Reassortment events in the evolution of hantaviruses

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Abstract
Hantaviruses (order Bunyavirales, family Hantviridae), known as important zoonotic human pathogens, possess the capacity to exchange genome segments via genetic reassortment due to their tri-segmented genome. Although not as frequent as in the arthropod-borne bunyaviruses, reports indicating reassortment events in the evolution of hantaviruses have been recently accumulating. The intra- and inter-lineage reassortment between closely related variants has been repeatedly reported for several hantaviruses including the rodent-borne human pathogens such as Sin Nombre virus, Puumala virus, Dobrava-Belgrade virus, or Hantaan virus as well as for the more recently recognized shrew-borne hantaviruses, Imjin and Seewis. Reassortment between more distantly related viruses was rarely found but seems to play a beneficial role in the process of crossing the host species barriers. Besides the findings based on phylogenetic studies of naturally occurring strains, hantavirus reassortants were generated also in in vitro studies. Interestingly, only reassortants with exchanged M segments could be generated suggesting that a high degree of genetic compatibility is required for the S and L segments while the exchange of M segment is better tolerated or is particularly beneficial. Altogether, the numerous reports on hantavirus reassortment, summarized in this review, clearly demonstrate that reassortment events play a significant role in hantavirus evolution and contributed to the currently recognized hantavirus diversity.

Keywords Hantavirus · Reassortment · Evolution

Introduction

First reports that genome segment exchange, in other words genetic reassortment events, could have been occurring in the evolution of hantaviruses are rather old and emerged soon after the discovery of hantavirus cardiopulmonary syndrome-causing Sin Nombre virus (SNV) in the United States [1, 2]. Until recently, reassortment (more precisely its absence) was even used as one of the taxonomical species demarcation rules. In general, it was considered as rather exceptional process in the hantavirus evolution. However, reports on conflicting findings in the segment-specific evolutionary trees suggesting reassortment have been recently accumulating across both the “old” rodent-borne hantaviruses as well as the more recently recognized new hantaviruses found in non-rodent hosts. In light of these findings, it seems that reassortment is more common among hantaviruses than previously reported. Moreover, it is highly likely that the recent progress in the utilization of next-generation-sequencing (NGS) technologies leading to massive increase of full genome sequences will bring more findings in this area, too. It is, therefore, time to review and summarize the current knowledge on reassortment findings in the recently established family Hantviridae.

Hantaviruses (order Bunyavirales, family Hantviridae) are enveloped, single stranded RNA viruses with segmented genome of negative polarity. The genome is composed of three segments, small (S) segment encoding nucleocapsid protein (N protein) [3], medium (M) segment encoding glycoprotein precursor (GPC) co-translationally cleaved into the envelope glycoproteins Gn and Gc [4], and large (L) segment encoding the L protein primarily serving as the viral RNA-dependent RNA polymerase (RdRp) [5].

Virus entry into cells is mediated by binding to a cell surface receptor. Integrins are considered to be the main
receptors for hantaviruses at least in vitro [6–8] but other molecules, complement decay accelerating factor (DAF) [9], and globular heads of complement C1q receptor (gC1qR) [10], were reported to mediate hantavirus infection in cultured cells, too. The entry then proceeds through clathrin-dependent endocytosis shown for the prototypical Hantaan virus (HTNV) [11] and/or other pathways including micropinocytosis, clathrin-independent receptor-mediated endocytosis, or other routes [12, 13]. Viral particles are then trafficked to late endosomes. Low Ph-triggered, Gc-mediated virus cell membrane fusion releases viral genetic material into the cytoplasm. Transcription of the viral mRNAs includes the process of cap-snatching and involves localization of N and L proteins to cytoplasmic processing bodies (P bodies) where they use the caps of host mRNAs destined for degradation [14]. The hantavirus RNA synthesis is initiated by the prime-and-realign mechanism [15]. The endoplasmic reticulum-Golgi intermediate compartment (ERGIC) is considered to be the site of viral replication [16]. The virions are assumed to bud into the cis-Golgi and then transported to the plasma membrane for release, presumably via recycling endosomes [17].

