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Letter to the Editor

Genetic Interaction between Dobrava and Saaremaa Hantaviruses: Now or Millions of Years Ago?

In their paper (3), Klempa and coauthors reported results on (phylo)genetic analyses of hantaviruses occurring in two species of *Apodemus* mice, Dobrava virus (DOBV)-Af (from DOBV associated with *A. flavicollis*) and DOBV-Aa (associated with *A. agrarius*), and concluded that "DOBV-Af and DOBV-Aa are distinct but also subject to genetic exchanges that affect their evolutionary trajectories." From these grounds, they then criticize our view that DOBV-Aa is a distinct hantavirus species, Saaremaa virus (SAAV) (1, 7, 9). (When we first discovered SAAV, it was designated a genetic lineage of DOBV [5, 8], and it was only with accumulating data that we came to the conclusion that it represents a distinct, new hantavirus.) We feel that the claim of Klempa et al. for currently occurring genetic exchanges between DOBV and SAAV is not well grounded and their view lacks an evolutionary dimension.

Although DOBV and SAAV are closely related, there are three most important differences. (i) While DOBV causes severe hemorrhagic fever with renal syndrome (fatality up to 12%), SAAV causes a milder form of the disease, similar to nephropathia epidemica (references 2 and 4 and our unpublished data). (ii) DOBV and SAAV are clearly differentiated by classical serology (reference 1 and our unpublished data). (iii) DOBV is lethal to suckling mice, while SAAV is not (J. Klingström and Å. Lundkvist, unpublished data).

In fact, in answering the crucial question of whether DOBV and SAAV are distinct entities or not, Klempa et al. came up with some controversy themselves: on the one hand, the two types are distinct; on the other, they are subject to genetic interactions (reassortment and recombination). Following this logic, DOBV and SAAV should, at the same time, be reproductively isolated and capable of "breeding".

In our opinion, we are dealing here with a case of host switching, which occurred in the evolution of these hantaviruses (7, 9, 11), and we most recently obtained the crucial piece of evidence to support our view (6). Phylogenetic analysis revealed a discrepancy in the relationships of DOBV, SAAV, and Hantaan virus and their respective rodent hosts. This discrepancy is consistent with the transmission of (pre)DOBV/SAAV between *A. flavicollis* and *A. agrarius*, which resulted in the ecological and reproductive isolation of the two hantaviruses. Crucially, the time point of the host switching, 2.7 to 4.0 million years ago, was closer to the present than the estimated time of split between the two *Apodemus* species (<6.5 million years ago).

We would also like to address two specific points. (i) The claim for the reassortment between DOBV and SAAV is based on the lack of monophyly of all of the SAAV S segment sequences. Klempa et al., using a TREE-PUZZLE program, showed for their set of the S segment sequences that the Estonian lineage of SAAV is not monophyletic with the Russian-Slovakian lineage (see Fig. 1 in reference 3). We shared this opinion until a year ago, when the set of S sequences in our analyses was smaller. However, when more strains became available (e.g., DOBV strains from Greece and Russia and an SAAV strain from Denmark), all SAAV S sequences turned monophyletic and, therefore, there was no longer any contradiction in the S- and M-segment-based phylogenies. This conclusion was still valid when the new DOBV strain from East Slovakia, Esl/400Af, was added to the data set. For these calculations we used the distant matrix approach (Fitch-Margoliash method). TREE-PUZZLE gave either monophyly of all SAAV strains (HKY model of nucleotide substitutions) or multifurcation of DOBV and SAAV lineages (TN model). Thus, the "reassortment claim" is not supported by the phylogenies seen with the use of larger data sets.

(ii) The claim for the recombination between DOBV and SAAV. Recombination points suggested for the M segment of one of the SAAV strains (see Fig. 3 in reference 3) are not sharp, as one would expect for a recent recombination (10), but "diffuse" (which would be a logical result of genetic drift with the time passing). The idea of currently occurring recombination also demands a better explanation of the fact that the recombination seems to occur not only between the sympatric DOBV and SAAV from the same location in East Slovakia but between the Slovakian and Estonian viruses as well.

