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Molecular aspects of Rift Valley fever virus and the emergence of reassortants

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Abstract

Rift Valley fever phlebovirus (RVFV) is a mosquito-transmitted pathogen endemic to sub-Saharan Africa and the Arabian Peninsula. RVFV is a threat to both animal and human health and has costly economic consequences mainly related to livestock production and trade. Competent hosts and vectors for RVFV are widespread, existing outside of endemic countries including the USA. Thus, the possibility of RVFV spreading to the USA or other countries worldwide is of significant concern. RVFV (genus *Phlebovirus*) is comprised of an enveloped virion containing a three-segmented, negative-stranded RNA genome that is able to undergo genetic reassortment. Reassortment has the potential to produce viruses that are more pathogenic, easily transmissible, and that have wider vector or host range. This is especially concerning because of the wide use of live attenuated vaccine strains throughout endemic countries. This review focuses on the molecular aspects of RVFV, genetic diversity of RVFV strains, and RVFV reassortment.

Keywords Rift Valley fever · Phlebovirus · Replication · Genetic diversity · Reassortment

Introduction

Rift Valley fever phlebovirus (RVFV) is the etiological agent of a mosquito-transmitted disease endemic in sub-Saharan Africa which has spread to the Arabian Peninsula. Outbreaks of the disease result in high morbidity and mortality among young ruminants including sheep, goats, cattle, and pseudoruminants such as camels, and frequent abortions in pregnant animals [1]. Thus, RVFV is responsible for great economic losses to livestock production and rural farming communities. Moreover, the virus can cause disease in humans ranging from mild flu-like symptoms to severe hepatitis, blindness, encephalitis, and in some cases death [1]. Inactivated and live attenuated vaccines have historically been used for veterinary use during outbreaks in endemic countries [2]. Live attenuated vaccines are controversial because of the concern of reversion back to virulence, either by mutation or by genomic segment reassortment with the circulating wild-type strains. Other RVFV vaccines have been developed using various platforms such as genetically modified live, recombinant, vectored and replicon systems in order to make safer, more effective, and DIVA (differentiating infected from vaccinated animals) compatible vaccines [3]. However, there is still no fully licensed veterinary or human vaccine for use in non-endemic countries.

Transmission of RVFV can occur through contact with infected blood, milk, or tissue, or by RVFV-infected mosquito bites. RVFV-competent mosquito vectors fall under 73 species of mosquitoes in the 8 genera of the family *Culicidae* [4, 5]. Enzootics/epidemics typically occur following periods of heavy rainfall which creates favorable breeding conditions for mosquito vectors. During inter-epidemic periods, RVFV circulates in nature via horizontal transmission between mosquito vectors and livestock, or wildlife reservoirs such as indigenous wild ruminants. Trans-ovarial or vertical transmission is also widely reported [5]. Field testing for RVFV by immunological assays in endemic areas

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and in vivo studies has shown that RVFV can infect a wide range of animals [6]. Although no definitive wildlife reservoir has been clearly identified, there is evidence suggesting RVFV may be maintained in buffalo [7, 8]. Competent vectors and host species exist outside of endemic geographic regions including the USA [9–11]. Interestingly, cell lines from multiple tissues derived from animals native to North America such as white tailed deer, coyote, and swine were found to support RVFV replication [12]. Thus, RVFV is not only a concern for endemic countries, but presents a risk worldwide.

Molecular properties of RVFV

Classification

RVFV belongs to the genus *Phlebovirus*, along with Punta Toro, Uukuniemi, sandfly fever-like phleboviruses and other species. The genus *Phlebovirus* has been historically classified under the family *Bunyaviridae* along with *Orthobunyavirus, Hantavirus, Nairovirus*, and *Tospovirus*. In 2016, the International Committee on Taxonomy of Viruses created a new order, *Bunyavirales*, which contains nine new families and reorganized the previous genera, thus replacing *Bunyaviridae* [13]. *Phlebovirus* is currently classified under the family *Phenuiviridae* (Fig. 1).

Virion organization

Virion structure

Using cryo-electron tomography, RVF viral particles were determined to be spherical with an average particle diameter of 103 ± 3 nm [14], consistent with previous studies which identified pleomorphic particles with varying diameters ranging from 90 to 110 nm by negative staining [15]. Each of the three viral RNA segments forms ribonucleoprotein (RNP) complexes (viral core; 66 nm diameter) with the viral nucleocapsid protein (N) and the viral RNA-dependent RNA polymerase (L) which are packaged in a lipid bilayer (envelope) obtained during budding into the Golgi complex during virion assembly [16]. The envelope is made up of dimers of the viral glycoproteins, Gn and Gc, organized with icosahedral symmetry and projecting as 12-nm-long cylindrical spikes on the envelope [14, 17, 18]. The 12 pentamers and 110 hexamers are arranged on the viral surface with icosahedral symmetry having a T12 triangulation number [14]. Located 2.2 nm apart, the pentons (12 nm diameter) are positioned around the fivefold symmetry axis and hexons (14 nm diameter) organized around the threefold, quasi threefold, and twofold axes [14, 19]. The virus particle is estimated to contain 720 glycoproteins of both Gn and Gc with each penton made of 10 glycoproteins, and each hexon made of 12 glycoprotein molecules of Gc or Gn monomers, homodimers, or heterodimers [13]. A gap of 4.5 nm exists between the viral envelope and the RNP core [14]. The RNP has a string-like structure and forms a pan-handle structure due to the complementarity of the 5′ and 3′ genomic termini [20].

