Antiviral Research 170 (2019) 104569

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

High-throughput screening for negative-stranded hemorrhagic fever viruses using reverse genetics

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ARTICLE INFO

Keywords:
(3–10 max.): reverse genetics
High-throughput screening
Antiviral screening
Life cycle modelling
Minigenome systems
Viral hemorrhagic fever

ABSTRACT

Viral hemorrhagic fevers (VHFs) cause thousands of fatalities every year, but the treatment options for their management remain very limited. In particular, the development of therapeutic interventions is restricted by the lack of commercial viability of drugs targeting individual VHF agents. This makes approaches like drug repurposing and/or the identification of broad range therapies (i.e. those directed at host responses or common proviral factors) highly attractive. However, the identification of candidates for such antiviral repurposing or of host factors/pathways important for the virus life cycle is reliant on high-throughput screening (HTS). Recently, such screening work has been increasingly facilitated by the availability of reverse genetics-based approaches, including tools such as full-length clone (FLC) systems to generate reporter-expressing viruses or various life cycle modelling (LCM) systems, many of which have been developed and/or greatly improved during the last years. In particular, since LCM systems are capable of modelling specific steps in the life cycle, they are a valuable tool for both targeted screening (i.e. for inhibitors of a specific pathway) and mechanism of action studies. This review seeks to summarize the currently available reverse genetics systems for negative-sense VHF causing viruses (i.e. arenaviruses, bunyaviruses and filoviruses), and to highlight the recent advancements made in applying these systems for HTS to identify either antivirals or new virus-host interactions that might hold promise for the development of future treatments for the infections caused by these deadly but neglected virus groups.

1. Introduction

Viral hemorrhagic fever (VHF) is a clinical syndrome characterized by non-descript initial symptoms such as malaise and fever, which progresses at later stages to include vascular damage, capillary permeability, and coagulation abnormalities, leading to a terminal phase with shock-like symptoms and multiorgan failure (Feldmann and Geisbert, 2011; Paessler and Walker, 2013; Schnittler and Feldmann, 2003). It is regularly caused by infection with members of one of four RNA virus groups, of which three (i.e. the filoviruses, bunyaviruses and arenaviruses) are negative-sense single-stranded RNA viruses. Among the filoviruses, Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV) and Bundibugyo virus (BDBV) are all causative agents of Ebola virus disease, while Marburg virus (MARV) and Ravn virus (RAVV) cause Marburg virus disease. Both are characterized by hemorrhagic manifestations in severe cases. These zoonotic agents are transmitted either directly from fruit bats, which are presumed to be the natural reservoir, or through other host species (particularly non-human primates), and frequently show secondary human-to-human transmission, primarily as a result of close contact with infected individuals, their tissues or secretions/excretions. They cause sporadic outbreaks of VHF in West and Central Africa with an average case fatality rate (CFR) of 40–80% (Burk et al., 2016).

Among bunyaviruses, three families regularly cause VHF. These include Crimean-Congo hemorrhagic fever virus (CCHFV, *Nairoviridae* family), which causes infections with a CFR of between 5% and 30% (Bente et al., 2013) and is associated with particularly prominent hemorrhagic manifestations. CCHFV is widespread throughout much of Africa, Asia and Eastern Europe, where it is transmitted primarily via the bite of infected hard ticks (particularly *Hyalomma spp.*) or through contact with infected livestock, and shows frequent nosocomial transmission (Whitehouse, 2004). Rift Valley fever virus (RVFV, *Phenuiviridae* family) is a mosquito-borne bunyavirus that is widely distributed throughout Africa and the Arabian Peninsula. While infection normally causes a self-limiting febrile disease in humans, it can lead to severe symptoms, including VHF, with a CFR of around 2% (Atkins and

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https://doi.org/10.1016/j.antiviral.2019.104569
Received 23 May 2019; Received in revised form 28 June 2019; Accepted 25 July 2019
Available online 26 July 2019

Freiberg, 2017). Several members of the Old World hantaviruses (*Orthohantaviridae* family; e.g. Hantaan virus (HTNV), Seoul virus (SEOV) and Dobrava-Belgrade virus (DOBV)) cause hemorrhagic fever with renal syndrome (HFRS), which is endemic throughout much of Asia. The severity of the infection varies widely depending on the specific virus but can reach CFRs of 5–15% for Hantaan virus (Jonsson et al., 2010). In all cases, hantaviruses are transmitted to humans through the secretions/excretions of asymptomatically infected rodent host species (Jiang et al., 2016).

Causative agents of VHF among the arenaviruses include members of both the Old World complex (i.e. Lassa virus (LASV) and Lujo virus (LUJV)) and the New World complex (i.e. Junín virus (JUNV), Machupo virus (MACV), Sabiá virus (SABV), Chapare virus (CHAV) and Guanarito virus (GOTV)). These are transmitted to humans from distinct rodent reservoirs. By far the highest infection rates are documented for Lassa fever, which is endemic in West Africa where it is thought to cause up to 500,000 cases annually with an estimated CFR of 1–2% (McCormick et al., 1987). Among the New World arenaviruses JUNV (the causative agent of Argentine hemorrhagic fever), MACV (Bolivian hemorrhagic fever) and GOTV (Venezuelan hemorrhagic fever) are the most prevalent and are found in geographically distinct regions of South America (Charrel and de Lamballerie, 2003). However, they all cause clinically very similar illnesses with a high frequency of severe disease, including hemorrhagic symptoms, and CFRs of up to 20–30% (Carrion et al., 2012).

Currently there are few specific treatments available for VHF-induced illnesses in humans. A notable exception is the off-label use of the nucleoside analogue ribavirin in the treatment of Lassa fever, Crimean-Congo hemorrhagic fever and HFRS (Mardani et al., 2003; Oestereich et al., 2016; Rusnak et al., 2009). However, its efficacy in these infections remains unclear due to a lack of high quality clinical data, and indeed some more recent studies have cast doubt on its therapeutic value (Bausch et al., 2010; Ceylan et al., 2013; Koksal et al., 2010). Further, while convalescent plasma containing high-titer neutralizing antibodies has been shown to be highly effective in treating JUNV infection, this therapy is likely highly specific for this single virus and cannot be easily scaled for wide-spread implementation (Kenyon et al., 1986; Salazar et al., 2012).

