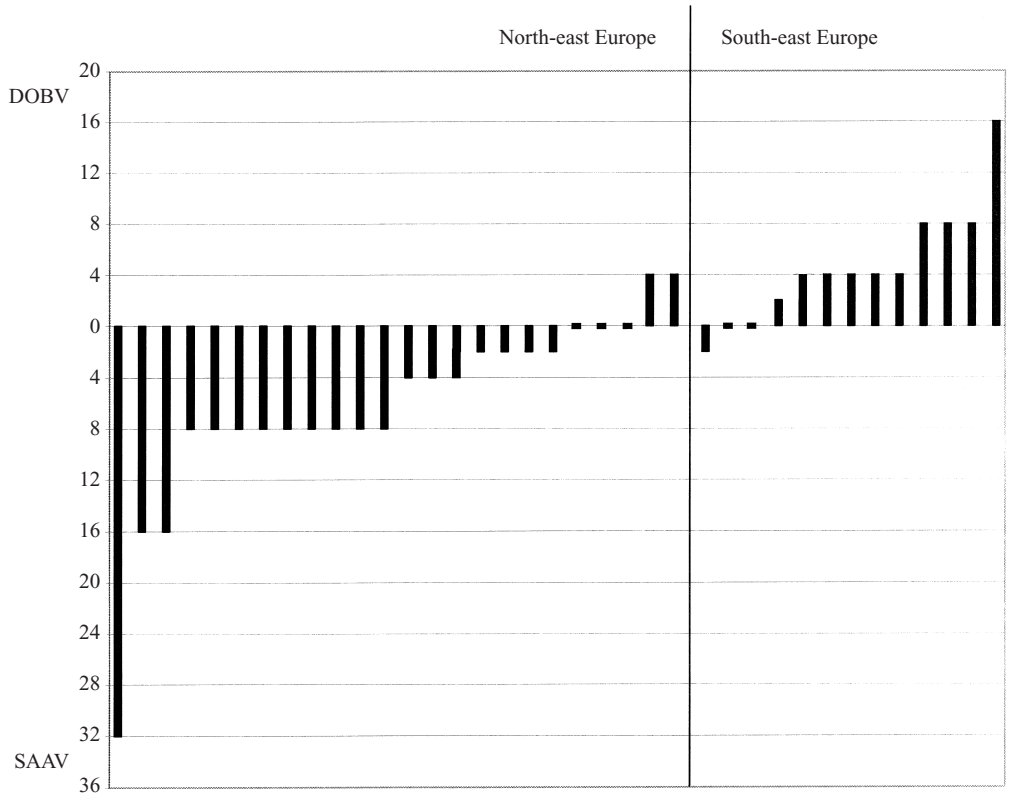

Fig. 1. FRNT analyses of human sera originating from north-eastern *vs.* south-eastern Europe. The bars indicate the ratio of neutralizing end-point titers to DOBV *vs.* SAAV for each serum sample.

Thus, most samples from Saaremaa island showed a remarkably high specificity for SAAV, which was not observed for the samples from the other parts of the country.

Two Estonian sera (both from the south-eastern Jõgevamaa county) reacted fourfold higher to DOBV; a result which may indicate the circulation of this hantavirus in the area.

DISCUSSION

The present study revealed a clear serological distinction between DOBV and SAAV as 23 human sera reacted with at least fourfold higher neutralizing end-point titre to one of the two viruses, including four sera that neutralized SAAV at high titres but did not at all react with DOBV (or HTNV, SEOV or PUUV). Thus, DOBV and SAAV meet the classical criteria for unique hantavirus serotypes. These findings confirmed the earlier suggestions for a distinction between DOBV and SAAV [5, 12], based on different primary rodent reservoirs, and up to 6·1% diversity for the amino acid sequence of the complete glycoprotein precursor. Using the criteria currently selected to define hantavirus species [15] and taking into account the presently available data on ecology, genetics, and serology, DOBV and SAAV should be considered as distinct species. Similar data were recently reported for the closely related HPS-causing New York (NYV) and Sin Nombre (SNV) hantaviruses [17], which also have unique mammalian hosts and geographical distribution, but most similar G1 and G2 surface proteins (93 and 97% identity, respectively). In that study, sera from HPS-patients showed 4- to 32-fold higher titres to either of the two viruses and the results were accordingly interpreted as NYV and SNV define unique serotypes.

The epidemiology of HFRS in central and eastern Europe is complex due to the existence of several rodent species known to be potential carriers of pathogenic hantaviruses, and the cases range from clinically severe, usually attributable to DOBV-like infections, to milder, more typical of PUUV-like infections. Here we show that the actual picture is even more complicated due to the presence of SAAV.