Genome organization

The genome is composed of a tri-segmented, negative-stranded RNA that encodes for seven proteins (Fig. 2). The 6.4 kb large (L) segment encodes the viral RNA-dependent RNA polymerase (RdRp; L protein). The 3.2 kb medium (M) segment encodes a single open reading frame (ORF) which produces the envelope glycoproteins Gn and Gc, and non-structural proteins: NSm (14 kDa) and the 78 kDa Gn/

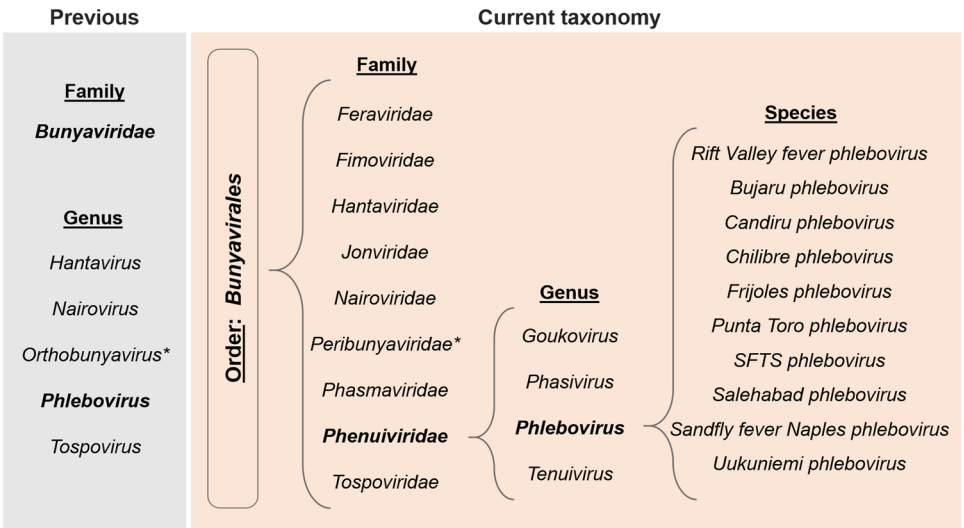

Fig. 1 Classification of Rift Valley fever virus. Current reclassification was designated in 2016 by the International Committee on Taxonomy of Viruses. The genus *Orthobunyavirus* was reclassified under the family *Peribuyaviridae* (*). *SFTS* severe fever with thrombocytopenia syndrome

