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Review

Viruses of the family *Bunyaviridae*: Are all available isolates reassortants?

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ABSTRACT

Viruses of the family *Bunyaviridae* (the bunyaviruses) possess three distinct linear, single-stranded, negative sense or ambisense RNA segments (large, medium, and small). Dual infections of arthropod and perhaps vertebrate and plant hosts provide substantial opportunity for segment reassortment and an increasingly recognized number of the nearly 300 viruses in this family have been shown to be reassortants. Reassortment of RNA segments (genetic shift) complements genetic drift (accumulation of point mutations) as a powerful mechanism underlying bunyavirus evolution.

Here we consider the possibility, if not likelihood, that most if not all bunyaviruses currently recognized may represent reassortants, some of which may be reassortants of existing viruses, and some of which may be reassortants of extinct viruses. If this hypothesis is correct, then the roots of the family and genus trees of bunyaviruses as currently described (or ignored) are incomplete or incorrect.

Contents

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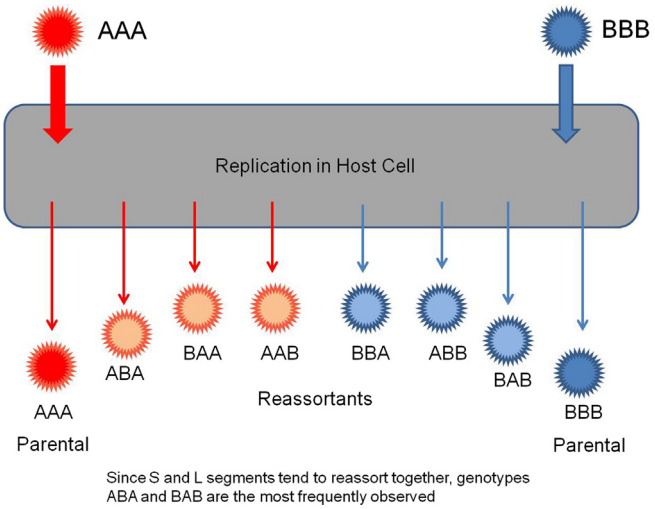

Fig. 1. Potential reassortants resulting from the dual infection of a cell by two different "parental" bunyaviruses: virus A (genome S_A, M_A, L_A), and virus B (genome S_B, M_B, L_B). Color indicates segment origin – A=red; B=blue – and intensity corresponds with the predominating genome origin: 2 segments from A=light red; 2 segments from B=light blue (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

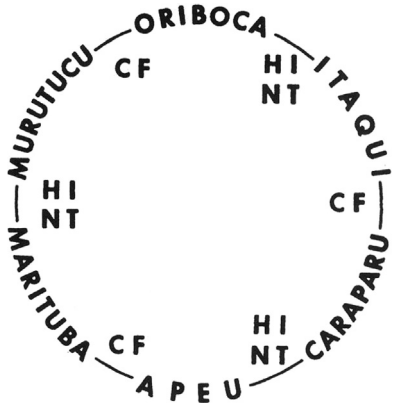

Fig. 2. Antigenic reactivity of six Group C orthobunyaviruses as determined by hemagglutination-inhibition, neutralization and complement-fixation (figure courtesy of the American Journal of Tropical Medicine and Hygiene).

Introduction

Viruses of the family *Bunyaviridae* (the bunyaviruses) possess three distinct linear, single-stranded, negative or ambisense RNA segments (large, medium and small). The small (S) RNA codes for the nucleocapsid protein, and in viruses of several genera also for a non-structural protein, NSs, which interferes with innate immunity. The surface spikes of the virions comprise two glycoproteins, Gn and Gc, embedded in a lipid bilayer. The glycoproteins are coded by the medium (M) RNA that generates a polyprotein, which is proteolytically processed, and in viruses of some genera also includes a non-structural protein (NSm) of unknown function. The large (L) RNA codes for the transcriptase and replicase protein, the large RNA-dependent RNA polymerase (RdRp or L protein). Bunyaviral L proteins, in addition to polymerase activity, have an endonuclease activity that cleaves cellular messenger RNAs for the production of capped primers used to initiate transcription of viral messenger RNAs (a feature known as 'cap snatching').

When two closely related bunyaviruses infect the same susceptible cell at the same time, their genome segments may be variously incorporated into the progeny viruses. For example, should two bunyaviruses, A and B, each with three genomic segments, co-infect a cell, progeny viruses may variously comprise a mixture of the L RNAs, M RNAs, and S RNAs of the two parent viruses, as well as progeny identical to the infecting parental viruses (Fig. 1). However, sequence relationships in natural reassortants may become diluted by longer periods of genetic drift, and putative assignments of "parent" and "reassortant" may be ambiguous, especially when a supposed parent can itself have been a reassortant. Another obvious possibility is that sequences may recombine to generate chimeric segments formed from homologous portions of homotypic or possibly heterotypic bunyaviruses, although this has so far only been described for a few viruses, most recently for the newly emerging severe fever with thrombocytopenia syndrome virus from China (He and Ding, 2012; Lam et al., 2013) but was also suggested previously for Crimean-Congo hemorrhagic fever virus (Lukashev, 2005; Deyde et al., 2006) or more convincingly for hantaviruses (Zhang et al., 2010; Zuo et al., 2011).

Bunyaviruses share several molecular characteristics; but based on their differences, the International Committee on Taxonomy of Viruses has classified them into five genera: *Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus,* and *Tospovirus* (King et al., 2012). Nearly 300 viruses have been placed in these taxa, including 97 species, 81 possible species, and a large number of isolates, encompassing 19 viruses in seven groups of related viruses and dozens of ungrouped viruses.

In the past, bunyaviruses were classified in "serogroups" using antigenic relationships determined principally by hemagglutination-inhibition (M segment determined; group placement), complement-fixation (S segment determined; group or complex placement), and neutralization (M segment determined; specific identification) tests; serologic makers determined by the L segment are not identified. Infrequently, a virus in one antigenic group reacted to a virus assigned to a different group. Over time, this quandary was solved by recognition that these links between groups implied that the groups were closely or distantly related. When electron microscopy and molecular markers confirmed these relationships, general taxonomic groupings at family, genus, and species levels became possible.

By the late 1970s monoclonal antibodies and molecular tools sufficient to detect subtle differences between bunyaviruses had been developed. Further and innovative modifications made possible the discrimination of minor yet significant details regarding the RNAs of bunyaviruses and their structure–function relationships (Bishop et al., 1980). Iroegbu and Pringle concluded from their studies of laboratory-produced reassortants of Batai, Bunyamwera, and Maguari viruses (all Bunyamwera group orthobunyaviruses) that there is no genetic barrier to exchange of genetic material between these viruses and that viruses of this group may constitute a single gene pool, discounting geographical and ecological limitations (Iroegbu and Pringle, 1981).