Importantly, a major limitation in the development of treatments for these agents has been the limited target market represented by each of them, which restricts commercial interest. This makes drug repurposing and/or the identification of broadly acting inhibitors targeting common mechanistic features of these viruses (or their interactions with the host) highly attractive approaches for therapeutic development. In addition, this kind of broad-spectrum antiviral approach for the treatment of VHFs would simplify therapy in resource limited areas by allowing treatment initiation for these clinically similar diseases (several of which occur frequently within the same geographic region) in advance of a detailed molecular diagnosis, which indeed may not always be available. In addition, compounds with such a broad activity spectrum would in theory also be expected to allow targeting of newly emerging members of these virus groups, of which a number have been identified in recent years.

Clearly efforts to identify and establish mechanism of action for existing drugs suitable for repurposing or to identify new drug targets common to a range of VHF pathogens must rely on high-throughput analysis of relevant drug or knock-down/knock-out libraries. Consequently, reverse genetics-based approaches are proving to be pivotal tools in this screening process. This is particularly the case since, with the exception of the full-length clone systems that are used to generate recombinant viruses, these systems (i.e. the life cycle modelling (LCM) systems) can be used safely under BSL1/2 conditions. In this review, we seek to outline availability and recent technical advancements with regards to reverse genetics tools in the field of VHF virus research and highlight their application in the context of a number of recent high-throughput screens (HTS) of relevance for antiviral development against this important group of pathogens.

2. Genome organization and life cycle

Despite their clinical similarities, the viruses associated with VHF exhibit quite divergent features regarding their genome organization and protein coding mechanisms (Fig. 1). The filovirus genome consists of a non-segmented single-stranded RNA molecule of negative polarity that encodes for at least seven viral proteins (Fig. 1A). In contrast, the arena- and bunyaviruses both have segmented genomes but encode for their viral proteins using different strategies (Fig. 1B and C). The arenavirus genome is bisegmented with each segment (S and L segments) encoding two proteins in an ambisense arrangement, i.e. one positive-sense open reading frame (ORF) and one negative-sense ORF. These two ORFs are separated by a secondary structure-forming intergenic region (IGR) that regulates the termination of transcription (Albarino

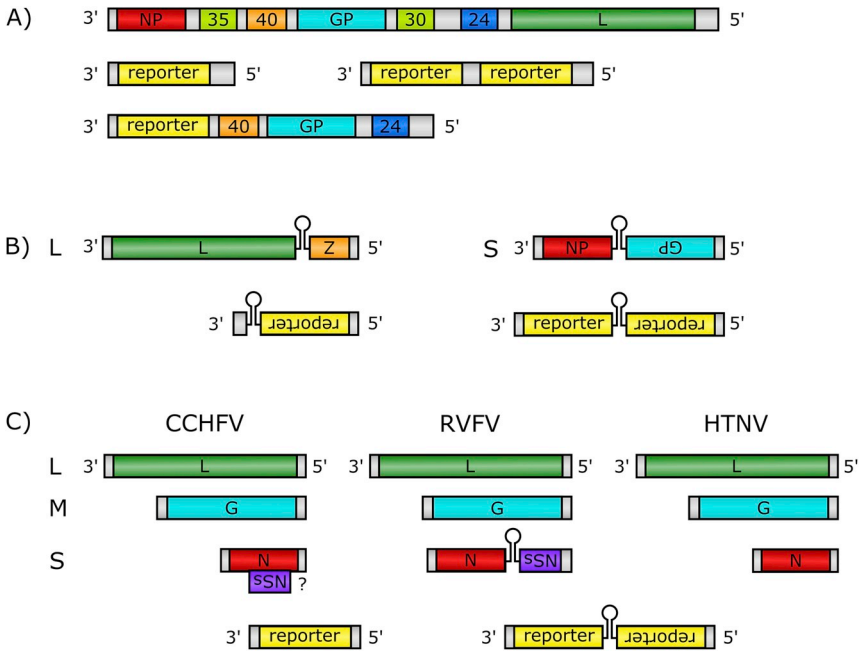


Fig. 1. Overview of the genome organization of negative-sense hemorrhagic fever viruses and the structure of their minigenomes. The genome organization of A) filoviruses, B) arenaviruses, and C) bunyaviruses (CCHFV, RVFV, HTNV), as well as their respective minigenomes (monocistronic, bicistronic or tetracistronic) are shown. All genomes/minigenomes are shown in 3′-5′ (viral RNA) orientation. Grey indicates non-coding regions/untranslated regions, while hairpin-forming intergenic regions in ambisense segments are illustrated. The RNP proteins are shown in either red (nucleoprotein) or green (dark: polymerase; light: accessory proteins). Viral proteins necessary for morphogenesis, budding and entry are shown in orange (matrix protein), turquoise (glycoprotein) or dark blue (nucleocapsid condensation), while other accessory proteins are depicted in purple. Minigenomes encode either one or more reporter genes (yellow) replacing viral genes.

et al., 2007; Pinschewer et al., 2005). Bunyavirus genomes are trisegmented (S, M and L segments) and segments generally encode only a single ORF each in negative polarity (Fig. 1C) (Bouloy and Weber, 2010; Zivcec et al., 2016). An obvious exception is the ambisense S segment of RVFV, which uses an ORF arrangement similar to that of the arenaviruses. Furthermore, CCHFV has been suggested to utilize an unusual approach in which positive- and negative-sense ORFs on the S segment directly overlap without an intergenic region (Barnwal et al., 2016).