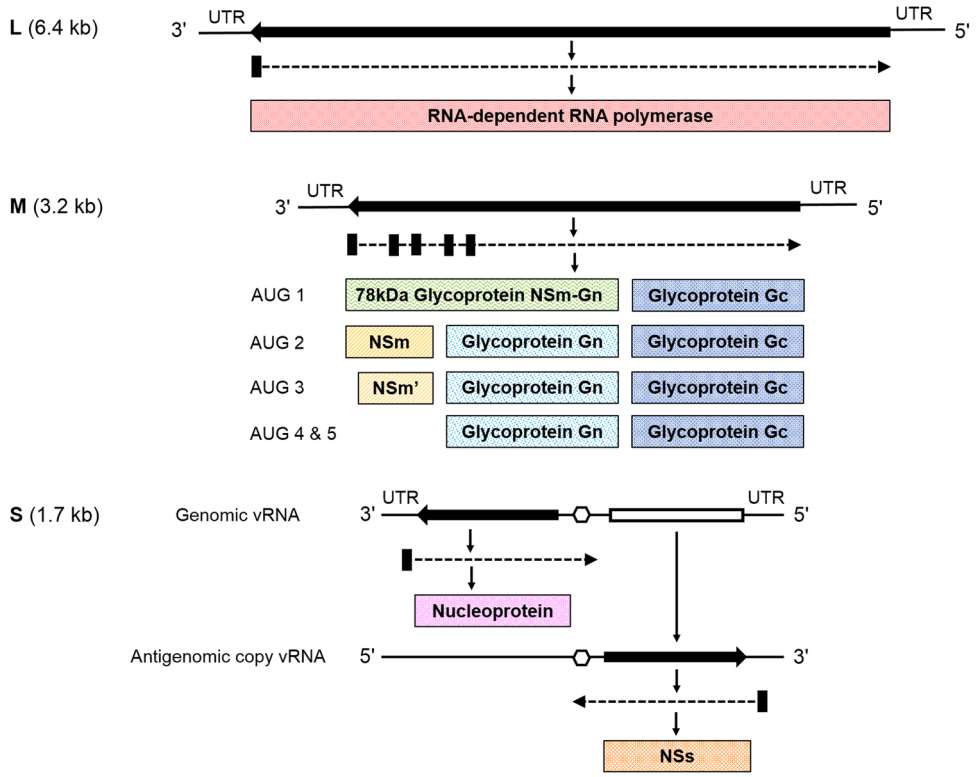

Fig. 2 Genomic organization of Rift Valley fever virus. The encoded open reading frames are shown as solid black arrows on the large (L), medium (M), and small (S) segments of the viral genome. Untranslated regions (UTR) flank each segment. The length of each genomic segment is indicated in kilo bases (kb). Transcribed mRNAs are depicted as dashed arrows with the start codons (AUG) indicated as black boxes, and the resulting proteins shown as shaded boxes. Proteins encoded by the M segment are produced by posttranslational cleavage of translated polyproteins. The nucleoprotein is generated by an mRNA transcribed from the negative-sense genomic viral RNA (vRNA). The non-structural S protein (NSs) is represented on the genomic-sense S vRNA as a white box and is produced from an mRNA transcribed from the antigenomic copy vRNA generated during viral replication. The octagon represents an intergenic transcription termination site

NSm fusion protein; these proteins are produced by alternative usage of multiple AUG start codons at the 5′ end of the mRNA and co-translational cleavage of the respective precursor proteins [21–24]. Translation initiation from the first AUG results in the synthesis of the 78 kDa Gn/NSm protein, and the NSm protein is produced from the second AUG. Protease cleavage at the N-terminus of Gn releases a mature NSm protein. Translation from the third AUG was recently reported to generate a truncated NSm protein termed NSm′ [25]. This protein lacks 38 amino acids from the N-terminus of NSm and when compared with NSm, is predominantly expressed in infected Vero cells [25]. Translation from the fourth or fifth AUG produces Gn and Gc, both of which have a single ectodomain with 1 or 4 potential *N*-glycosylation sites, respectively [21–24]. The 1.7 kb small (S) segment is ambisense, with the nucleoprotein N encoded in the genomic-sense, and NSs in the antigenomic orientation. Transcription termination sites are present at both viral-sense and antiviral-sense RNA located between the N and NSs ORFs. Short untranslated regions (UTRs) flank each genomic segment which hybridize to form circular RNP structures and are important for both transcription and replication [26].

Viral protein functions

Gn and Gc are important for viral attachment and entry [14]. The envelope glycoprotein Gn is responsible for recruitment of RdRp to the Golgi membranes [27]. Gn is also independently involved in the packaging of N into virions [27]. The glycoproteins are also involved in efficient viral release from infected cells, for which the primary stimulus is the encapsidated genome [27, 28]. The N protein encapsidates genomic RNA by protein–RNA and protein–protein interactions and forms the RNP [13, 17]. The L protein forms part of the RNP core and plays a vital role in the primary transcription of mRNAs transcribed from the viral genome soon after viral entry and directs the genomic RNA replication in the cytoplasm [27, 29]. Gn, Gc, and N make up the major

antigenic proteins and are therefore the common targets for diagnostic and vaccine development.

The non-structural proteins (NSm, 78 kDa Gn/NSm, and NSs) are associated with virulence. Both, NSm, and the 78 kDa protein were shown to be dispensable for viral replication [30]. However, it was observed that infection of *Culex quinquefasciatus* with RVFV lacking the 78 kDa/NSm displayed reduced transmissibility and infection rate whereas it was completely unable to infect *Aedes aegypti* mosquitoes [31]. NSm is targeted to the mitochondrial membrane and plays a role in delaying the onset of virus-induced apoptosis [32, 33]. NSs is also dispensable for the viral life cycle but is a major virulence factor for RVFV due to its ability to suppress host antiviral responses [34]. NSs is localized to both the cytoplasm and the nucleus where it forms filamentous structures [35]. One of the earliest known functions of NSs is its ability to inhibit IFN function [36]. It was later found that NSs specifically blocks transcription from the IFN-β promoter by interacting with the sin3A-associated protein (SAP30) through the transcription factor YY1. This interaction allows the repressor complex of SAP30, YY1, and Sin3A-associated corepressor factors to stay on the IFN-β promoter, resulting in the inhibition of transcriptional activation of the IFN-β promoter [37].

Another major virulence function of NSs is its ability to suppress general host gene expression by inhibiting the essential transcription factor TFIIH. NSs independently interacts with two subunits of TFIIH: p44, resulting in its sequestration [38] and p62, resulting in its proteasomal degradation [39–41]. Besides its effect on the host transcription machinery, NSs also prevents the induction of antiviral genes in infected cells by promoting the proteasomal degradation of dsRNA-dependent protein kinase (PKR) [28, 42]. PKR is activated in virus-infected cells, which results in increased phosphorylation of the translation initiation factor eIF2α which in turn results in translation suppression and a general inhibition of viral gene expression [43, 44]. Thus, inhibition of PKR by NSs allows RVFV protein synthesis to continue in virus-infected cells. A study by Kalveram et al. (2013) revealed that the host transcription inhibition and PKR degradation functions of NSs are independent of each other [45].