Some relationships, however, remained unclear. In the classic and informative investigation of six Group C viruses (family *Bunyaviridae*, genus *Orthobunyavirus*), Oriboca, Itaqui, Caraparu, Apeu, Marituba, and Murutucu, were shown to be variously related to each other: Oriboca and Itaqui by hemagglutination-inhibition and neutralization, Itaqui and Caraparu by complement-fixation, Caraparu and Apeu by hemagglutination-inhibition and neutralization, Apeu and Marituba by complement-fixation, Marituba and Murutucu by hemagglutination-inhibition and neutralization, and Murutucu and Oriboca by complement-fixation, as shown in Fig. 2 (Shope and Causey, 1962). As other Group C viruses were isolated and identified, their relationships also were determined by then-standard antigenic tests. It seemed reasonable to expect that viruses shown to be related based on the results of one kind of test would cross-react to varying extents in other kinds of tests. How then could they react by one test and not by another test and still be related? Indeed, not only did some not cross-react

Table 1
Possible genome segment organization of reported heterotypic reassortant bunyaviruses[a].

Serogroup or genus	Virus	S segment	M segment	L segment	Reference
Group C	Apeu	S_{MTBV}	M_{CARV}	L_{NA}[a]	Nunes et al. (2005)
Group C	Murutucu	S_{ORIV}	M_{MTBV}	L_{NA}	Nunes et al. (2005)
Group C	Itaqui	S_{CARV}	M_{ORIV}	L_{NA}	Nunes et al. (2005)
Group C	Restan	$S_{(MURV/ORIV)}$	$M_{(MURV/MTBV)}$	L_{NA}	Nunes et al. (2005)
Simbu	Jatobal	$S_{(OROV)}$	M_{unique}	$L_{(OROV)}$	Saeed et al. (2001a)[b]
Simbu	Iquitos	S_{OROV}	M_{unique}	L_{OROV}	Aguilar et al. (2011)
Simbu	Aino B7974	$S_{PEAV\ CSIRO\ 110}$	M_{AINOV}	$L_{PEAV\ CSIRO\ 110}$	Yanase et al. (2010)
Simbu	Shamonda	$S_{SATV/DOUV}$	M_{Y7V}	$L_{(SATV/DOUV)}$	Yanase et al. (2012), Goller et al. (2012), Saeed et al. (2001b)
Simbu	Schmallenberg[c]	$S_{\{SHAV(SATV)\}}$	$M_{(SATV/DOUV)}$	$L_{\{SHAV/SATV\}}$	Yanase et al. (2012), Goller et al. (2012)
Bunyamwera	Ngari [Garissa]	S_{BUNV}	M_{BATV}	L_{BUNV}	Bowen et al. (2001), Gerrard et al. (2004), Briese et al. (2006)
Bunyamwera	Potosi	$S_{CVV/MAGV}$	$M_{(KRIV/MDV)}$	$L_{(CVV/MAGV)}$	Briese et al. (2007)
Bunyamwera	Main Drain	$S_{(MAGV/NORV)}$	$M_{(KRIV/POTV)}$	$L_{(MAGV/CVV)}$	Briese et al. (2007)
Bunyamwera	Macaua	S_{unique}	$M_{(TAIAV/WYOV)}$	L_{unique}	Chowdhary et al. (2012)
Bunyamwera	Tucunduba	S_{TAIAV}	M_{WYOV}	L_{TAIAV}	Chowdhary et al. (2012)
Bunyamwera	Cholul	S_{CVV}	$M_{POTV/KRIV}$	$L_{\{POTV/CVV\}}$	Blitvich et al. (2012)
Phlebovirus	Aguacate	S_{ARMV}	$M_{(DURV)}$	$L_{(ARMV/IXCV)}$	Palacios et al. (2011)
Phlebovirus	Granada	S_{MASV}	M_{unique}	L_{MASV}	Collao et al. (2010)
Tospovirus	$L_GM_TS_G$	S_{GRSV}	M_{TCSV}	L_{GRSV}	Webster et al. (2011)

Virus name abbreviations are: MTBV=Marituba virus, CARV=Caraparu virus, ORIV=Oriboca virus, OROV=Oropouche virus, SHAV=Shamonda virus, SATV=Sathuperi virus, DOUV=Douglas virus, BUNV=Bunyamwera virus, BATV=Batai virus, CVV=Cache Valley virus, KRIV=Kairi virus, MAGV=Maguari virus, NORV=Northway virus, WYOV=Wyeomyia virus, TAIAV=Taiassui virus, POTV=Potosi virus, Y7V=Yaba 7 virus, SBV=Schmallenberg virus, ARMV=Armero virus, DURV=Durania virus, IXCV=Ixcanal virus, MASV=Massilia virus, GRSV=groundnut ringspot virus, TCSV=tomato chlorotic spot virus, $L_GM_TS_G$=$L_GM_TS_G$ virus.

[a] NA=No sequence available; unique=divergent sequence that does not allow assignment; ()=distant relationship with common ancestor to said virus segment(s); {A/B}=indicates a virus A that itself is considered to be a reassortant of virus B. In bunyaviruses, which have three genome segments, assignments may be made based on the assumption that consistent relationships for two segments should represent the background in which the third (divergent) segment reassorted; unless reassortment among three viruses occurs, which, to our knowledge, has not yet been identified. We list the closest recognizable relative for each segment, although in some cases several possibilities may exist (see Fig. 3). Reassortments between strains of a single virus (homotypic reassortment) are not considered in this table.

[b] According to GenBank entry JQ675603.

[c] It is unclear whether SBV is a reassortant (or possibly a 'revertant' – a SHAV that again picked up a SATV/DOUV-related M RNA). However, the SBV M RNA appears to be ancestral to SATV/DOUV (see Fig. 3, [(Goller et al. (2012))]), while its S RNA appears to be a descendant, and its L RNA a twin to SATV/DOUV, with the latter two segments being most closely related to SHAV. Thus, because SHAV has an apparent genotype $S_{SATV/DOUV}$, M_{Y7V}, $L_{SATV/DOUV}$, SBV may also be a parent of SHAV, providing its S and L RNAs.

with certain relatives, others cross-reacted completely by one test and not at all by another. In 2005, Nunes et al. (2005) published a study of the 13 Group C viruses recognized at that time. Sequencing the entire S RNAs and part of the M RNAs (705 nt) of each of these viruses, they found that the S RNA of Caraparu virus isolate BeH-5546 was nearly identical to the S RNA of Oriboca virus while the M RNA matched that of Caraparu virus, and concluded that Caraparu virus isolate BeH-5546 is a reassortant virus. However, without knowledge of the L RNA relationships an assignment of any "donor/recipient" relation must remain tentative (Table 1). These and other data indicated that the Group C viruses include many natural reassortants. Nunes et al. also corroborated earlier serologic (antigenic) studies and suggested that in order to more fully understand the epidemiology of these and other arboviruses, a combination of serologic and genetic methods is necessary.