When transmitted to humans, hantaviruses can cause severe disease. Transmission occurs usually through inhalation of aerosolized rodent excreta and rarely via biting by infected animals [18], but the human intestinal tract is a possible entrance port, too [19]. Hantaviruses present on the American continent, such as SNV or Andes virus (ANDV) cause hantavirus (cardio)pulmonary syndrome. HTNV and Seoul virus (SEOV) in Asia and Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV) in Europe are most common hantaviruses causing hemorrhagic fever with renal syndrome. Both diseases share the main pathogenetic mechanisms involving changes in blood coagulation, vasodilatation, and disturbances in the barrier function of the capillaries, resulting in capillary leakage and inflammatory processes in the affected organs [18].

Hantaviruses produce chronic and asymptomatic infection in their reservoir hosts, small mammals. Besides with hantaviruses typically associated rodents, other small mammals such as shrews, moles, and bats were identified as hantavirus reservoir hosts during the last decade [20–22]. Very recently, using a large-scale meta-transcriptomic approach, hantavirus-related sequences were identified even in reptiles, ray-finned fish, and jawless fish [23]. Although there are accumulating exceptions, hantaviruses are in general considered to be host-specific. A particular hantavirus is usually transmitted only by one or few closely related host species. This association is at least partially reflected also in their phylogeny, particularly among the rodent-borne hantaviruses. Therefore, hantaviruses have been considered to have co-evolved with their hosts over millions of years [24]. Recent phylogenetic analyses including the more recently discovered shrew-, mole-, and bat-borne hantaviruses revealed a complex evolutionary history where not only virus-host co-divergence but also cross-species transmission and ancient reassortment events played a role. Furthermore, these analyses also suggest that shrews, moles, or bats might have been the hosts of ancestral hantaviruses [25–28].

Reassortment is defined as exchange of gene segments between viruses that co-infect the same cell, which can result in the formation of progeny viruses that are genetically distinct from both parental viruses. Therefore, reassortment can create viral progeny conferring important fitness advantages. On the other hand, successful reassortment between two parental strains during co-infection requires a high degree of genetic compatibility including intricate packaging signals and RNA–RNA and/or RNA–protein interactions [29].

Reassortment is particularly well known for influenza A virus where it is associated with the antigenic shift and emergence of new pandemic strains [30]. However, all viruses with segmented genomes possess the capacity to exchange genome segments. Reassortment has been well documented for several other pathogenic viruses such as reoviruses, arenaviruses, or bunyaviruses [29, 31, 32]. Clearly, the ability of important human pathogens to reassort not only has implications for their ongoing evolution but can also lead to changes in their virulence and transmission efficiency and, therefore, has impact on public health.

Reassortment within the order Bunyavirales

Bunyaviruses (order Bunyavirales) with their tri-segmented genome are obvious candidates for reassortment playing role in their evolution. Indeed, reassortment seems to be rather frequently reported within the order, especially within the family Peribunyaviridae (former genus Orthobunyavirus). Particularly interesting is the fact that there are frequent reassortment events found between distinct viruses (i.e., heterotypic reassortment). Briese et al. [32] even suggested that most if not all currently recognized bunyaviruses in fact represent reassortants of existing or extinct viruses. The high frequency of reassortment might be explained by the fact that many of these viruses are arthropod-borne viruses (arboviruses) and are, therefore, capable of alternately replicating in hematophagous arthropods and vertebrates. Dual infections of arthropod hosts provide considerable opportunity for reassortment of the genome segments. Particularly mosquitoes and culicoids (unlike ticks) feed frequently, providing a greater opportunity for dual infections in them as well as in their vertebrate hosts. Another interesting phenomenon is the super-infection resistance which may prevent secondary infection by closely related bunyaviruses and thereby reduce the frequency of co-infections. However, it may actually promote opportunities for segment reassortment between more distantly related bunyaviruses [32].
Numerous examples of natural occurrences of reassortment can be found across the order and were recently systematically reviewed by Briese et al. [32]. For instance, Jatobal virus and Iquitos virus of the Simbu serogroup of orthobunyaviruses are both reassortants containing S and L segments of Oropouche virus and a unique M segment of a yet unrecognized Simbu serogroup virus [33, 34]. Complex reassortment scenarios were reported also for Shamonda and Schmallenberg viruses [35]. Similarly, several viruses of the group C orthobunyaviruses such as Apeu, Murutucu, and Itaqui, represent reassortants with various combinations of segments from Marituba, Caraparu, and Oriboca viruses [36]. Another well-documented example within the Bunyamwera serogroup viruses is the hemorrhagic fever causing Ngari virus (and its isolate Garissa virus) which is a reassortant containing S and L segments of Bunyamwera virus and M segment of Batai virus. This is particularly interesting because Ngari virus can be associated with large outbreaks of severe illness in East Africa while its parents are reported to cause rather mild symptoms in humans but more severe symptoms including abortions and teratogenic effects in livestock [37–40].