NSs also interacts with the host pericentromeric DNA sequences, thus inducing chromosome cohesion and segregation defects [46]. Another interesting observation is the NSs-induced nuclear relocalization of the host Poly(A) binding protein 1 (PABP1) during RVFV infection. PABP1 binds to the polyA-tail of cellular mRNAs and enhances host mRNA translation by bridging the polyA-tail with the 5′cap-binding eIF4F complex. RVFV-transcribed viral mRNAs, like other phleboviruses, do not have a polyA-tail and hence is independent of PABP1 function. By relocalizing PABP1 during RVFV infection, NSs ensures the inhibition of host mRNAs translation, thereby increasing the chances of viral mRNA translation by freeing up the host cell translation machinery [47]. Besides these functions, the multifunctional protein NSs also induces an increase in reactive oxygen species, activates the p53 signaling pathway, inhibits mRNA export from the nucleus, induces DNA damage response via the ataxia-telangectasia mutated (ATM) signaling pathway, and also inhibits cell cycle arrest either at the G_0/G_1 or S phase [48–52].

Replication and packaging

RVFV replication occurs in the cell cytoplasm and generally takes approximately 10–12 h to complete one cycle (Fig. 3a). Replication has been shown to start locally, near the endosomal fusion site [53]. The RNPs are released into the cytosol and transcribed, translated, and replicated within the first 6 h of infection, with an estimated 40 min doubling time [53]. Recruitment of genome segments to the Golgi occurs at 6–8 hpi, most likely through interactions between N and the cytoplasmic tail of Gn [27, 53, 54]. Gc is targeted to the endoplasmic reticulum (ER) by virtue of an ER-targeting motif, while Gn is retained at the Golgi by means of a Golgi-retention signal, thus allowing co-localization of both glycoproteins at the Golgi apparatus. The cytoplasmic tail of Gn recruits the RNP and the RdRp, and after assembly, the progeny virions bud from the Golgi [55–57]. While previous work suggested the packaging mechanism for RVFV is a selective process [58, 59], more recent evidence supports a non-selective process [53]. A recent study by Schreur and Kortekaas (2016) showed that RVF virions have heterogeneous segment composition, with 40% containing no genome segments, up to 25% lacking M, and only around 10% of virions contained all three genome segments [53]. Other studies have also demonstrated the plasticity of RVFV packaging through the efficient production of RVF replicon variants with two and four segmented genomes [60–62]. Furthermore, during coinfection with different virus strains, it is possible for replicated genomic segments from the parent infecting viruses to be packaged into virions, generating reassortant viruses (Fig. 3b). It is also known that during assembly, both negative- and positive-sense viral genomic RNAs are incorporated into the virion, thus allowing for the immediate transcription of both the N and NSs mRNAs [29, 63].

Genetic diversity of RVFV

Sequencing and phylogenetic studies conducted over the last 20 years have shown the RVFV genome to be overall highly conserved, with most variances occurring as random single site mutations throughout the genome with no well-defined