The important point is that such reassortment can introduce radical phenotypic changes caused by new combinations of segments. For instance, a virus that is highly infectious but not highly pathogenic (and provided each of these traits is determined by separate segments) could mix with a virus that is not highly infectious but is highly pathogenic to result in reassortant progeny viruses that not only have low infectivity and low pathogenicity but also those that are highly infectious and highly pathogenic.

Nathanson, Gonzalez-Scarano, and their associates, showed that the L RNA segment of bunyaviruses is the major determinant of neuroattenuation and that in vitro phenotypes (temperature sensitivity and altered plaque morphology) segregated with the L RNA segment. Attenuation in mice and infectivity for mosquitoes of some virus clones mapped to the M RNA segment, suggesting that the virus glycoproteins, which are involved in virus entry, also play a role in virulence (Endres et al., 1991). These seminal findings initiated an era of subsequent studies of the correlation of structure and function.

Neither genome segmentation nor reassortment is an exclusive feature of bunyaviruses. Among other families of viruses with segmented genomes (*Orthomyxoviridae*, *Arenaviridae*, and *Reoviridae*), reassortment of the H and N surface glycoproteins of influenza A virus (family *Orthomyxoviridae*, genus *Influenzavirus A*) or additional reassortment of any of their other six genome segments are well known and the reason seasonal recurring vaccination against the latest reassortant influenzavirus is necessary. For viruses with more than three RNA segments, the number of potential reassortments is enormous. That orbiviruses (family *Reoviridae*, genus *Orbivirus*) have 10 double-stranded RNA segments and rotaviruses (family *Reoviridae*, genus *Rotavirus*) have 11 provides a basis for complex reassortment and high phenotypic variability among these viruses.

Of the viruses transmitted by hematophagous arthropods, the arboviruses, most have RNA genomes and many have multiple genome segments. Arboviruses are capable of alternately replicating in hematophagous arthropods and vertebrates and generally are cytolytic for their vertebrate hosts but cause little or no cytopathogenicity in their arthropod hosts. Bunyaviruses of the genera *Orthobunyavirus*, *Nairovirus*, and *Phlebovirus* may replicate in and be transmitted by hematophagous mosquitoes, ceratopogonids, ticks, phlebotomine flies, or other hematophagous arthropods, but some appear to have narrow host ranges, particularly in regard to their arthropod hosts. Viruses of the genus *Tospovirus* are transmitted by non-hematophagous arthropods and infect plant hosts; no arthropod vectors are identified for members of the genus *Hantavirus*, that are strictly associated with their specific rodent host.

Thus, dual infections of arthropod hosts (which do not produce antibody) provide considerable opportunity for reassortment of the genome segments, and several of the nearly 300 viruses placed in the family *Bunyaviridae* or which may be members of this family have been shown to be reassortants. A smaller proportion of recognized tick-borne viruses than of dipteran-borne viruses are known to be reassortants. This may reflect differences in the biology of ticks and mosquitoes or culicoids. Ticks are relatively long-lived and feed infrequently (hard ticks). Mosquitoes and culicoids may be short-lived, but they feed frequently, providing a greater opportunity for dual infections in them. However, as shown by Bishop and Beaty (1988), a phenomenon of super-infection resistance may prevent secondary infection by closely related bunyaviruses. Indeed, this may actually promote opportunities for segment reassortment between more distantly related bunyaviruses.

For reassortment between two or more viruses to be feasible, their distributions must be sympatric, both spatially and temporally. If two related viruses occur only in Africa and North America, they cannot reassort unless one of them is moved to the habitat of the other. Such movement can be unintentional (by infected migrating vertebrates, including humans, by movement of infected arthropods through travel or trade, or after escape from virology laboratories) or intentional (by criminal or bioterroristic act), but when it does occur, it can result in a potential or actual problem. It is when two related bunyaviruses share an ecologic niche that reassortment is much more likely. In temperate regions, because vector availability may be seasonal, the reliance of bunyaviruses on vector transmission often ensures that different bunyaviruses that occur in the same geographic location are, by necessity, being transmitted at the same time. Because bunyaviruses cause many human infections, with notable exceptions mainly uncomplicated febrile illnesses, the opportunities for reassortment to occur in humans, or in other vertebrates, also are ample because of the huge populations of arthropods that can occur in some geographic areas and the relatively high rates of arbovirus infections in those arthropods.

In 1981 Ushijima et al. provided evidence for naturally-occurring reassortant bunyaviruses of the Patois group (Ushijima et al., 1981). Also in 1981 Akashi et al., working with Japanese and Australian Simbu viruses, provided evidence for the occurrence of natural reassortants of these bunyaviruses (Akashi et al., 1997), and in 2010 Yanase et al. reported that various isolates of viruses that had been identified as either Aino or Peaton Simbu group bunyaviruses were actually reassortants, the L and S RNAs sorting together and the M RNAs reassorting more independently (Yanase et al., 2010) (Table 1). These observations have now been applied to bunyaviruses other than orthobunyaviruses but the examples below for orthobunyaviruses are used here because so much is now known about them and, in particular, about their replicative mechanisms.

Examples of known natural occurrences of reassortment

Reassortment among Simbu serogroup orthobunyaviruses

The first isolation of Oropouche virus (Simbu group) was from a febrile human in Trinidad (Anderson et al., 1961) but the earliest documented epidemic of Oropouche fever occurred in 1961 in Pará State, Brazil, infecting more than 11,000 people (Bishop et al., 1980). Subsequently, at least 27 outbreaks were recognized, the largest of which involved an estimated 102,000 people in 1980 and infections with this virus have been documented to occur in Panama, Peru, Ecuador and Bolivia (Baisley et al., 1998). Oropouche virus is recognized to be transmitted principally by biting midges, *Culicoides paraensis* (Roberts et al., 1981), whose abundance provides opportunity for dual infections in the epidemic areas. Oropouche fever re-emerged in the eastern Amazon region 26 years after the last epidemic and was caused by genotype II, a lineage previously found only in Peru and western Brazil.