As for other negative-sense RNA viruses, the members of all three groups encode for an RNA-dependent RNA polymerase (L) as well as a nucleoprotein (NP or N) and a surface glycoprotein (GP or G) (Fig. 1). In addition, the filoviruses and arenaviruses also encode for a distinct matrix protein (VP40 or Z, respectively). The polymerase cofactor VP35, the transcriptional activator VP30, and the nucleocapsid-associated protein VP24 are all unique to filoviruses (Fig. 1A), while phleboviruses and nairoviruses also encode a nonstructural protein NSs on their ambisense S segment that mediates interactions with the host cell (Fig. 1C) (Ly and Ikegami, 2016). The minimal requirements for transcription and replication of negative-sense RNA viruses are defined by the components of the ribonucleoprotein complex (RNP). For both bunyaviruses and arenaviruses, the viral RNA-dependent RNA polymerase (L) and the nucleoprotein (NP/N) are sufficient for these processes (Ikegami et al., 2005; Lee et al., 2000). However, viral RNA synthesis of filoviruses also requires both the polymerase cofactor VP35 and the transcriptional activator VP30 (Enterlein et al., 2006; Muhlberger et al., 1999; Volchkov et al., 2001). Interestingly, in the case of MARV, VP30 is not required for RNA synthesis in the context of shorter genome analogues (i.e. minigenomes), although the reasons for this remain unclear (Muhlberger et al., 1998).

Also common to all RNA viruses is that each genome segment is flanked by 3′ and 5′ non-coding regions (NCR) that are involved in the regulation of transcription and replication. Consequently, these elements play a critical role in the development of reverse genetics-based approaches that model these processes. Filoviruses contain a bipartite genomic promoter in their 3′ NCR (leader) and an antigenomic promoter in the 5′ NCR (trailer) region, for which a bipartite structure has not yet been described (Weik et al., 2005). Transcription of filovirus genes is regulated by well conserved transcription start and stop signals, and while the transcriptional promoter has not yet been mapped, it must also be located within the leader region (Weik et al., 2005). Interestingly, while the filovirus genome ends show a high degree of terminal complementarity, experimental evidence indicates that at least the leader region is capable of forming a hairpin structure independently, and that this retains both genomic replication and transcription promoter activity (Crary et al., 2003; Hoenen et al., 2010). Similarly, the arenavirus and bunyavirus genome ends also appear to interact with the viral polymerase independently of one another, despite their sequence complementarity. However, for these viruses the 5′-end of the RNA appears to be specifically necessary for activation of the corresponding promoter located at the 3′-end of the RNA (Gerlach et al., 2015; Pyle and Whelan, 2019).

The life cycles of arena-, filo- and bunyaviruses have been described in detail elsewhere (Hallam et al., 2018; Hoenen et al., 2019; Walter and Barr, 2011), but importantly they share a number of key commonalities. Briefly, entry is driven by interaction of their respective glycoproteins with specific cellular surface receptors or attachment factors, which trigger internalization into the endolysosomal pathway (Miller and Chandran, 2012; Rojek and Kunz, 2008). Cell entry and release of the viral genome into the cytoplasm is then mediated in a pH-dependent manner. Following release of the encapsidated genome, viral RNA synthesis takes place in the cytoplasm, and in the case of filo- and arenaviruses occurs in cytosolic inclusion bodies (Baird et al., 2012; Hoenen et al., 2012). First, the negative-sense ORFs are directly transcribed by protein components brought in as part of the incoming nucleocapsid (primary transcription) and produce an initial wave of viral proteins that support the initiation of further secondary transcription and also viral replication, which leads to the generation of complementary RNA (cRNA) antigenomes. These not only serve as templates for the replication of new viral RNA (vRNA) genomes, but also for the transcription of any ambisense ORFs. At late stages in the virus infection cycle newly formed nucleocapsids are then transported by a combination of viral and host factors to the budding sites, where assembly and virus particle release takes place. In the case of filo- and arenaviruses this process occurs at the plasma membrane, while budding and assembly of bunyaviruses takes place in the Golgi complex, with subsequent transport of the particles to the cell membrane via cellular export pathways (Elliott, 1997; Feldmann et al., 2013; Hallam et al., 2018).

3. Reverse genetics systems

The many commonalities between the VHF virus life cycles have made it possible to establish reverse genetics systems for these viruses based on a common set of principles that either allow us to specifically model individual steps or the whole virus life cycle, as well as to generate recombinant viruses. In particular, minigenome systems allow the modelling of viral RNA synthesis, while newer transcription and replication-competent virus-like particle (trVLP) systems can also be used to model assembly, morphogenesis and entry. In contrast, full-length clone (FLC) systems facilitate the generation (‘rescue’) of fully infectious recombinant viruses and thus allow analysis of the whole virus life cycle.

Despite the differing levels of complexity and applications of the various reverse genetics-based systems, the overall design and considerations necessary when establishing such systems are quite similar. In general, there are three different platforms available based on the polymerase used for transcription of the initial genome (or genome analogue) RNAs. These are based on the T7 bacteriophage polymerase or the cellular DNA-dependent RNA polymerases PolI or PolII. The T7 polymerase system is currently the most commonly used, and most systems have so far at least initially been constructed based on this approach. This polymerase has to be exogenously supplied (via either transient or stable expression) but has the advantage that it generates precise and unmodified 5′ transcript ends, although a variable number of G residues are sometimes also included following the minimal core promoter sequence to increase promoter strength, and these are incorporated into the final product. Whether this is a viable strategy or not depends on the viral tolerance for variability at these sites. However, the bigger challenge is posed by the 3′ transcript ends, which due to imprecise termination are quite heterogeneous in length and sequence, and can impair the efficiency of viral RNA synthesis. Therefore, a Hepatitis Delta virus (HDV) ribozyme sequence is commonly included upstream of the T7 terminator in order to ensure authentic 3′ viral genome ends. While there are undoubtedly limits to the processivity of the T7 polymerase, experience with filovirus systems have shown that these are clearly in excess of 20 kb.

Despite the relative popularity of the T7-based systems, more recently reverse genetics systems have also been established based on mammalian PolI and PolII, both of which are expressed endogenously. However, PolI relies on species-specific promotor elements, and while systems have been developed for both rodent and human cells, this can potentially limit its applications. PolI produces unmodified RNA transcripts with comparatively precise initiation and termination sites (Heix and Grummt, 1995). In contrast, PolII generates 5′-capped and 3′-poly (A) modified RNAs and thus requires not only a 3′ HDV ribozyme sequence, but also an additional hammerhead ribozyme at the 5′-end, in order to generate authentic genome ends. Given the length of naturally occurring mRNAs in the cell, processivity with this polymerase can be expected to far exceed the needs of any viral genome transcription system. Further, based on their wide-spread use in protein expression, very strong synthetic versions of the PolII promoter have been

developed, allowing for robust RNA synthesis using this system. However, transcript splicing can be an issue.