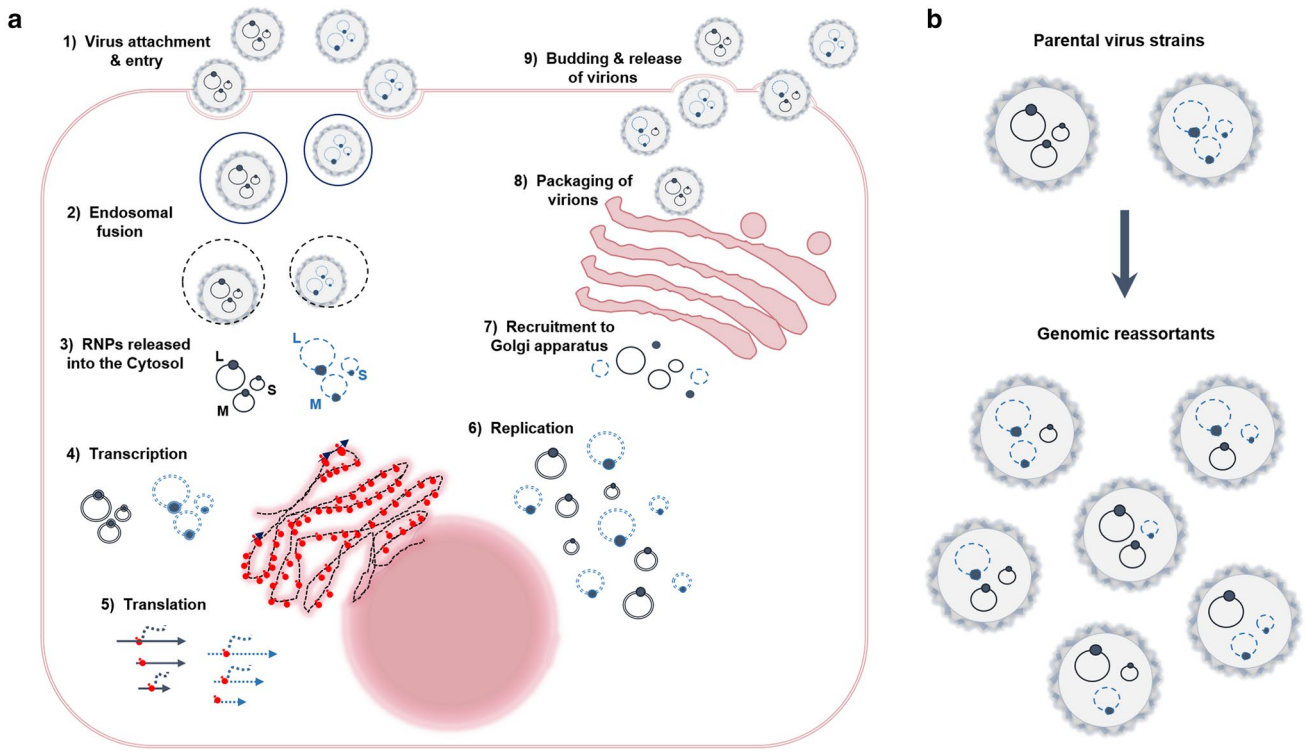

Fig. 3 Rift Valley fever virus replication cycle. **a** Coinfection with two different virus strains is shown. Following attachment and entry into a host cell, the viral ribonucleoproteins (RNPs) which consist of viral genomic RNAs complexed with nucleoprotein and viral RNA-dependent RNA polymerase are released into the cytosol via endosomal fusion. Circularization of the linear, single-stranded genome segments occurs via hybridization of the flanking untranslated regions. Transcription, translation, and replication occur in the cytoplasm. Newly replicated viral genomic segments and proteins are recruited to the Golgi for assembly and packaging of virus particles. Mature virions are released from the infected cell. **b** Possible virus reassortants following coinfection with two virus strains are represented by solid- or dotted-line genomic segments

variable regions. This makes differentiation between strains without genome sequencing difficult, since there are also no well-defined serotypes. The first phylogenetic study based on classification by NSs of 18 different RVFV strains yielded only two genomic lineages: Egyptian and sub-Saharan [64]. Subsequent studies including partial and whole genome sequences have expanded the major lineages to Egypt, West Africa, and East Central Africa [65], then to 7 lineages designated A through G [66, 67], and more recently to 15 lineages classified as A through O [68]. The varying number of lineages identified can be explained by the varying number of analyzed sequences, the use of partial or whole genome sequences, and the methods used for analysis.

The first comprehensive whole genome analysis consisted of 33 RVFV isolates from various African countries and Saudi Arabia over a period spanning 1944–2000, with varying pathogenicity [66]. Included were samples collected during two large outbreaks in 1977–1978 Egypt and 1987 Mauritania, as well as, from 1970 Zimbabwe localized epizootic events. This study illustrated that while the virus strains were ecologically and biologically diverse, the overall genetic diversity was low. Pairwise identity of genome segments between isolates were similar, with nucleotide and amino acid differences of 5% and 2% for M, and 4% and 1% for the L and S segments, respectively. A later study evaluating partial M sequences of 203 isolates also found similar genetic pairwise differences [68].

Interestingly, higher diversity was observed among isolates from Zimbabwe during a low endemic period compared to the large outbreaks in Egypt and Mauritania, suggesting multiple strains concurrently circulating in Zimbabwe, and a single predominant strain in Egypt and Mauritania [66]. In contrast, a genetic analysis of a wide variety of samples collected during the Kenya 2006–2007 RVFV outbreak showed two lineages circulating during that time [69]. This translated to a pairwise nucleotide difference of over 1%, as compared to 0.2% reported for epidemics in Egypt and Mauritania [66]. Thus, these studies demonstrate the conserved nature of the RVFV genome, despite differences in virulence, and that multiple RVFV strains can be found co-circulating during both epidemic and inter-epidemic periods.

RVFV reassortment

Viral reassortment is the exchange of genomic segments between two or more virus strains during coinfection of a host cell (Fig. 3). This phenomenon can occur among segmented RNA viruses, including members of the *Phlebovirus* and *Orthobunyavirus* genera. Genomic reassortment among RVFV strains is also known to occur and has been demonstrated experimentally and documented among field isolates. The results from those studies are discussed in the following sections.