Among phleboviruses, multiple inter-lineage reassortment events were reported for Rift Valley virus [41] and for severe fever with thrombocytopenia syndrome virus [42, 43]. Moreover, some phleboviruses such as Aguacate [44] or Granada [45] are considered to be heterotypic reassortants. Interestingly, reassortment events have been so far repeatedly reported only for Crimean-Congo hemorrhagic fever virus [46–49] but for no other members of the Nairovirus family.

**Hantaviruses: mostly intra-species (homotypic) reassortment**

It is interesting to note that hantaviruses are, in contrast to other bunyaviruses, not transmitted or hosted by arthropods but are tightly associated with small mammals as their reservoir hosts. Based on the concept of Briese et al. [32], this fact should reduce the extent of co-infections and thereby also reduce the probability of reassortment events. Indeed, most of the reports on reassortment in hantaviruses are limited to inter-lineage events within the same virus species usually carried by a single reservoir host (Table 1). The phenomenon of heterotypic reassortment frequently seen in orthobunyaviruses or phleboviruses seems to be very rare or at least could not be well-documented yet.

First findings indicating reassortment events in hantaviruses were reported for SNV soon after its discovery. Li et al. [1] analyzed complete S and M segment sequences of two virus isolates from eastern California and found that while their M segment sequences differed from one another by only 1%, the S segments differed by 13%. They concluded reassortment as the most likely explanation for their data. These findings were then confirmed and further extended by the analyses of SNV sequences obtained from deer mice (*Peromyscus maniculatus*), the principal host of SNV, trapped in Nevada and eastern California. Phylogenetic analyses of all three segments indicated that several segment exchanges were possible but those involving M segment were found most frequently. Conflicting signals in the S and M segment-based phylogenetic trees suggesting reassortment were also found in a more recent study involving SNV sequences obtained from deer mice collected in Colorado, New Mexico, and Montana from 1995 to 2007 [50].

Occurrence of reassortment events is well documented also for the most common European hantavirus, Puumala virus (PUUV) associated with bank voles (*Myodes glareolus*). In a series of systematic studies performed in central and northern Finland, notably high frequency of reassortment, 19.1–32%, could be observed [51–53]. Moreover, one interesting phenomenon could be noticed. The studies in central Finland identified reassortants between two phylogenetic clusters within the same, Finish lineage [51, 53]. In this case, basically all six possible segment combinations were found and the most common were those schematically designated as $S_B M_A L_A, S_A M_B L_B, S_B M_A L_B$ where the S, M, and L capital letters stand for the genomic segments and the subscripted A and B letters indicate origin of the given segment to one of the two hypothetical, phylogenetically distinct parents. ND indicates that the origin was not determined.