The host-switching hypothesis does not totally exclude the possibility of genetic exchange (in the past!) between the newly established ancestral SAAV in *A. agrarius* and the still very closely related (if not almost identical) ancestral DOBV in *A. flavicollis*—in biotopes where the two pairs of hosts and viruses coexisted. However, as in every case of origination of a new species, the crucial prerequisite is its reproductive isolation. Consequently, all of the genetic exchanges between the two diversifying hantaviruses should have stopped; otherwise, SAAV in *A. agrarius* (DOBV-Aa) would never have become distinct.

This reasoning leads us to the conclusion that Klempa et al. could have been right were they not two to four million years late.

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Authors' Reply

Our paper (4) describes phylogenetic aspects of the hantavirus species Dobrava (DOBV) present in at least two different *Apodemus* species, *A. flavicollis* (DOBV-Af) and *A. agrarius* (DOBV-Aa). It shows that the virus strain Saaremaa could be the result of reassortment processes between members of the DOBV-Af and DOBV-Aa lineages in their evolutionary history rather than the representative of a new, unique hantavirus species that is distinct from DOBV. The title of the Letter to the Editor by Plyusnin et al. is somewhat misleading, since the genetic interactions proposed by us did not occur between two independent virus species as one could infer from their title and, even more important, in no part of our paper (4) we had postulated that the observed genetic exchanges are "now" or "currently" occurring. Nevertheless, Plyusnin et al. take this claim of "currently occurring" genetic exchanges as the basis for their explanations regarding at what time during evolution genetic exchange processes could have had occurred. In our opinion, there are not enough data available that can be used to accurately estimate the point at which the postulated genetic exchanges between DOBV-Af and DOBV-Aa occurred nor is there information that can decisively demonstrate that genetic exchange between the two lineages has ceased.

DOBV-Aa in general, and the Saaremaa strain in particular, is not sufficiently different from DOB-Af to allow it to be differentiated according to the criteria recommended by the International Committee on Taxonomy of Viruses (2), which are ecological, genetic, and serological and do not include pathogenicity criteria. On the basis of this recommendation we have considered the pros and cons in the definition of DOBV-Aa (or even Saaremaa) as a distinct virus species (4, 9). The different hosts of DOBV-Af and DOBV-Aa and the sympatric occurrence of members of these virus lineages support the idea of different species, but other criteria are not fulfilled, namely, those of sufficient amino acid sequence differences in the nucleocapsid and glycoproteins as well as the absence of genetic exchanges between the virus lineages and of the formation of natural reassortants. A fourfold difference in two-way cross-neutralization tests of immune sera was exhibited by a majority of human sera tested; however, a substantial number of serum samples did not exhibit this difference. Inasmuch as there are no nucleotide sequence data from these infected persons available, it is not possible to finally explain this reactivity pattern. Moreover, no results are available that cross-compare the serologic reactivities of laboratory animals experimentally infected by defined virus strains.

The occurrence of genetic exchange (reassortment and very probably also recombination events) in the evolution of the DOBV species is one important factor in deciding the taxonomic status of the two virus lineages. Plyusnin et al. point out that they also had believed in genetic reassortment processes before 1 year ago but that the introduction of new, additional sequences changed their conclusions. Unfortunately, this claim cannot be confirmed by us. Those authors did not forward any exact information about the new sequences they have considered, and in fact, no additional complete S and M segment sequences from Greece, Russia, and Denmark can be found in GenBank as of today (17 March 2003). They also do not present any details of the results of their analysis, including statistical support, branch lengths, or tree rooting, so their claim cannot be reasonably discussed. The methodological bases of their analyses are also unclear. Neither a "distant matrix approach" nor distance-based methods in general are implemented in TREE-PUZZLE (www.tree-puzzle.de), despite allusions to those capabilities by the authors.