Experimental evidence

Viral reassortment was originally utilized to map the mutations which conferred attenuation of the RVFV MP-12 strain [70]. Reassortants isolated from Vero cells coinfected with MP-12 and wild-type ArD38661 were tested in mice, which revealed attenuating mutations on each of the three genome segments. Similarly, Turell et al. [71] used several monoclonal antibodies to investigate the potential for reassortment to occur in the mosquito vector by using a *Culex* mosquito and hamster model and the RVFV strains ZH501 [72], ArD38661 [73] and MP-12 [74]. Both studies detected only S and M reassortants, as there were no monoclonal antibodies to differentiate the L segment. Despite this limitation, the study by Turell et al. (1990) yielded several important findings regarding RVFV reassortment [71]. First, reassortment was shown to occur in mosquitoes intrathoracically injected with two different virus strains. This was demonstrated between two pathogenic strains (ZH501 and ArD38661), as well as, a pathogenic and attenuated strain (ArD38661 and MP-12). Second, prior infection with one strain (MP-12) was shown to interfere with replication of a second homologous strain in the mosquito (ArD38661). Third, reassortants were isolated from mosquitoes that intermittently fed on a hamster infected with the ArD38661 strain and subsequently fed on a hamster infected with ZH501, as well as, from mosquitoes fed on a hamster coinfected with both strains. Finally, reassortant viruses were transmitted to naïve hamsters by infected mosquito bites. Thus, this study showed that reassortment can occur between two RVFV strains during coinfection, at the vector level. Furthermore, these two studies demonstrated reassortment between a pathogenic and attenuated strain, ArD38661 and MP-12, respectively.

A more recent study used an in vitro model system to investigate the risk of RVFV MP-12 strain to reassort with other related viruses of the family *Phenuiviridae* of the genus *Goukovirus*, and Arumowot virus, an unclassified *Phlebovirus* [75]. The results showed that coinfected cell cultures with either of the two viruses did not produce any viable reassortants with RVFV MP-12. However, reassortment did occur between RVFV MP-12 and a genetic MP-12 variant in coinfected C6/36 mosquito cells. Collectively, these in vitro studies demonstrate that reassortment can readily occur and is a conserved event between RVFV strains.

Examples in nature

Examples of RVFV reassortment that have occurred under natural conditions from 5 separate field and case studies have been reported (Table 1). The first identification

Table 1 Identified reassortants from field isolates

Isolate ID	Isolation year	Origin	Source	Reassorted segment	References
ArD38661	1984	Senegal	Mosquito	S	[65]
AnK6087	1984	Guinea	Bat	S	[65]
H D 47502	1987	Mauritania	Human	S	[65]
ArD104769	1993	Senegal	Mosquito	L	[65]
AnD106417	1993	Senegal	Zebu	L	[65]
73HB1230	1973	CAR	Human	L	[66]
2007000608	2007	Kenya	Bovine	M	[69]
SA184/10	2010	South Africa	Human	M	[68]
TANTan00107	2007	Tanzania	Human	L/M	[76]
211HMMRRO1987	1987	Mauritania	Human	M/S	[76]
11ANMMRHG1998	1998	Mauritania	Sheep	M/S	[76]
SA75	1975	South Africa	Human	M/S	[76]
3574	1974	South Africa	Sheep	M/S	[76]
76370	1970	Zimbabwe	Bovine	M/S	[76]
Beijing-01	2016	Angola	Human	S	[67]

CAR Central African Republic

of RVFV reassortment from field isolates was by Sall et al. [65]. In that study, 20 isolates were subjected to phylogenetic analysis, revealing 3 major lineages based on geographic regions: West Africa, Egypt and Central-East Africa. Two tests used to analyze the incongruence in topology identified five reassortants. The reassortants identified were isolated from various sources between 1984 and 1993 in Senegal, Guinea, and Mauritania (Table 1) and consisted of reassortment of the S or L genomic segments [65].

Whole genome sequence analysis of 33 RVFV isolates suggested reassortment was not common since there was less incongruence in their topology; however, there was some evidence indicating the occurrence of reassortment events [66]. From the seven phylogenetic lineages (A–G), strain 73HB1230 was found to have S and M segments belonging to lineage B, while the L segment grouped with lineage A. Furthermore, there was phylogenetic evidence of an earlier reassortant event with the S segment of this strain. In addition, there was support for reassortment among M segments of lineages D and E although a later analysis by Grobbelaar et al. (2011) did not find clear evidence to support this as a reassortment event [68].

Grobbelaar et al. [68] performed partial sequencing of the M segments of an additional 170 RVFV field isolates, expanding on the previous 33 sequenced genomes [66]. Altogether, the 203 isolates were collected over a period of 67 years (1944–2010) from Saudi Arabia and 16 countries of Africa. Following phylogenetic analysis, five identified divergent isolates were further sequenced and analyzed for possible genetic reassortment. From that analysis, an M segment reassortant (SA184/10) was identified. The reassortant was isolated from a patient with an accidental needle stick injury while vaccinating sheep, which could have been potentially exposed to both the live attenuated Smithburn vaccine strain and wild-type virus. Phylogenetic investigation revealed that the M segment of the isolated virus grouped with the Smithburn vaccine strain of lineage K, and the S and L segments were closely related to SA54/10 which was isolated from another patient in the same area. In addition, there was evidence of historical reassortment events for three other isolates: H1739 and H1825 from the first recorded human deaths caused by RVF in 1975, and 95EG Cow-2509 isolated from an aborted fetus of a cow vaccinated with 95EG in Egypt [68].