Decades of multiple studies by international, national, state, local, and institutional investigators have demonstrated the relatively high risk of bunyaviral and other arboviral infections, particularly in tropical regions. For example, among 1227 persons sampled by Baisley et al., 33.7% were positive for IgG antibody to Oropouche virus (Baisley et al., 1998). More recently, Forshey et al. (2010) conducted a clinic-based syndromic surveillance system in 13 locations in Ecuador, Peru, Bolivia, and Paraguay. Acute-phase serum samples collected from febrile participants were tested for virus and acute- and convalescent-phase sera were tested for pathogen-specific IgM by ELISA. In all, 20,880 participants were included in the study, with evidence for recent arbovirus infection detected in 6793 (32.5%). Orthobunyavirus (Oropouche virus, Group C viruses, and Guaroa virus) infections were observed in approximately 3% of febrile episodes; this rate may be an underestimation if only serologic tests are relied upon.

Jatobal virus was isolated in 1985 from a South American coati (*Nasua nasua*) in Pará State, Brazil and was classified as a distinct member of the Simbu serogroup of orthobunyaviruses on the basis of neutralization tests. On the basis of nucleotide sequencing however, the authors found that the S RNA of this virus is very similar ($>95\%$ identity) to that of Oropouche virus, in particular the Peruvian genotype of Oropouche virus. In comparison, limited nucleotide sequencing of the M RNA-encoded G2 protein genes of Jatobal and Oropouche viruses, showed that they were dissimilar ($<66\%$ nucleotide identity) and neutralization tests confirmed the lack of cross-reactivity between the viruses. These results suggested that Jatobal virus is likely a reassortant containing the S RNA of Oropouche virus (Table 1, Fig. 2). It is interesting and instructive that Jatobal virus is in comparison to Oropouche virus attenuated in hamsters, which suggests that the S RNA of Oropouche virus is not directly involved in hamster virulence (Saeed et al., 2001a).

Another isolate, named Iquitos virus, is also a reassortant of Oropouche virus (Aguilar et al., 2011). This virus possesses the S and L segments of Oropouche virus and the M segment of a novel Simbu group virus (Table 1). It was first isolated in 1999 from a febrile patient in Iquitos, Peru, and later (2005 and 2006) identified as the cause of outbreaks of what were called “Oropouche fever” in Iquitos. Prior infection with Oropouche virus does not seem to protect against disease caused by Iquitos virus.

Akabane virus, a medically-important member of the Simbu serogroup is a teratogenic pathogen in Australasia causing abortions, stillbirths, premature births, and congenital abnormalities with arthrogryposis-hydranencephaly syndrome in cattle, sheep and goats and encephalomyelitis in cattle. Kobayashi et al. (2007) sequenced and phylogenetically analyzed field isolates of Akabane virus from Japan, Taiwan, Australia, and Kenya, and an isolate of Tinaroo virus, which is found in Australia and Japan. Discrepancies among the phylogenetic trees of the S, M and L RNA segments indicate genomic reassortment events among these field isolates, with the M RNA segment encoding the glycoproteins that induce neutralization antibodies being the most variable.

In 2011, malformed calves, lambs and goat kids were observed in Germany. The etiologic agent was named Schmallenberg virus (Simbu group; genus *Orthobunyavirus*, family *Bunyaviridae*), after the locale where the first cases were found. The virus has spread to many other countries in Europe and has become a costly and painful experience. Soon after discovery, the virus was shown to be a member of the Simbu group, ostensibly related most closely to Akabane virus, which was not known to occur in Europe.