We have never claimed that the proposed recombination events are occurring "now" or "currently" (4). The longer evolutionary history is one factor which could explain the absence of sharp recombination breakpoints in the presented analysis. Also, one cannot expect a sharp signal because one of the parent lineages was absent from the data set, which is clearly indicated by the missing curve that would otherwise have come up in the bootscanning analysis (4). Furthermore, the "sharpness" of the signals is hampered by the application of a window-based technique such as bootscanning and is dependent on the size and the overlap of the signals.

To explain why two distantly related virus species, Hantaan virus and DOBV, have been found in the same host species, *A. agrarius*, while closely related virus lineages, DOBV-Af and DOBV-Aa, are hosted by two different rodent species, *A. flavicollis* and *A. agrarius*, a host switch of ancient DOBV from *A. flavicollis* to (European) *A. agrarius* has been suggested (10). We also believe that a host switch occurred in the evolution of DOBV-Af and DOBV-Aa, but this problem was not the subject of our article and does not affect our presented results (4). At the current stage of knowledge, insufficient data (complete viral nucleotide sequences) are available to define the scenario and the direction of a possible viral host switch between different *Apodemus* species. Plyusnin et al. refer to their "crucial piece of evidence" to support the idea of a host switch (5). In their comparative analysis of phylogenetic trees from rodent and hantavirus sequences, those authors included only Saaremaa sequences but not the other available DOBV-Aa sequences (5); inclusion of these sequences in the analysis would have changed their "tanglegram" crucially. Moreover, those authors claim in this paper, as well as in its adjacent erratum, to have computed bootstrap support values from 10,000 puzzle steps by using TREE-PUZZLE. However, the TREE-PUZZLE package is not able to infer bootstrap values from puzzling step trees. We suppose that the cited values (5) are PUZZLE support values, which should not be mistaken with bootstrap values since they are derived differently (6; TREE-PUZZLE manual [www.tree-puzzle.de]).

DOBV-Af and DOBV-Aa are closely related virus lineages that have undergone exchange of genetic material in the form of reassortment and probably recombination in the past. Such events must be very rare, or it would not be possible that they

can be maintained as distinct genetic lineages. Thus, the virus lineages were (and are) largely reproductively isolated. There are no means currently of decisively dating the observed genetic exchanges between the two DOBV subtypes; however, it should be noted that a genetic interaction between almost identical ancestral strains at the very beginning of their divergent evolution as suggested by Plyusnin et al. would be not detectable in today's studies. Reassortment and recombination events can only be verified by sequence comparisons when the parental lineages became different enough in their (independent) evolution. Dual infection within a single rodent provides the opportunity for genetic exchanges between virus lineages. Incidental hantavirus infections of heterologous rodent species are well documented in nature; such "spillover" infections can involve host species that serve as natural reservoirs to their own hantavirus and are able to allow the formation of reassorted or recombinant viruses.

Though not relevant for taxonomical and evolutionary considerations, the possibility of different virulence characteristics associated with DOBV-Af and DOBV-Aa is of considerable clinical interest. One could speculate that the amino acid differences in the viral proteins could contribute to this property. As far as we know, the only clinically well-characterized patients from regions where DOBV-Aa strains were found in parallel in the rodent population are from Central Europe, and exclusively mild clinical courses have been described there (7, 8). However, before further speculating, one would need to obtain hantavirus genomic sequences from those patients and to compare them with the viral sequences from different *Apodemus* species. Moreover, we know from studies on Hantaan virus that the exchange of as little as one amino acid in the viral glycoprotein can change the virulence dramatically (1, 3). It is widely accepted that changes in pathogenicity caused by a few amino acid substitutions are without relevance for taxonomical considerations.

To better understand the phylogeny of hantaviruses, occurrence of genetic exchanges, and species definition, analysis of larger sets of complete nucleotide sequence data is needed. In the investigation of DOBV evolution one probably needs also to include virus sequences obtained from more species of the genus *Apodemus.* Such investigations should be more helpful than speculations of whether one could date processes to two or four million years before the present. The ready proposal of new species within the genus *Hantavirus* without compelling support for species status would generate confusion rather than scientific benefit.

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