Another M reassortant was identified from an analysis of 31 samples collected during the Kenya 2006–2007 RVFV outbreak [69]. This was later confirmed by Freire et al. (2015) using a set of tools initially used to study reassortment of influenza viruses. Using this analysis framework, pairs of segments were compared revealing five additional M and S reassortants, as well as, a new L/M reassortant, TANTan00107, shown in Table 1 [76].

More recently, an S reassortant was identified from a man working in Angola in 2016 during a non-epidemic period [67]. Similar to the previous studies, phylogenetic analysis revealed seven lineages, A–G. The reassortment occurred between lineages A and E, with the L and M segments most closely related to a South African isolate from sheep in 2009, and S grouping with several strains spanning regions from Egypt to South Africa and Madagascar from the 1970s to 2004 (lineage A). The patient was a fork-lifter in a rural area, frequently exposed to mosquitoes with no history of contact with livestock or febrile humans.

Together, these data (summarized in Table 1) show that reassortment between RVFV strains occurs naturally in the field, and multiple examples of reassortants for each segment type have been identified. Furthermore, reassortants have been isolated from a wide range of sources, and during epidemic and inter-epidemic periods or regions.

Frequency of RVFV reassortment

In earlier studies, 7 out of 50 (14%) viruses isolated from Vero cells coinfected with the RVFV MP-12 and ArD38661 strains identified as reassortants [70]. Reassortants were also found from all ten mosquitoes simultaneously coinfected with those same strains, although the percentage of reassortants was not reported [71]. Reassortants made up 27% (32/119) of viruses recovered from mosquitoes coinfected with ZH501 and ArD38661 [71]. However, because those studies lacked the ability to differentiate the L segment, the percentages reflect only S and M reassortants.

More recently, a study with the RVFV MP-12 strain and a genetic variant showed out of 47 plaques isolated from coinfected C6/36 mosquito cells, 83% were reassortants [75]. The reassortants recovered were further differentiated by segment and consisted of 36% L reassortants, 15% M, and 32% were S. It is not known whether reassortment occurs preferentially for a particular segment, but experimental [75] and field data (summarized in Table 1) indicates that reassortment can occur among any of the three genomic segments. Furthermore, there is evidence that RVFV uses a non-selective packaging strategy, suggesting that reassortment between segments may also be non-selective [53].

While experimental infections in mosquitoes suggest mosquitoes are permissive for generation of RVFV reassortants (27% collectively), fewer reassortants (only 2) have been recovered from infected mosquitoes from the field (Fig. 4; Table 1). This most likely reflects the bias of optimized experimental conditions which may not be representative of those encountered in the field, as well as the sources and number sampled. For example, infection of mosquitoes by Turell et al. (1990) was performed by intrathoracic inoculation which bypasses the midgut infection/escape barrier which could correlate to a higher reassortant frequency

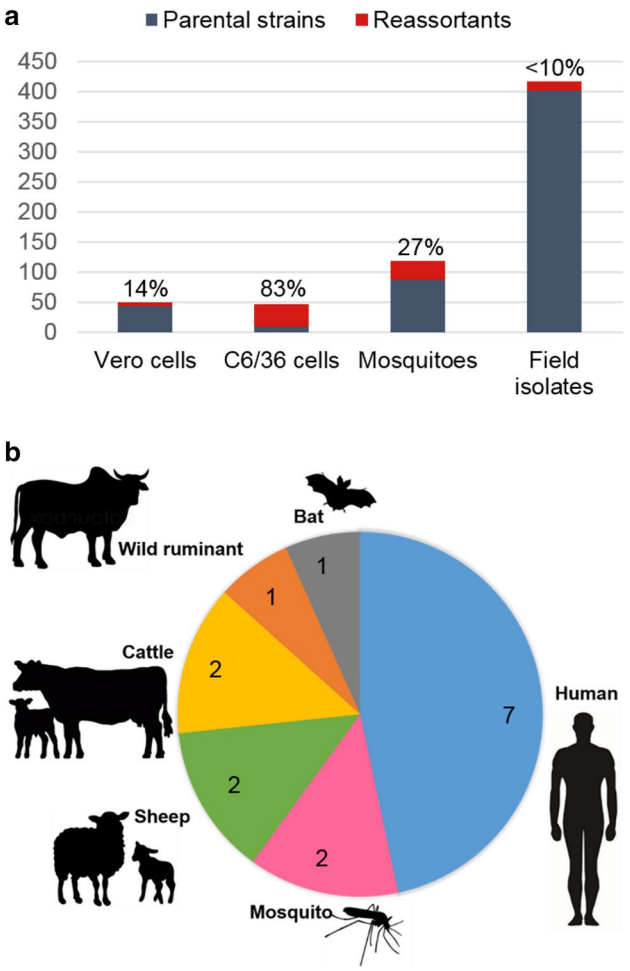

Fig. 4 Rift Valley fever virus reassortment. **a** Frequency of experimental and field reported reassortants. The number of reassortants out of total virus isolates evaluated is shown, with percentage of reassortants indicated. **b** Sources from which reassortants have been isolated in the field are shown; the number of reassortants isolated from each source derived from Table 1 are indicated