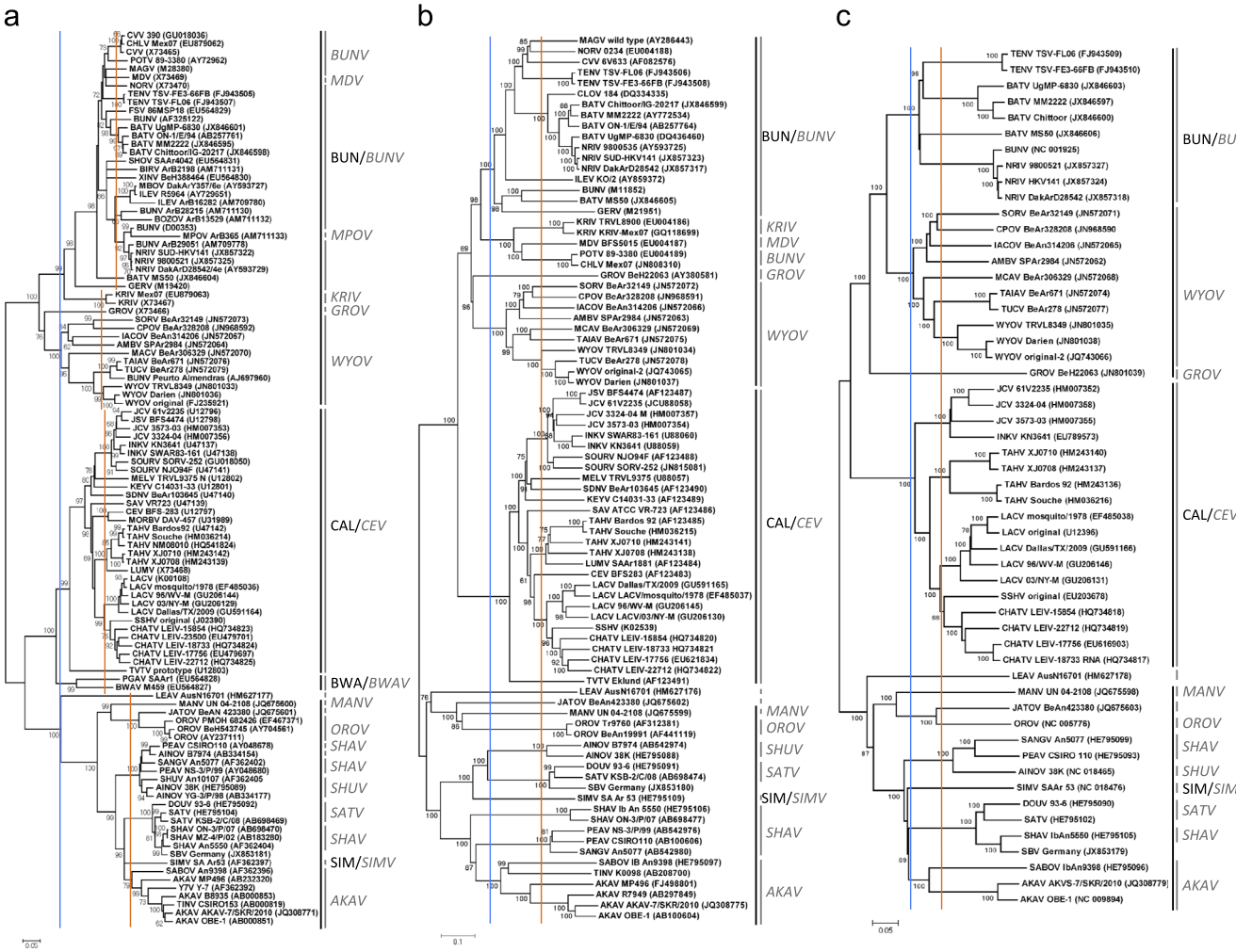

Fig. 3. Phylogenetic analyses of representative orthobunyavirus sequences, comprising complete coding sequence for S (a), M (b), and L (c) segments. In cases where sequences for multiple strains of a virus are available, selected highly divergent variants were included to indicate the respective span of genetic variability. Neighbor joining trees were constructed using a Jukes-Cantor model and consensus trees generated from 1000 pseudoreplicates are shown; bootstrap agreement values $>60\%$ are indicated at the respective nodes; scale bars indicate number of substitutions per site; GenBank Accession numbers are shown in parenthesis next to virus name abbreviation and strain (where known). Branches crossing the blue lines relate to most of the species registered by the International Committee on the Taxonomy of Viruses (ICTV; BUNV, MDV, MPOV, KRIV, GROV, WYOV, CEV, BWAV, MANV, AKAV, OROV, SATV, SIMV, SHAV, SHUV; gray font and gray lines to the right of the trees) that in part overlap with classically defined serogroups (Bunyamwera (BUN), California (CAL), Bwamba (BWA), Simbu (SIM)); black font and black lines to the right of the trees), with individual viruses branching to the right-hand side of the line; branches crossing the orange lines represent mainly individual viruses, with different strains or isolates of a virus branching to the right-hand side of the line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Subsequent investigations revealed that Schmallenberg virus may be a reassortant containing the M RNA of Sathuperi virus (from India and Japan) and the S and L RNAs of Shamonda virus (Nigeria) (Table 1, Fig. 3), neither of which virus has been detected in Europe (Yanase et al., 2012). The complexity caused to science by reassortment of these viruses is emphasized by the studies of Goller et al. (2012) who, agreeing with the work of Saeed et al. (2001b), suggested that the taxonomy of Shamonda virus should be revised to incorporate it in the species *Sathuperi virus* and that the remaining members of the previous species *Shamonda virus* should form a renamed species *Peaton virus* or *Sango virus* (Goller et al., 2012). These authors further indicated that the results of their studies showed that Schmallenberg virus belongs to the species *Sathuperi virus* and that Schmallenberg virus may not be a reassortant but may be an ancestor of the reassortant Shamonda virus, which includes the Schmallenberg L and S segments and "the M segment of an unclassified virus" (Yaba-7 virus (Saeed et al., 2001b), Table 1).

These detailed insights into the phylogeny of Schmallenberg virus could be the basis for the development of efficient, cross-protective vaccines. The overall results highlight the importance of conducting full-genome analyses to identify potential genetic reassortants and to investigate the evolutionary history of viruses with segmented genomes. It is not impossible that convergent evolution of viruses leads to the selection of genetically similar RNAs in more than one virus and so give the impression of reassortment between viruses from disparate geographic areas. However, such convergent evolution is readily discernible by sequence analysis.

Reassortment among Bunyamwera serogroup viruses

In late 1997 and early 1998, a large outbreak of hemorrhagic fever occurred in East Africa. Of 115 clinical samples collected from hemorrhagic fever patients in Kenya and southern Somalia, 27 (23%) showed serologic evidence of acute infection with Rift Valley fever virus (family *Bunyaviridae*, genus *Phlebovirus*), as determined by IgM detection, virus isolation, detection of virus RNA by reverse transcription-polymerase chain reaction (RT-PCR), or immunohistochemistry. However, two patients (one from Kenya and the

other from Somalia) whose illness met the hemorrhagic fever case definition yielded virus isolates that were not Rift Valley fever virus; the virus was tentatively named Garissa virus. Of the Rift Valley fever virus negative patients that met the hemorrhagic fever case definition during the Kenya and southern Somalia outbreak, 27% had evidence for Garissa virus infection by PCR or had IgM antibody to Garissa virus. Electron microscopy indicated that the two virus isolates were bunyaviruses. Regions of the L and S segments of the two virus isolates were successfully amplified and their nucleotide sequences shown to be nearly completely identical with those of Bunyamwera virus, a long recognized African mosquito-borne virus not previously associated with severe human disease. Unexpectedly, the virus M segment was not that of Bunyamwera virus. Further studies showed that the L, M and S segments of another orthobunyavirus, Ngari virus, were virtually identical to those of Garissa virus, showing also high sequence identity with L and S of Bunyamwera virus, while the M segment differed from Bunyamwera virus. It was concluded that Garissa virus is an isolate of Ngari virus, which in turn is a reassortant of Bunyamwera virus and another unidentified orthobunyavirus (Bowen et al., 2001; Gerrard et al., 2004). Later, Briese et al. reported that analysis of M segment sequences of two Bunyamwera group viruses, Batai and Ilesha viruses, revealed 95% deduced amino acid identity between Batai virus and Ngari virus M RNA and concluded that an African Batai virus was the donor of Ngari virus M segment sequence (Briese et al., 2006). In summary, Garissa virus is an isolate of Ngari virus, and both are reassortants of Bunyamwera virus L and S segments and the M segment of Batai virus (Table 1, Fig. 3).