Once a polymerase is selected, different systems also vary in the orientation of the genome (or genome analogue), which can be encoded in either genomic or antigenomic orientation to produce either a vRNA-like or cRNA-like RNA molecule, respectively. While minigenomes are frequently encoded in vRNA orientation, in some cases FLC systems have been shown to work more efficiently with genomes encoded in the cRNA orientation (Yun et al., 2013), which potentially limits vRNA/mRNA hybridization that can lead to dsRNA-mediated antiviral responses prior to encapsidation of the viral RNA transcript by the nucleoprotein.

The third major parameter where reverse genetics systems for different viruses vary is the cell line used, which is dependent on several different criteria. Since the T7 polymerase has to be exogenously expressed, T7-driven systems are often used together with stable expression cell lines (e.g. BSR-T7/5 or BHK-T7-9 cells) (Billecocq et al., 2008; Buchholz et al., 1999; Volchkov et al., 2001). While this reduces the number of plasmids that need to be transfected, it remains unclear whether this improves assay performance over transfection of a T7-encoding plasmid. Regardless, in both T7-driven and non-T7-driven systems, transfection efficiency remains one of the primary considerations that influence cell line selection. Further, for the FLC system, cells not only need to be transfected, but they also need be susceptible to infection to ensure viral propagation. As a result, in some systems a mixture of cell lines is used to ensure all these requirements are met (Towner et al., 2005).

Finally, in the case of life cycle modelling systems or reporter-expressing recombinant viruses, the choice of a suitable reporter and its expression strategy is critical and highly dependent on the intended application (reviewed in (Falzarano et al., 2014)). In general there are two groups of reporters that are currently favored: fluorescent reporters and luminescent reporters. For microscopic analyses of reporter activity, fluorescent reporters are mainly used. These kinds of reporters are suitable for high-content imaging and have the benefit of allowing multiple analysis points without the need for independent samples. Further, no additional reagents are needed for measurement/quantification, which can be a significant advantage during high-throughput analysis with respect to both cost and labor. In contrast, the use of luciferase reporters reduces the need for expensive and highly specialized equipment and potentially allows for more rapid screening, due to their increased sensitivity over fluorescent proteins. Especially the use of newer brighter luciferases, such as NanoLuc luciferase, instead of Renilla or Firefly luciferase, can further increase assay sensitivity. However, luciferases are not detectable without the addition of their respective substrates, increasing assay costs. While this is in many cases an end-point analysis, secreted luciferases (e.g. Gaussia luciferase) have also been developed that allow for multiple measurements over time, although they are not yet widely used.

3.1. Full-length clone systems

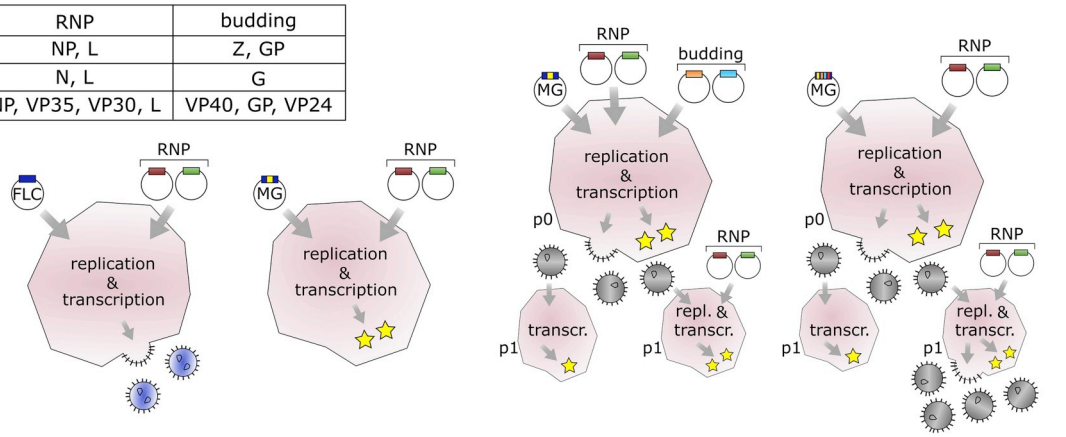
FLC systems allow the rescue of fully infectious recombinant viruses (Fig. 2) from a plasmid-expressed version of the complete viral genome. The usage of cell lines differs between the systems for the different viruses. To date, almost all FLC systems described for VHF causing arenaviruses and bunyaviruses employ baby hamster kidney cells (BHK-21, BHK-T7/5 or BHK-T7-9) for the initial generation of recombinant virus, although also HEK 293T cells have been used successfully for the generation of RVFV (Habjan et al., 2008). For filoviruses, however, a wider range of cell types has been used successfully, including BSR-T7/5 and Huh7 cells (Enterlein et al., 2009; Tsuda et al., 2015), as well as Vero cells, which appear to work well despite these cells being relatively difficult to transfect (Hoenen et al., 2013). Also mixtures of different cell lines combining an easily transfectable cell line, such as HEK 293T cells, with a highly infectable cell line, like Vero cells, have been used for the generation of recombinant filoviruses (Towner et al., 2005).

Despite the different transcription platforms, genome orientations and cell lines that can be employed, the actual rescue protocols for the generation of recombinant filoviruses, bunyaviruses and arenaviruses using FLC systems are very similar. All systems so far developed have relied on either T7 or PolI for the generation of the viral genome or, more often, antigenome (Tables 1–3), and indeed the main difference is only the number of plasmids that need to be transfected to reconstitute the complete genome and the viral RNP (Fig. 2). Following transfection of the genome and RNP-encoding plasmids, the polymerase (T7 or PolI) transcribes the full-length plasmid (or genome segment-encoding plasmids for segmented viruses) into a non-encapsidated “naked” vRNA or cRNA (depending on the orientation), which is then encapsidated by the viral RNP proteins to serve as a template for viral RNA synthesis. The plasmid-derived RNP proteins use these RNAs to establish viral transcription (and subsequent translation) of the genome-encoded viral proteins, which then leads to further transcription and replication as well as assembly and budding of virions that can be used to infect new cells. The initial plasmid-driven generation of genomic RNA and its illegitimate encapsidation by the RNP proteins, as well as the overexpression of the viral RNPs, are all artificial steps in this system (i.e. steps for which there are no equivalents in the viral life cycle), but these no longer play a role following passage since the virus produced is fully infectious.