because the reassortant viruses did not undergo the same selective pressure as orally fed viruses [71]. Concerning is the comparatively high number of reassortant viruses isolated from humans (Fig. 4b), especially given the fact that other reassortant bunyaviruses are associated with severe human illness [77, 78]. Still, it is not clear whether reassortment occurs more frequently in humans, ruminants, other mammals, or mosquitoes; or whether those RVFV reassortants isolated possess altered pathogenicity and/or host range.

Reversion to virulence

Reassortment of an attenuated RVFV with a pathogenic strain resulting in reversion to a virulent phenotype is of significant concern given the wide use of attenuated and modified live vaccine strains in endemic countries. For example, the attenuated live Smithburn vaccine strain has been widely used throughout Africa for more than six decades and, in fact, has been identified as reassorting with a wild-type field strain [68]. The live attenuated MP-12 strain has been pursued as a more desirable alternative to earlier live attenuated strains such as Smithburn. Reassortment between MP-12 and a pathogenic RVFV strain, ArD38661, was demonstrated both in vitro [70] and in vivo [71]. Reassortants isolated from MP-12/ArD3811 coinfected Vero cells and tested for virulence in mice were found to possess an attenuated phenotype for each of the three MP-12 genome segments [70]. Furthermore, genetic analysis showed multiple mutations on each segment of the MP-12 genome conferred attenuation, suggesting the risk of MP-12 reverting to a virulent phenotype via reassortment is low or unlikely [79, 80]. Nonetheless, another study showed the introduction of a few revertant mutations within the MP-12 L and M segments following 25 passages in Vero or MRC-5 cells [81]. Additionally, there is still the risk of other live attenuated vaccine strains that do not possess attenuations in each of the three genome segments to reassort with wild-type viruses.

Reassortment with related viruses

Reassortment of RVFV with other closely related viruses (e.g., other members of *Phenuiviridae*) is also a concern, especially with the co-circulation of multiple bunyaviruses in the field and the continued discovery of new viruses. For example, Ngari virus was detected during a RVFV outbreak in Mauritania in 2010, with serological evidence of a possible coinfection in a goat [82]. Ngari virus itself is a reassortant of Bunyamwera virus and Batai virus, both classified under the genus *Orthobunyavirus* [77, 78, 83], and is associated with hemorrhagic fever in humans, although clinical disease in animals is not currently known. Both Bunyamwera and Batai viruses infect humans, animals, and arthropods. The concern is the possibility that reassortment could lead to more virulent strains which are easily transmittable and which could have a wider vector or host range. An experimental approach to reassortment between the MP-12 strain and bunyavirus species closely related to RVFV was performed by Ly et al. (2017) using the Arumowot virus (AMTV) and the Gouleako goukovirus (GOLV), both transmitted by mosquitoes in Africa. The results of this study showed that AMTV and GOLV do not form detectable reassortant strains with the MP-12 strain in coinfected C6/36 cells, most likely due to their incompatibility among the N, L, and Gn/Gc proteins. While reassortment of RVFV with other related viruses may be less likely, as shown by Ly et al. (2017), it should be taken in to consideration and not overlooked [75].

Concluding remarks

While there is strong evidence that RVFV genetic reassortment does occur, there is much that remains unknown. More studies are warranted to address the following critical questions regarding RVFV reassortment.

- What is the frequency of reassortants recovered from naturally infected vectors (different mosquito species), ruminants (different species), non-ruminants, and humans?
- What is the potential of emergence of reassortants between wild-type RVFV and attenuated live RVFV vaccine strains (e.g., Smithburn, MP-12), or other members of *Phenuiviridae*?
- Is there preference based on a particular segment for reassortment?
- What are the phenotypic consequences of such reassortants? Are reassortants more fit? More virulent? Have a wider host range?
- Ultimately, how do all of the above criteria relate to risk to humans and animals?

These questions are not easily answered given the complexity of virus–vector–host interactions, and the constraints of working with a Select Agent. Experimental studies are laborious and ultimately require genetic modification of the viruses used or sequencing to confirm a reassortment event due to the low genetic diversity among RVFV strains. Data collection from larger sampling numbers will be necessary to more accurately assess frequency rates and risk. While certain animal species and virus–vector interaction studies are possible to address the consequence of reassortants, recapitulation of natural conditions in experimental studies with humans is not possible. Thus, study models such as non-human primate and well-documented field cases are necessary for addressing these questions in humans. As molecular virological techniques such as sequencing becomes more affordable and bioinformatic tools become more sophisticated, the elucidation of these questions should become more attainable. The limited number of earlier studies discussed in this review provide insight and pave the way to address these key questions regarding RVFV reassortment.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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