Briese et al. extended their genetic analyses to other orthobunyaviruses of the Bunyamwera serogroup, the results of which indicated that additional natural genome reassortments of these viruses occur (Briese et al., 2007). Whereas the relative phylogenetic positions of all three genome segment sequences were similar for Northway (from Alaska) and Kairi (Central and South Americas) viruses, the relative positions of Potosi virus and Main Drain virus M segment sequences (both from North America) diverged from those of their L and S segments (Table 1, Fig. 3). These findings indicate the occurrence of M segment reassortment in Potosi and Main Drain viruses and suggest that natural genome reassortment is a driving force in the evolution of viruses of the Bunyamwera serogroup. Further genetic analyses, focusing on other bunyaviruses, showed that the three genome segments of Wyeomyia group viruses (Wyeomyia, Taiassui, Macaua, Sororoca, Anhembi, Cachoeira Porteira, Tucunduba and Iaco viruses) showed that these viruses differ from other viruses in the Bunyamwera serogroup and from other orthobunyaviruses by having truncated NSs sequences that may not counteract the host's interferon response (Chowdhary et al., 2012). Additional findings suggest the occurrence of genome reassortment within the Wyeomyia group, identifying Macaua and Tucunduba viruses as M-segment reassortants that, in the case of Tucunduba virus, may have altered pathogenicity. They stressed the need for whole-genome sequence information to facilitate characterization of orthobunyaviruses and to resolve phylogenetic relationships of all their genetic segments. Obviously, the classical antigenic and serologic techniques originally used to identify viruses are inadequate for such sophisticated investigations and techniques such as polymerase chain reaction assays must be added to achieve the necessary detail and resolution of data. Furthermore, virus isolation remains indispensable as it provides researchers with material with which to determine biological characteristics and link phenotypes to genetic markers.

As reported by Blitvich et al., Cholul virus, a recent orthobunyavirus isolate from mosquitoes collected in the Yucatan Peninsula of Mexico, is a reassortant (Blitvich et al., 2012). The virus L RNA and M RNA are similar to the homologous regions of Potosi virus, with 98% and 96% nucleotide identity, respectively; however, its S RNA has 98% nucleotide identity to the homologous region of Cache Valley virus. This suggested that Cholul virus is a reassortant that combines L and M RNA segments from Potosi virus, a reassortant virus itself, with an S RNA segment from Cache Valley virus (Table 1, Fig. 3).

One of the best studied bunyaviruses is La Crosse virus, the evolutionary success of which is dependent on its ability to adapt to changing conditions of vector and vertebrate host via intramolecular genetic changes and segment reassortment. The latter appears to be related to vertical (transovarial) transmission of the virus. For example, Reese et al. (2008) showed that segment reassortment occurred in naturally infected *Aedes triseriatus* mosquitoes from Wisconsin and Minnesota in 2000, 2004, 2006 and 2007. Phylogenetic and linkage disequilibrium analyses revealed that approximately 25% of the analyzed infected mosquitoes and isolated viruses contained reassorted genome segments, suggesting that homotypic La Crosse virus segment reassortment is frequent in nature.

It has been conventionally thought among arbovirologists that vector-borne pathogens evolve towards a primarily benign relationship with their arthropod vectors, but the proof for such a hypothesis is erratic, with some vectors being effected and some not, depending on the mechanism of infection (horizontal or vertical). Lambrechts and Scott (2009) concluded that the degrees of virulence among vector-virus systems likely reflect different selective pressures imposed on arboviruses primarily transmitted horizontally, rather than vertically.

Reassortment in other bunyaviruses

Phleboviruses

Rift Valley fever, sandfly fever Sicilian, sandfly fever Naples, and the recently recognized virus causing severe fever with thrombocytopenia syndrome (Yu et al., 2011) are members of the bunyavirus genus *Phlebovirus*, and because they and many other phleboviruses cause febrile illnesses, these viruses have been recently examined at the molecular level, which, in addition to intratypic reassortment among Rift Valley fever virus lineages (Bird et al., 2007), also indicated reassortment between different viruses within this genus (Table 1). In brief, Aguacate virus is a likely reassortant that obtained its M RNA from an as-yet unknown virus (Palacios et al., 2011); Granada virus may also be a reassortant of another recently described virus, Massilia virus, which provided the L and S RNAs, and an as-yet unidentified phlebovirus which provided the M RNA segment (Collao et al., 2010); and some Candiru group viruses may also represent M RNA segment reassortants (Palacios et al., 2011).

Nairoviruses

The first evidence that nairovirus segments reassort was discovered by Hewson et al. studying Crimean-Congo hemorrhagic fever virus isolated from distant geographical locations (Hewson et al., 2004). This virus is transmitted between vertebrates by ticks and perhaps transported by trade in livestock or by long-distance carriage of virus by infected ticks on migrating birds. Evidence for multiple RNA segment reassortment events has been accumulated and intraviral reassortment appears to be relatively common. It has been implied that homotypic reassortment of Crimean-Congo hemorrhagic fever genomes may affect the pathogenicity of the virus. Reassortment of genomes between different nairoviruses (i.e., heterotypic reassortment) has not been reported.

Hantaviruses

Soon after the recognition of hantavirus pulmonary syndrome and its causative agent, Sin Nombre virus (genus *Hantavirus*), in the United States, reassortants of this virus were detected. The results of work by Henderson et al. (1995) suggested that M segment, and to a lesser extent L and S segment reassortants occur within Sin Nombre virus populations in deer mice (*Peromyscus maniculatus*), the principal rodent host of this virus, but that reassortment does not, or only rarely (Rodriguez et al., 1998), occur between Sin Nombre viruses and other rodent-borne hantaviruses found in the same geographic area but in different rodent hosts. The authors concluded that "as genetic distance increases, the frequency of formation of viable reassortants decreases, or that hantaviruses which are primarily maintained in different rodent hosts rarely have the opportunity to genetically interact", since co-infection events in their rodent hosts provide the only opportunity for reassortment due to the lack of an arthropod or other vector. However, whereas there is ample evidence for reassortment of RNA segments between strains of the same hantavirus (Black et al., 2009), there also is compelling evidence that reassortment of hantaviral RNA segments can occur when vertebrate host-switching occurs (Zou et al., 2008; Chu et al., 2011).

Other examples of natural reassortants of hantaviruses have been documented but they do not appear to be as commonly occurring as are reassortants of other bunyaviruses. Laboratory studies of artificially-induced hantaviral reassortants have demonstrated that whereas reassortants occur, they usually are between closely related hantaviruses (Rodriguez et al., 1998). Nonetheless, when reassortants of Andes and Sin Nombre viruses were produced in a series of laboratory studies, reassortants of only one type were found; these comprised the L and S RNAs of Sin Nombre virus and the M RNA of Andes virus. This reassortant virus was highly infectious and elicited high-titer, Andes virus-specific neutralizing antibodies, but it did not cause hantavirus pulmonary syndrome and was not pathogenic for hamsters, which otherwise is a characteristic of Andes virus. The authors concluded that presence of the Andes virus M RNA is not sufficient to produce the Andes virus phenotype (McElroy et al., 2004). Taken together, these observations suggest that whereas hantaviruses can form reassortants when closely related to each other and have the opportunity to co-infect susceptible cells, it is likely that this does not occur often in nature because their rodent hosts exist in distinct cycles and, of course, these viruses are not transmitted by arthropods but in a host-specific manner from rodent to rodent under usual conditions.