Not only have various mutagenesis studies been performed using FLC systems, they have also been used to generate reporter-expressing viruses, which then have significant potential applications in screening (see Tables 4–6). Reporters can be inserted using a variety of approaches into the viral genome: 1) as an additional transcriptional unit (ATU), 2) as a fusion protein directly coupled to the protein of interest, 3) as a fusion protein connected by a self-cleavable linker (e.g. P2A), or 4) as a replacement for a non-essential gene. However, not all approaches are feasible for all viruses/proteins. The insertion of an ATU has been used very successfully for filoviruses (Albarino et al., 2013; Ebihara et al., 2007; Towner et al., 2005). Importantly, however, using different positions for the insertion leads to different expression levels of the reporter and also to different levels of attenuation of the virus, presumably due to the transcriptional gradient of mononegaviruses (Ebihara et al., 2007). In contrast, the insertion of an ATU is not possible for arena- and bunyaviruses due to their lack of a start-stop transcriptional regulation (Pinschewer et al., 2005). For arenaviruses the expression of a reporter gene has been approached by the addition of a duplicate segment such that each segment copy encodes only one essential viral gene, with the other deleted to allow insertion of a foreign ORF, such as a reporter. This results in the generation of trisegmented arenaviruses that are strongly attenuated (Ortiz-Riano et al., 2013). It is unlikely that a similar approach to obtain additional coding capacity would be viable for RVFV or CCHFV, as they do not have any segments known to encode two essential gene products. Nonetheless, generation of 4-segmented bunyaviruses is certainly possible and was achieved for RVFV by splitting the glycoprotein subunits onto separate copies of the M segment (Wichgers Schreur et al., 2014).

However, bunyaviruses encode several non-essential gene products, and indeed for both NSs and NSm of RVFV these can be replaced with reporters (Gerrard et al., 2007; Ikegami et al., 2006; Wichgers Schreur et al., 2014). Nonetheless, this is another approach that does not work for all viruses, and in particular there are no viral proteins in filo- or arenaviruses that are not essential for the viral life cycle. Direct fusion of a reporter protein gene to a viral ORF should in theory be possible for all viruses discussed, but so far it has only been used for the insertion of fluorescent proteins into the genomes of MARV (Schudt et al., 2013) and EBOV (Hoenen et al., 2012). However, an interesting strategy to replace part of the bunyavirus glycoprotein with a reporter has also been developed (Shi et al., 2010), and although it has not yet been applied to any of the VHF-causing bunyaviruses a similar approach

	RNP	budding
Arenavirus	NP, L	Z, GP
Bunyavirus	N, L	G
Filovirus	NP, VP35, VP30, L	VP40, GP, VP24



	full-length clone system	minigenome	monocistronic trVLP		multicistronic trVLP	
			naïve p1	pretransfected p1	naïve p1	pretransfected p1
entry	+	-	+ (p1)	+ (p1)	+ (p1)	+ (p1)
primary transcription	+	-	+ (p1)	-	+ (p1)	-
replication	+	+	+ (p0)	+ (p0 & p1)	+ (p0)	+ (p0 & p1)
secondary transcription	+	+	+ (p0)	+ (p0 & p1)	+ (p0)	+ (p0 & p1)
assembly, budding	+	-	+ (p0)	+ (p0)	+ (p0)	+ (p0)

Fig. 2. Overview of existing reverse genetics systems. Shown are models of the various reverse genetics systems with an overview of the steps modelled in each of the respective systems. The RNP proteins or proteins necessary for budding and entry are specified for all viruses. The full-length clone (FLC) system is used for the generation of recombinant viruses (shown in blue), which have a fully authentic life cycle. Minigenome (MG) systems can be used to model replication and secondary transcription, which is reflected by the production of reporter protein (indicated by stars). Transcription and replication-competent virus-like particle (trVLP) systems model secondary transcription and replication in producer (p0) cells, as for the minigenome assay. However, they also lead to the production of trVLPs containing the encapsidated minigenome (shown in grey). These can then be used to infect target cells (p1 cells), where they then undergo primary transcription (naïve p1 cells) or secondary transcription and replication (pre-transfected p1 cells) to generate reporter activity reflecting these processes. The multicistronic trVLP system functions in the same way as the monocistronic trVLP system, except that trVLPs can also be passaged in pre-transfected target cells, owing to the expression of the budding components from the minigenome, which enables new trVLPs to be produced in pre-transfected p1 cells.

Table 1
Currently available reverse genetics-based tools for high-throughput screening of arenaviruses. Reporter-expressing viruses, as well as minigenome and trVLP systems, are indicated according to the polymerase system (T7 or PolI-based) used for initial (mini-)genome transcription. The reporters used in these systems are also indicated (GFP: green fluorescent protein; CAT: chloramphenicol acetyl transferase; FLuc: Firefly luciferase; GLuc: Gaussia luciferase; NLuc: NanoLuc luciferase). All trVLP systems are monocistronic.

experimental system	reporter-expressing viruses		life cycle modelling systems		
			minigenome systems		trVLP systems
					pre-transfected
	T7-based	PolI-based	T7-based	PolI-based	T7-based
LASV	ZsGreen (Welch et al., 2016)	GFP (Cai et al., 2018)	CAT (Capul et al., 2011) FLuc (Hass et al., 2004)	ZsGreen (Welch et al., 2016) GLuc (Welch et al., 2016)	
JUNV		GFP and GLuc (trisegmented) (Ortiz-Riano et al., 2013)	GFP (Albarino et al., 2009); (Dunham et al., 2018) NLuc (Dunham et al., 2018)		CAT (Casabona et al., 2009) NLuc (Dunham et al., 2018) GFP and GLuc (Rathbun et al., 2015)
MACV			GFP (Kranzusch et al., 2010)		

might indeed be feasible. Nonetheless, in all cases such direct tagging approaches need careful evaluation as the fusion must not disturb the function of the respective viral protein. To overcome this, in some instances a self-cleaving P2A linker has been used to insert a fluorescent protein, and this has been shown to work for arena- (Cai et al., 2018), filo- (Albarino et al., 2015) and bunyaviruses (Welch et al., 2017) (for an overview of the different reporter viruses see Tables 1–3).