### Table 1 Summary of the reported naturally occurring intra-species reassortment events among hantaviruses

| Virus          | Reassortment scenarios$^a$ | References |
|---------------|--------------------------|------------|
| Sin Nombre virus | $S_B M_B L_{ND}$          | [1]        |
| Puumala virus  | Mostly $S_B M_B L_A$, $S_B M_A L_B$, rarely $S_B M_B L_A$, $S_A M_B L_A$ | [2]        |
| Puumala virus  | $S_B M_B L_A$, $S_B M_A L_B$ | [50]       |
| Dobrava-Belgrade virus | $S_B M_B L_{ND}$          | [55, 58]   |
| Hantaan virus  | $S_B M_A L_B$             | [60]       |
| Seoul virus    | $S_B M_A L_{ND}$          | [61]       |
| Imjin virus    | $S_B M_A L_A$             | [62]       |
| Seewis virus   | $S_A M_{ND} L_B$          | [63]       |

$^a$Schematic representation of the reassortment pattern. S, M, and L capital letters stand for the S, M, and L genomic segments. Subscripted A and B letters indicate origin of the given segment to one of the two hypothetical, phylogenetically distinct parents. ND indicates that the origin was not determined.
Finland, reassortment was detected between more distantly related groups of viruses representing two distinct, previously defined lineages, the Finnish and the North Scandinavian lineage. This fact did not reduce the frequency of reassortment observations, it was actually 32%, the highest value among the three studies. However, of the six possible segment combinations, only two were found, those where both S and L segments originated from the same genetic lineage [52]. This pattern is typically found in the heterotypic reassortment events described for orthobunyaviruses [32] and also with in vitro generated hantavirus reassortants (see below).

In a phylogenetic study focused on PUUV sequences obtained from bank voles captured in Central Europe [54], authors noticed that the strains from eastern Slovakia clustered as expected with the sequences from Bohemian Forrest (Czech Republic) and Bavarian Forrest (Germany) only in the M but not S segment analyses. In the S segment analyses, the Slovak strains surprisingly clustered with the strains found in bank voles from Belgium, France, the south of the Netherlands, and regions in north-western Germany. This topology incongruence indicates the occurrence of a reassortment event. Since only partial sequences were analyzed, homologous recombination cannot be completely ruled out, too. Unfortunately, analysis of only partial sequences is a limitation of most of the studies analyzing sequences obtained directly from the hantavirus reservoir hosts. It seems to be a general consensus that the conflicting signals are interpreted as hints for reassortment but not for homologous recombination.

It is also interesting to note that besides the recent PUUV study [54], indications of reassortment, and/or recombination were reported from eastern Slovakia also for other hantaviruses [55, 56]. This phenomenon might be directly associated with the fact that eastern Slovakia has been involved in several end-glacial colonization routes of rodents. The region is a contact area for three phylogeographic clades of bank voles; the Carpathian, Western, and Eastern clade [57]. The region, therefore, seems to be a melting pot providing ample opportunity for the sympatric occurrence of several virus lineages consequently leading to co-infections followed by reassortment and/or recombination events.

Fig. 1: Schematic representation of all potential reassortants resulting from the co-infection of a cell by two hypothetical parental hantaviruses a (blue) and b (red). Envelope color corresponds to the color of the encoding M segment. S, M, and L capital letters stand for the S, M, and L genomic segments. Subscripted A and B indicate origin of the given segment to one of the two parents. The reassortants generated in the in vitro experiments and also most frequently found among the naturally occurring reassortants are marked by grey background. “Diploid” viruses containing both parental versions of at least one segment were transiently observed in the in vitro experiments. Four examples out of the 13 possible “diploid” patterns are shown in the bottom part of the figure.
schematically designated as $S_A M_B L_A$ could be found [60]. Differences in the clustering patterns of S and M segment-based phylogenetic trees suggested inter-lineage reassortment also among SEOV lineages in central south China [61].

Indications for reassortment events were recently reported also for the shrew-borne hantaviruses. Imjin virus (MINV) is a shrew-borne hantavirus identified in the Ussuri whitetoothed shrews (Cricodura lasiura) in the Republic of Korea and China. The reassortment pattern $S_A M_B L_A$ was recently identified in a study from the Republic of Korea [62]. Intriguing differences between the L-segment and S-segment phylogenies implying multiple reassortment events were observed also in extensive phylogenetic analysis of Sorex araneus shrew-borne Seewis virus [63].