Our results further indicated that SAAV might be the most common DOBV-like infection in north-eastern Europe and that DOBV is the most common cause of DOBV-like infection in south-eastern Europe. Interestingly, and in contrast to the common

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Table 1. *Human sera originating from various regions in Estonia analysed by FRNT*

Origin	No.	FRNT end-point titres DOBV	SAAV
Saaremaa island			
Est (Saaremaa)	674	< 40	640
Est (Saaremaa)	678	40	320
Est (Saaremaa)	701	40	320
Est (Saaremaa)	711	160	640
Est (Saaremaa)	712	40	80
Est (Saaremaa)	719	40	160
Est (Saaremaa)	715	40	320
Est (Saaremaa)	723	< 40	320
Est (Saaremaa)	728	40	1280
Est (Saaremaa)	2108	< 40	320
Est (Saaremaa)	2123	40	640
Est (Saaremaa)	2129	< 40	320
Vormsi island			
Est (Vormsi)	27	160	320
Est (Vormsi)	33	40	160
Mainland Estonia			
Est (Raplamaa)	2137	40	80
Est (Võrumaa)	1716	40	320
Est (Lääne-Virumaa)	1258	< 160	80
Est (Pärnumaa)	2248	40	320
Est (Ida-Virumaa)	1320	< 40	80
Est (Harjumaa)	883	40	320
Est (Jõgevamaa)	636	640	160
Est (Jõgevamaa)	666	2560	640
Est (Läänemaa)	949	640	640
Est (Läänemaa)	985	10240	10240

pattern, two serum samples from south-eastern Estonia showed fourfold higher titres to DOBV. This result may indicate that, in addition to SAAV, also DOBV causes human infections in north-eastern Europe, which is in line with the presence of *A. flavicollis* also in this area [5]. Other, less likely explanations could be that the two Estonian individuals from which the serum samples showed fourfold higher titres to DOBV may have been infected outside Estonia, or that the immune responses in some individuals do not mirror the causative agent. Five serum samples were not separable by FRNT as they had equally end-point titres to SAAV and DOBV. One possible explanation is a lower specificity of the immune responses in certain individuals, which is then mirrored in the inability to differentiate these two closely related viruses. Other causes may also be possible, e.g. an exposure to both serotypes, a decrease of virus-specificity over time after the infection, a decreased specificity due to repeated freeze-thawing of the sample, or for the samples with low but equal titres – a still unknown but closely related hantavirus.

The detailed analysis of the Estonian serum samples revealed another interesting observation. Most samples from Saaremaa island, located to the west of mainland Estonia, showed a remarkably high specificity for SAAV, which was not found for the samples from the neighbouring island Vormsi or mainland Estonia. This might indicate an independent evolution of SAAV over a long time period at this geographically separated location. Further search for DOBV- and SAAV-like strains from mainland Estonia is in progress.

The recently reported human DOBV-like infections in Germany and Slovakia, as well as the presence of SAAV in *A. agrarius* from Slovakia and Slovenia, indicated that SAAV might cause HFRS also in central and south-eastern Europe [9, 12, 14]. SAAV (also referred to as DOBV-Aa) has been suggested as the major cause of DOBV-like disease in central Europe, although direct evidence remains to be obtained [14]. Our results were to some extent in line with the suggestions above i.e. the geographical distribution of human SAAV infections might cover large areas of eastern Europe, as three serum samples from south eastern Europe showed equal or higher titres to SAAV as compared to DOBV. It should be stressed, that since all previous serological studies have regarded SAAV and DOBV as identical hantaviruses, the FRNT analyses have so far been based only on one of the viruses (usually DOBV/Slovenia).

The current data indicate that the pathogenicity for humans of DOBV and SAAV may differ. The most severe HFRS cases have been reported from the Balkans (reported fatality rate among hospitalized patients 9–12%), where DOBV is believed to be dominant [1, 3, 8]. In contrast, in areas where one might expect SAAV to be dominating, no fatalities associated with DOB-like viruses have been reported [7, 14, 16]. However, no fatal cases were registered during the 1995–6 outbreak in Bosnia-Herzegovina, an area where DOBV might be dominating [4, M. Hukic and Lundkvist, unpublished).

It should be noted that SAAV and DOBV have been shown to co-circulate both in Slovenia and in Slovakia and that the actual number of SAAV *vs.* DOBV infections is unknown. Therefore, further studies on the distribution of human infections/disease caused either by DOBV or SAAV are urgently needed in large parts of Europe.

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