Despite their advantages and the many successful examples of viable reporter-expressing viruses that have been produced, the use of these systems is only possible in high containment (BSL 3/4)

Table 2
Currently available reverse genetics-based tools for high-throughput screening of bunyaviruses. Reporter-expressing viruses, as well as minigenome and trVLP systems, are indicated according to the polymerase system (T7 or PolI-based) used for initial (mini-)genome transcription. The reporters used in these systems are also indicated (GFP: green fluorescent protein; CAT: chloramphenicol acetyl transferase; RLuc: Renilla luciferase; FLuc: Firefly luciferase; GLuc: Gaussia luciferase; NLuc: NanoLuc luciferase). All trVLP systems are monocistronic.

experimental system	reporter-expressing viruses		life cycle modelling systems		
			minigenome systems		trVLP systems
					pre-transfected
	T7-based	PolI-based	T7-based	PolI-based	T7-based
CCHFV	ZsGreen (Welch et al., 2017)		GLuc (Bergeron et al., 2010)	GFP (Flick et al., 2003b) CAT (Flick et al., 2003b)	NLuc (Zivcec et al., 2015) RLuc (Devignot et al., 2015)
RVFV	GFP (Bird et al., 2008); (Harmon et al., 2016) Katushka (Islam et al., 2016) RLuc (Ikegami et al., 2006)	GFP (Billecocq et al., 2008)	FLuc (Ikegami et al., 2005) RLuc (Zamoto-Niikura et al., 2009)	CAT (Gauliard et al., 2006)	RLuc (Habjan et al., 2009); (Piper and Gerrard, 2010) RLuc (transcription-deficient) (Klemm et al., 2013)
HTNV				CAT (Flick et al., 2003a)	

Table 3
Currently available reverse genetics-based tools for high-throughput screening of filoviruses. Reporter-expressing viruses, as well as minigenome and trVLP systems, are indicated according to the polymerase system (T7 or PolII-based) used for initial (mini-)genome transcription. The reporters used in these systems are also indicated (GFP: green fluorescent protein; RFP: red fluorescent protein; CAT: chloramphenicol acetyl transferase; RLuc: Renilla luciferase; FLuc: Firefly luciferase; GLuc: Gaussia luciferase; CLuc: Cypridina luciferase; NLuc: NanoLuc luciferase). trVLP systems are monocistronic unless otherwise indicated (bicistronic: 2cis; tetracistonic: 4cis).

experimental system	reporter-expressing viruses	life cycle modelling systems			
		minigenome systems		trVLP systems	
				pre-transfected	naïve
	T7-based	T7-based	PolII-based	T7-based	T7-based
EBOV	GFP (Ebihara et al., 2007); (Towner et al., 2005) mCherry (Hoenen et al., 2012) ZsGreen (Albarino et al., 2015) FLuc (Hoenen et al., 2013) GLuc (Uebelhoer et al., 2014)	CAT (Muhlberger et al., 1999) CAT (2cis) (Brauburger et al., 2014) FLuc (Modrof et al., 2002) GLuc (Tao et al., 2017) GFP-CAT (fusion) (Prins et al., 2010) GFP/mCherry (dual reporter) (Mehedi et al., 2013) FLuc (replication-deficient) (Hoenen et al., 2010)	GFP (Nelson et al., 2017b)	GFP (Watanabe et al., 2004) GFP (4cis) (Schmidt et al., 2018) RLuc (4cis) (Watt et al., 2014)	RLuc (Hoenen et al., 2006) NLuc (4cis) (González-Hernández et al., 2019)
MARV	GLuc (Uebelhoer et al., 2014) GFP (Albarino et al., 2013); (Schmidt et al., 2011) mCherry (Mittler et al., 2018) RFP (Schudt et al., 2013)	CAT (Muhlberger et al., 1998) CLuc (Alonso and Patterson, 2013) GLuc (Alonso and Patterson, 2013)		RLuc (Wenigenrath et al., 2010)	RLuc (Krähling et al., 2010)

laboratories. This of course poses a challenge for HTS, as these laboratories usually do not have the technology or specialized expertise necessary for performing these screens and vice versa.

3.2. Life cycle modelling systems

Given the containment requirements for work with infectious virus (including reporter-expressing viruses), LCM systems have gained popularity as a non-infectious alternative for such screening approaches. These systems are based on genome analogues (called minigenomes) and can be used to model specific aspects of the virus life cycle safely under BSL1/2 conditions (depending on local regulations).

3.2.1. Minigenome systems

The simplest of the LCM tools are the minigenome systems (sometimes also known as replicon systems), which exclusively model viral RNA synthesis. Instead of full-length genomic or antigenomic viral RNA, these systems make use of a genome analogue where some or all of the viral ORFs have been removed and replaced by a reporter ORF. Typically, minigenome constructs retain both the terminal 3′ and 5′ viral NCRs and thus all the cis-acting elements and signals for efficient transcription and replication. Minigenome constructs based on arenavirus segments or the RVFV ambisense S segment also retain the IGR of these segments in order to facilitate transcription termination (Albarino et al., 2007; Pinschewer et al., 2005). While it is possible to drive the replication and transcription of such constructs with RNP proteins derived from helper virus infection, this is in practice rarely done, except

Table 4
HTS studies with drug or siRNA libraries using reverse genetics for arenaviruses as a screening platform.