**Heterotypic or “ancient” reassortment events**

All the above described reassortment events can be considered as intra- or inter-lineage events between closely related strains of the same virus. In most cases, these events also occur within a single reservoir host. However, there are few reports which describe reassortment events between distinct viruses or phylogenetic incongruences at deep nodes. Two of those reports are originating from south America where numerous rodent species have been identified to harbor unique hantavirus strains. Ape Aime-Itapúa virus (AAIV) was identified in Akodon montensis from Paraguay [64]; in the S segment tree, AAIV clusters with Jabora virus (JABV) associated with A. montensis. In contrast, in the M segment analyses AAIV shows a strong relationship with Pergamino virus, originally identified in Argentina in A. azarae [64, 65]. However, JABV by itself also shows conflicting positions in the S and M segment trees. The whole JABV clade clusters in the M segment tree with Maporal virus associated with the fulvous pygmy rice rat (Oligoryzomys fulvescens). However, in the S segment analyses, JABV clusters only with AAIV and occupies the most ancestral position within the South American hantaviruses [65]. These findings indicate that reassortment events involving distantly related hantaviruses are directly connected with or play a role in the processes of host-switching.

Very surprising findings were reported by Zou et al. [66]. Sequence analysis of cell culture isolates originating from A. agrarius mice and Rattus norvegicus rats revealed that spill over infections of HTNV from its reservoir host, A. agrarius, to R. norvegicus rats might be quite common. Most unexpectedly, two isolates, originally generated in 1988 from R. norvegicus rats, were shown to contain M and L segments of SEOV but the S segment of HTNV. This finding raises the question that, if such reassortants of HTNV and SEOV are possible, what could be their consequences for the public health and why are they not detected more frequently. It remains to be seen whether such viruses remained unnoticed because sequence analyses of more than one segment were not routinely performed until recently or whether those two isolates represent highly exceptional findings.

Recently, Bruges virus as second hantavirus in addition to Nova virus was identified to be harbored by the European mole (Talpa europaea). Occurrence of two highly divergent viruses in the same reservoir host shows that at least one of the viruses was involved in host-switching processes. Phylogenetic analyses of all three genomic segments showed tree topology inconsistencies at deep nodes, suggesting that Bruges virus may have emerged from ancient reassortment events. The virus appeared to be most closely related to hantaviruses associated with hosts from the Muridae family, to hantaviruses carried by shrews and moles, or showed ancestral position to both these groups in the S, M, and L segment-specific analyses based on complete coding sequences, respectively [67].

**In vitro generated reassortants**

Most of the reports claiming occurrence of reassortment in hantaviruses are based on phylogenetic analyses of naturally occurring strains, mainly obtained from the reservoir hosts. The basis for these claims are conflicting tree topologies, sometimes accompanied by more advanced phylogenetic analyses including hypothesis testing or recombination analysis on concatenated sequences. In other words, the claims are based on descriptive bioinformatic analyses showing the reassortment only indirectly. However, there are several reports bringing the ultimate proof that hantaviruses are capable to exchange genome segments during co-infections through in vitro experiments (Table 2).

Rodriguez et al. [68] showed already in 1998 that mixed infection of Vero E6 cells with two distinct strains of SNV can lead to generation of reassortant viruses in 8.5% of 294 progeny plaques tested. Most of the reassortants had the patterns $S_A M_B L_A$ and $S_A M_B L_B$. On the other hand, only one virus reassortant was observed among 163 progeny virus plaques from mixed infections between SNV and Black Creek Canal virus (BCCV), an HPS-causing virus from Florida, which has the cotton rat (Sigmodon hispidus) as its natural host; the reassortant carried the M segment of SNV and S and L segments of BCCV. Interestingly, in both experiments about 30% of the progeny virus plaques appeared to be transiently diploid, i.e. containing both versions of at least one segment.

Similar experiments were performed with SNV and ANDV by Rizvanov et al. [69]. Again, also diploid viruses were observed (20/337 progeny plaques) and all monoploid reassortant viruses (10/337 progeny plaques) contained the S and L segments of SNV but ANDV M segment. Despite having from ANDV only the M segment, the reassorted virus
showed replication efficiency in Vero E6 cells resembling ANDV rather than SNV.