Tospoviruses

Unlike arboviruses, tospoviruses do not replicate in hematophagous arthropods. Instead, they replicate in and are transmitted by plant-feeding arthropods, such as thrips of the genera *Frankliniella* and *Thrips*, and cause diseases in numerous plant hosts. Because their genomes comprise three RNA segments and they share other characteristics with bunyaviruses, they have been placed in a distinct genus (*Tospovirus*) of the family.

Reassortment of genome segments of tospoviruses had been demonstrated using field and laboratory-produced strains of tomato spotted wilt virus and results of genomic analyses supported a hypothesis that tomato spotted wilt virus utilizes genome reassortment to rapidly adapt to new host genotypes (Qiu and Moyer, 1999). However, reassortment of genome segments had not been documented between distinct viruses of the plant- and insect-infecting tospoviruses. Recent studies by Webster et al. showed that tospoviruses reassort their genome segments under natural conditions, as do other bunyaviruses (Webster et al., 2011). They found that the RNA sequences of an unnamed virus that causes severe symptoms in Florida tomatoes comprises the L and S RNAs of groundnut ringspot tospovirus and the M RNA of tomato chlorotic spot tospovirus (Table 1). Neither of the parental viruses is known to occur in the U.S. but this reassortant is and can be transmitted by western flower thrips (*Frankliniella occidentalis*). The authors concluded that this reassortant virus was introduced to the U.S. as such.

Bunyavirus genome stability

How do bunyaviruses maintain genomic stability when in nature many of them alternately replicate in arthropods and vertebrates? In one series of experiments, Moutailler et al. serially passaged Rift Valley fever virus in hamster cells (BHK-21) or in mosquito cells (Aag2) and alternately in these cells (Moutailler et al., 2011). Single host-passaged viruses became avirulent for vertebrates and infection with them protected cells against infection with virulent virus. After about a dozen passages in single hosts of either type, they found that the NSs gene encoding the virulence factor of this virus had large nucleotide deletions. Because this gene is non-essential for virus replication and viruses with such deletions replicate more rapidly than do those with the gene, such mutants became predominant (Moutailler et al., 2011; Bouloy et al., 2001). Conversely, genetic changes were not detected in viruses passaged alternately between arthropod and vertebrate cells. They concluded that alternating replication is necessary to maintain the virulence factor carried by the RVFV NSs-coded phosphoprotein. Whether or not these findings can be extrapolated for all vector-borne bunyaviruses, has yet to be determined.

Conclusions

Chastel suggested that asymptomatic infections in humans might serve as a "Trojan horse" for the introduction and spread of mosquito-borne arboviruses in non-endemic areas (Chastel, 2011). That might also be true for other hosts, other viruses, and other potential epidemiologic situations. Given that large and gregarious or migrating populations of humans and other vertebrates (or plants in regard to tospoviruses) might be in close contact with large populations of arthropod vectors of viruses, either could serve as virus reservoirs for at least brief periods of time. Such conditions become more threatening and urgent when two or more closely related bunyaviruses are circulating because those are the exact conditions under which reassortants emerge.

Geographic distances between viruses and vectors prevent reassortment. However, should ecologic conditions change or a relatively benign bunyavirus somehow be transported from its natural econiche to a different but suitable econiche, it could produce reassortants with a closely related, endemic, and relatively benign bunyavirus already established there. As noted above, reassortment of these viruses might result in progeny virus that is not at all benign. Genotypic drifts and shifts might make the situation more severe in terms of increases in pathogenicity.

Aside from geographic overlap and common susceptible host species and cell types, for reassortment to take place it also requires biological compatibility of the newly mixed components to produce viable progeny. Regardless of which of the two parental M segments is assembled into progeny virions, any of the host cell types that present one of the potentially multiple receptors targeted by one of the parental viruses in arthropod or mammalian hosts will be susceptible to infection. Once virus is internalized into the cell, either a productive or an abortive infection may ensue, depending on the capacity of viral, and viral and host cell components to interact functionally. Examples of specific interactions of viral and host components include the processing

of the viral polyprotein, assembly and egress of mature particles, and modulation of cellular innate immune system pathways. With respect to interactions between viral components, the S segment-coded nucleocapsid protein has to recognize and package all three RNA segments into ribonucleocapsid complexes (RNP), and to mediate interaction with the viral polymerase. The receptor-binding surface glycoproteins Gn and Gc, coded by the M segment, play crucial roles in virus assembly and budding through interaction with each other and possibly with host proteins that mediate their passage through the endoplasmic reticulum and the Golgi apparatus, and also by interacting with the nucleocapsid protein-wrapped RNPs. These functions are primarily mediated by the short cytoplasmic tail regions of these proteins (Shi et al., 2007; Överby et al., 2007). Interactions of the also M segment-coded non-structural protein (NSm) are not known and no function is assigned to this product as of yet. The viral polymerase, coded by the L segment, needs to recognize promoter regions at all three segment termini to initiate replication and transcription, and during copying it has to interact with nucleocapsid proteins.

The need for compatible viral components should favor reassortment between very closely related viruses, as it has been indicated through in vitro experiments by Iroegbu and Pringle for bunyamwera group viruses (Iroegbu and Pringle, 1981), and Rodriguez et al. for hantaviruses (Rodriguez et al., 1998): the more closely related the bunyaviruses, the more likely they are to produce viable reassortants. However, as mentioned earlier, co-infection in nature also is restricted by superinfection resistance, which permits co-infection by closely related viruses only in cases where the second virus infects rapidly after the first one and before superinfection resistance becomes effective. The balance between these two constraints, compatibility of newly mixed components and superinfection resistance, thus opens an evolutionarily productive window for reassortment: it limits reassortment between viruses that would not result in significant evolutionary shift, while still permitting adaptation of a virus to changing ecology by incidental reshuffling of the existing genetic inventory from variant strains (e.g. in La Crosse virus), and also supporting reassortment between differing viruses more likely to produce novel genetic traits.