Virus	reverse genetics-based screening platform used	scope	compounds/genes identified	references
LCMV	rLCMV/GFP-P2A-NP	30,400 small molecule library (purchased from Life Chemicals)	F3406 inhibits LCMV entry	Ngo et al. (2015)
JUNV	trVLP system (Gaussia luciferase and GFP)	87,321 compounds (novel lead-like molecules and FDA-approved drugs)	3 undisclosed compounds and tetrandrine; inhibit entry of arenaviruses (but not other tested virus groups)	Rathbun et al. (2015)
JUNV	primary screen: minigenome system (NanoLuc luciferase) secondary screen: trVLP (NanoLuc luciferase)	34 drugs (Nelson et al., 2017a)	AVN-944 (non-competitive IMPDH inhibitor), also inhibits Tacaribe virus	Dunham et al. (2018)
LASV	rLASV-GFP (GFP-P2A-NP)	ribavirin and favipiravir	proof-of-concept only using established positive controls	Cai et al. (2018)
LASV/EBOV	primary screen: LASV (ZsG) and EBOV minigenome system (Gaussia luciferase) secondary screen: rLASV/ZsG (ZsG-P2A-VP40), rEBOV/ZsG (ZsG-P2A-VP40)	27 commercially available nucleoside analogues	6-azauridine inhibits both LASV and EBOV; 2′-deoxy-2′-fluorocytidine (2′-dFC) inhibits LASV	Welch et al. (2016)

Table 5
HTS studies with drug or siRNA libraries using reverse genetics for bunyaviruses as a screening platform.

Virus	reverse genetics-based screening platform used	scope	compounds/genes identified	references
RVFV	rRVFV-GFP (GFP replacing NSs)	Genome-wide RNAi screen (22,909 genes)	Wnt/β-catenin-signaling pathway; also showed proviral activity for La Crosse virus and California encephalitis virus	Harmon et al. (2016)
RVFV	rRVFV-Katushka (Katushka replacing NSs)	28,437 small chemical compounds (Chemical Biology Consortium Sweden)	6 chemical compounds that inhibit RVFV infection	Islam et al. (2016)
CCHFV	rCCHFV/ZsG (ZsG-P2A-NP)	38 nucleoside analogues and 2 IMPDH inhibitors	2′-deoxy-2′-fluorocytidine (2′-dFC) inhibits CCHFV	Welch et al. (2017)

Table 6
HTS studies with drug or siRNA libraries using reverse genetics for filoviruses as a screening platform.

Virus	reverse genetics-based screening platform used	scope	compounds/genes identified	references
LASV/EBOV	primary screen: LASV (ZsG) and EBOV minigenome system (Gaussia luciferase) secondary screen: rLASV/ZsG (ZsG-P2A-VP40), rEBOV/ZsG (ZsG-P2A-VP40)	27 commercially available nucleoside analogues	6-azauridine inhibits both LASV and EBOV; 2′-deoxy-2′-fluorocytidine (2′-dFC) inhibits LASV	Welch et al. (2016)
EBOV	EBOV minigenome system (Firefly luciferase)	960 compounds (Prestwick Chemical Known Bioactives library)	cephaeline dihydrochloride, doxorubicin hydrochloride, ellipticine, emetine dihydrochloride, patulin and salinomycin	Jasenosky et al. (2010)
EBOV	rEBOV-GFP (ATU between NP and VP35)	1990 compounds (small molecule diversity set library - National Cancer Institute)	NSC 62,914 (antioxidant properties, scavenger for reactive oxygen species) inhibits EBOV, RVFV, LASV and VEEV	Panchal et al. (2012)
EBOV	rEBOV-GFP (ATU between NP and VP35)	Selective estrogen receptor modulators (SERM) compounds	estrogen receptor-antagonists like clomiphene and toremifene inhibit EBOV, SUDV, MARV	Johansen et al. (2013) Johansen et al. (2015)
EBOV	rEBOV-GFP (ATU between NP and VP35)	2600 approved drugs and molecular probes	class II cationic amphiphilic drugs targetting late stage of EBOV entry	Johansen et al. (2015)
EBOV	EBOV minigenome system (Renilla luciferase); additionally MARV minigenome system (Renilla luciferase)	200,000 small molecules from commercial sources (library from UT Southwestern Screening Core)	compounds sharing an amino-tetrahydrocarbazole scaffold (e.g. GSK983, SW835)	Luthra et al. (2018a); Luthra et al. (2018b)
EBOV	primary screen: minigenome (NanoLuc luciferase) secondary screen: rEBOV-Firefly luciferase	genome-wide siRNA screen (21,566 genes)	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (*de novo* pyrimidine synthesis)	Martin et al. (2018)
EBOV	tetracistronic trVLP system (Renilla luciferase)	8354 small molecules with known targets, FDA-approved drugs, natural compounds and kinase inhibitors	several known entry inhibitors were reidentified, bisadocyl inhibits morphogenesis/egress	Lee et al. (2018)
EBOV/MARV	primary screen: minigenome system (EBOV and MARV; Gaussia luciferase) secondary screen: rEBOV-Gaussia, rMARV-Gaussia	9 nucleoside analogues	6-azauridine (inhibitor of *de novo* pyrimidine synthesis) inhibits EBOV and MARV	Uebelhoer et al. (2014)

perhaps as an initial check for minigenome functionality, as it negates many of the advantages of these systems, and in the case of VHF pathogens, particularly their safety. Thus plasmid-based RNP protein expression is greatly favored. Again, while the RNP components that need to be provided vary in number and identity, the overall process is analogous between the different virus systems (see Fig. 2). Following transfection of the minigenome and RNP encoding plasmids, the polymerase (T7, PolI or PolII) transcribes the minigenome plasmid into RNA, which must be illegitimately encapsidated by the respective nucleoprotein in order to serve as a template for further viral RNA synthesis by the viral RNP proteins. If this occurs successfully, transcription and replication of the minigenome is initiated by the viral polymerase and the reporter gene is transcribed and subsequently translated. Reporter activity from classical minigenomes containing both the 3′ and 5′ NCRs reflects the RNA synthesis process in total, but cannot discriminate between transcription and replication. However, for filoviruses it is possible to dissect the RNA synthesis process using a replication-deficient minigenome (Hoenen et al., 2010). This construct contains a deletion within the antigenomic promoter and thus no longer supports replication of the minigenome, thereby allowing the analysis of the transcription process in isolation. However, given the contribution of replication to the generation of new targets for transcription, reporter activity in replication-deficient minigenome systems is several orders of magnitude lower than in an equivalent classical minigenome system. Unfortunately, since promoter activity of arena- and bunyaviruses genomes requires both the 3′ and 5′ termini (Gerlach et al., 2015; Pyle and Whelan, 2019), it is unlikely that a similar approach would be possible for these virus groups. Nonetheless, dissection of RNA synthesis is still possible for bunyaviruses, as transcription can be selectively blocked by inactivation of the cap-snatching endonuclease function of the polymerase, allowing only replication of the minigenome (Klemm et al., 2013).