The very same reassortment pattern was achieved also in the study of McElroy et al. [70]. In line with the previously mentioned study [69], the virus containing the S and L segments of SNV and M segment of ANDV (designated SAS) had in vitro growth and plaque morphology characteristics similar to those of ANDV. Most important results were obtained in the in vivo experiment. The SAS reassortant virus was highly infectious and elicited high-titer, ANDV-specific neutralizing antibodies in Syrian hamsters. However, the virus did not cause lethal HPS indicating that the ANDV M genome segment alone is not sufficient to confer the lethal HPS phenotype described for ANDV [71].

Reassortant could be later generated also between a pathogenic PUUV and a non-pathogenic Prospect Hill virus (PHV). The reassortant contained the glycoprotein coding M-segment derived from PUUV and the S and L segments from PHV. The reassortant together with parental viruses were characterized also in terms of their ability to modulate in vitro innate immune responses including induction of type I and type III interferon and interferon-stimulated gene MxA. In all experiments, the reassortant revealed the same characteristic innate antiviral response pattern as PHV, which is considered to be a non-pathogenic hantavirus. These data are not only consistent with the previous studies on SNV and ANDV reassortants but also led the authors to the conclusion that such reassortant viruses carrying M segment of the pathogenic virus together with S and L segments of non-pathogenic virus (such as PHV) could be used as attenuated vaccines [72].

Reassortment patterns were found in 65 out of 207 analyzed progeny clones (31.4%). As in the previous in vitro studies, only reassortant having S and L segments from the same parental virus and exchanged M segment were generated. In this case, both versions (schematically designated as $S_A M_B L_A$ and $S_B M_A L_B$ throughout this review) were found. Analogously to the study of Handke et al. [72], the reassortants were (together with the parental viruses) analyzed for the differential induction of innate immune responses in the established cell lines A549 and HuH7. The contrasting phenotypes of the parental viruses were found to be maintained by the reassortants carrying the respective S and L segments of the parental virus and were not influenced by the origin of the M segment.

Also in this reassortment experiment, significantly high proportion of the analyzed clones (65/207; 31.4%) were designated as diploids containing both parental versions of at least one segment [73]. In fact, diploid viruses were observed in all studies on in vitro generated reassortants mentioned above. These findings indicate that the hantavirus assembly process is not tightly controlled and more than three genome segments can be packed into the viral particle. This imperfect segment packaging is perhaps directly involved in the ability of hantaviruses to reassort.

**Conclusions**

Although not as frequent as in other arthropod-borne bunyaviruses, reassortment seems to be more common among hantaviruses than initially recognized. The intra- and interlineage reassortment between closely related variants seems to occur whenever co-infections of two virus variants are possible due to their sympatric occurrence.

On the other hand, heterotypic reassortment between more distantly related viruses occurs less frequently but seems to play a supporting role in the process of crossing the species barriers and host switching when, e.g., the newly
acquired M segment encoding the envelope glycoproteins might help the virus to establish persistent infection in the new host. Topologic incongruences in the deep nodes of the segment specific trees suggest that such events occurred in the past and contributed to the currently recognized hantavirus diversity.

All the reported in vitro reassortment experiments have in common that only reassortants with exchanged M segments could be generated. This finding suggests that a high degree of genetic compatibility including packaging signals and RNA–RNA and/or RNA–protein interactions is required particularly for the S and L segments while the exchange of M segment is better tolerated or is particularly beneficial.

Altogether, the numerous reports on hantavirus reassortment varying from naturally occurring intra-lineage reassortants to in vitro generated inter-species reassortants, as summarized in this review, clearly demonstrate that reassortment events play a significant role in hantavirus evolution. Consequently, it will be highly beneficial to invest in obtaining complete sequences of all three genomic segments in the future studies. It can be assumed that advancement of the next-generation sequencing technologies will generate more high-quality data which are needed to further elaborate the current accumulating evidence of reassortment as a significant driving force in hantavirus evolution.

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Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

Ethical approval This article does not contain any studies involving animals or studies with human participants performed by the author.

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