As biological closeness or distance between viruses is thus a reigning factor in the capacity of two viruses to reassort, it has been proposed as a criterion potentially useful for taxonomic classification. However, genetic diversity (as much as has been determined) is quite different among current taxonomic entities. For example, whereas reassortment occurs among members of the Bunyamwera group viruses (species *Bunyamwera virus*), but not between them and La Crosse virus (species *California encephalitis virus*), reassortment appears to be frequent between viruses of three species of the Group C viruses (Fig. 1); Oriboca, Itaqui (species *Oriboca virus*), Caraparu, Apeu (species *Caraparu virus*), Marituba, Murutucu, and Restan (species *Murutucu virus*). Thus, known reassortment competence and current taxonomy are not always congruent.

From examination of the various reassortments mentioned previously, it appears that most recognized reassortants of orthobunyaviruses and of other bunyaviruses possess the L and S RNAs derived from one virus and the M RNA from another virus. This may reflect the more intricate interplay between polymerase, nucleocapsid protein, and RNA segments that entails a closer match than required for the surface glycoproteins, whose interactions are focused on their short cytoplasmic tails and may rely on mechanisms more universally conserved among different viruses. However, given our still limited knowledge of bunyaviral genome sequences, productive reassortment of other segment combinations must not be excluded. For example, whereas the S and M segments of Guaroa virus show a similar phylogenetic history, that of the L segment appears to be different (Fig. 3). In fact, as has already been indicated by the controversy about the phylogenetic roots of Schmallenberg virus, detailed analysis of phylogenetic relationships, especially among currently analyzed Group C viruses poses more questions than it actually answers.

In Table 1 we summarize possible scenarios for reported heterotypic reassortments. However, unless exchange among contemporary viruses is suggested by convincingly high sequence identity, this involves indirect exchanges through extinct or still unidentified last common ancestors and interpretation of relationships may not be straightforward. For example, although Group C viruses Caraparu, Oriboca, and Marituba may be considered parental based on the consistent positioning of their S and M segment sequences in phylogenetic trees, without knowledge of their L segment phylogeny such assignments must be considered tentative. Nevertheless, comparing S segments of Restan, Murutucu, and Oriboca viruses, it appears that the Restan virus sequence appears ancestral to that of Murutucu virus, which in turn branches ancestrally to that of Oriboca virus (Nunes et al., 2005). This challenges a parental role for Oriboca virus, at least for the S segment, and suggests Oriboca and Murutucu viruses as possible (reassorted) descendants of a Restan virus S segment progenitor. For the M segment sequences, Restan virus branches again ancestrally to Murutucu virus, which, however, appears ancestral to Marituba virus, while Oriboca virus is placed in a different clade as a twin to Itaqui virus (Nunes et al., 2005). Without knowledge of their L segments, this could be compatible with Restan virus being the parental virus for these two segments, which, however, map to disparate clades and suggest Restan virus as a potential reassortant virus combining S and M segments from two phylogenetically different ancestors. It is currently also unclear to what extent genetic drift influences such relations, given that the evolutionary accumulation of mutations may differ between viruses and environments. For example, some Group C virus may spread mainly over a narrow geographic range, encountering a limited variety of mosquito and mammalian hosts, whilst other viruses may spread over a larger area into diverse habitats and hosts that may exert very different selective pressures. Furthermore, the direction and order of exchange even for closely related sequences may remain ambiguous; for example, the Bunyamwera group Potosi virus may have acquired its M RNA directly from Kairi virus or via the reassortant Main Drain virus, but Main Drain virus could likewise have acquired its M RNA directly from Kairi or via the reassortant Potosi virus. Similarly, Wyeomyia group Macaua and Taiassui viruses are related via their M RNAs, but which was the other reassortment partner?

In the context of this short mini-review we did not discuss details of the possible mechanisms of how genome segments segregate into progeny virions – by random chance incorporation of, possibly more than three, RNAs to result in at least one functional copy of S, M, and L per (viable) virion, or by segment, and thus sequence specific segregation that ensures a single complete (haploid) genome per virion. Although a purely random packaging strategy appears less likely due to its intrinsic inefficiency, there is some evidence that argues against a purely segment specific process either. Rodriguez at al. reported for experimental reasortants of Sin Nombre hantavirus a significant number of partially diploid plaques derived from first-generation progeny of dually infected cells (Rodriguez et al., 1998). Although the partially diploid viruses did not propagate stably and disappeared during passage, it remains unclear to what extend non-stoichiometric constellations of homologous segments may occur in bunyaviruses, as they are not readily identifiable by common serologic or PCR-based analyses.

In conclusion, it seems that there are still links missing from the big picture either because they do not exist since they became

extinct, or because they are not yet known. Close examination indicates intricate relations not only between individual virus segments, but also on a larger scale between species. For example, whereas the Bunyamwera group and California encephalitis viruses appear to share for their L and M segments a more recent common ancestor with each other than with the Simbu group viruses, for their S segment sequences California encephalitis viruses share a more recent common ancestor with the Simbu group viruses than with the Bunyamwera group viruses (Fig. 3). Interestingly, although beyond the scope of this short mini-review, there may even be relations to other families of segmented viruses deducible as indicated by the polyphyletic branching of bunyavirus L-segment sequences (Vieth et al., 2004) that indicates a common ancestry of phlebovirus and tenuivirus L sequences, or those of nairoviruses and arenaviruses, a relationship also discernable from the analysis of Crimean-Congo hemorrhagic fever virus N protein sequence that is more closely related to arenaviral than to bunyaviral N sequences (Carter et al., 2012).

Such findings raise the fundamental question as to what extent all currently circulating bunyaviruses may represent ancient or more recent reassortant viruses. This may include reassortment of yet unrecognized or already extinct reassortants or original parental viruses. If currently unrecognized but extant, then additional efforts at virus discovery might detect them. Although we can no longer find them if extinct, we might be able to create the parental viruses based on contemporary sequence variants using laboratory devised reassortants. Such research to rationally generate extinct parental viruses based upon extant descendant genomes might, however, be considered as controversial. Based on the premise that evolution is selecting for viruses that are relatively benign in their vertebrate hosts, such work would in essence be creating potentially “new” viruses with greater pathogenicity than are currently circulating, a possible biosafety hazard.

What then characterizes true parental prototypes, if they exist at all? Given the recent revolution in sequencing technology, which allows rapid determination of any organism's genome without prior knowledge of its composition, it is likely that we will gain a more detailed understanding of the dynamics of bunyavirus evolution over the next few years that may solve at least some of the discussed mysteries. Although detailed knowledge about bunyaviral genomes will not be “the holy grail”, it will certainly provide new insights into virus evolution, and build the basis to formulate questions beyond the basics of reassortment. It may be that all the bunyaviruses we now consider “prototype” strains are reassortants of ancient viruses. Thus: Where is Square One?

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