In addition to basic monocistronic minigenomes there are also a number of variations in minigenome design that have been developed in recent years. These include extension of the monocistronic minigenome to a bicistronic dual reporter-encoding minigenome by incorporation of a second reporter ORF. Such systems have so far been successfully established for MARV (Alonso and Patterson, 2013), EBOV (Brauburger et al., 2014) and LASV (Welch et al., 2016) (see Tables 1 and 3). Among the bunyaviruses such dual reporter-expressing minigenomes would likely only be possible based on the ambisense S segment of RVFV, as it is difficult to imagine an approach by which a reporter gene could be successfully incorporated as an overlapping ORF sequence within another intact ORF (e.g. for the CCHFV S segment), and all other bunyavirus segments contain only a single transcriptional unit. For filoviruses tetracistronic minigenomes have also been developed as a mean to express additional viral proteins, and thereby facilitate analysis of further steps in the viral life cycle beyond viral RNA synthesis (see next section).

3.2.2. Transcription and replication-competent virus-like particle systems

Transcription and replication-competent virus-like particle (trVLP) systems (sometimes also called infectious virus-like particle (iVLP) or transcription-competent virus-like particle (tcVLP) systems) represent an advancement of the classical minigenome systems in that, in addition to modelling viral RNA synthesis, they also allow the modelling of assembly, budding and entry (Fig. 2, Table 7). In order to achieve this, cells are not only transfected with the components for the minigenome system, but also with plasmids encoding the viral proteins necessary for budding and entry. For bunyaviruses this is solely the glycoprotein, while for arenaviruses both a dedicated matrix protein (Z) and glycoprotein are required, and for filoviruses not only are the glycoprotein and matrix protein (VP40) needed, but also the nucleocapsid-associated protein (VP24). Supplying these proteins leads to the generation of virus-like particles containing fully encapsidated minigenomes. These can then be used to infect target cells, which can either be pre-transfected (with expression plasmids encoding the viral RNP proteins) or naïve (non-transfected). When pre-transfected cells are used, much higher reporter activities are observed, as both replication and secondary transcription of the minigenome take place in these cells, but primary transcription cannot be modelled in this system (Hoenen et al., 2006; Krähling et al., 2010). In order to model primary transcription, a naïve trVLP system is needed. Here the target cells are not pre-transfected, and thus only primary transcription of the minigenome can take place. However, the reporter activity is much lower in such systems, making detection of the reporter challenging (Devignot et al., 2015; Hoenen et al., 2006; Krähling et al., 2010). To date, monocistronic trVLP systems have been successfully developed for filoviruses (EBOV and MARV) (Hoenen et al., 2006; Krähling et al., 2010; Watanabe et al., 2004; Wenigenrath et al., 2010), arenaviruses (JUNV) (Casabona et al., 2009; Dunham et al., 2018; Rathbun et al., 2015) and bunyaviruses (RVFV and CCHFV) (Devignot et al., 2015; Habjan et al., 2009; Zivcec et al., 2015) (for an overview, see Tables 1–3). As for minigenome systems, trVLP systems also differ in the cell lines and the polymerase systems used for the plasmid-driven generation of vRNA, although most systems are based on T7-driven expression of the minigenome. Only for RVFV has a PolI-driven trVLP system been established (Habjan et al., 2009). The majority of trVLP systems use either cell lines stably expressing T7 (i.e. BSR-T7/5) or highly transfectable HEK 293/293T cells for the trVLP generation, however, for the production of CCHFV trVLPs Huh7 cells were shown to work best (Devignot et al., 2015). Target cell selection also varies for different viruses and systems, but the most commonly used cell lines for filovirus trVLP systems are HEK 293T (Watanabe et al., 2004) and Huh7 cells (Wenigenrath et al., 2010), while arena- and bunyavirus trVLP systems use Huh7 (Devignot et al., 2015; Dunham et al., 2018) and BSR-T7/5 cells (Habjan et al., 2009). Reporters used to date for these systems include not only several different luciferases, but also fluorescent proteins like GFP (Habjan et al., 2009; Piper and Gerrard, 2010; Watanabe et al., 2004).

For filoviruses, the trVLP system has been improved in recent years by using a tetracistronic minigenome (Figs. 1–2) that not only encodes for the reporter, but also for the proteins necessary for morphogenesis and budding (i.e. VP40, $GP_{1,2}$ and VP24) (Watt et al., 2014). This results in a more regulated expression of these proteins, as they are transcribed from the minigenome in a manner that is dependent on viral RNA synthesis, as is the case during the authentic viral life cycle (Watt et al., 2014). This is beneficial since the overexpression of the budding components, especially VP24 and VP40, can be problematic as they are inhibitors of viral RNA synthesis (Hoenen et al., 2010; Watanabe et al., 2004). Supporting the benefits of such an approach, preparations of monocistronic trVLPs have been observed to contain particles with very different morphologies, and indeed most of the produced material is non-infectious, possibly due to overexpression of VP40 and GP (Spiegelberg et al., 2011). By comparison, use of the tetracistronic trVLP system leads to an approximately 500-fold increase in specific trVLP infectivity (Watt et al., 2014). To date, tetracistronic filovirus trVLP systems have been developed based on both fluorescent and luciferase reporters, which makes them suitable not only for HTS, but also for high-content imaging-based screens (Lee et al., 2018; Schmidt et al., 2018). Further, while this approach can be used to model all aspects of the viral life cycle, including primary transcription in naïve target cells (González-Hernández et al., 2019), the trVLPs produced can also be continuously passaged in pre-transfected target cells allowing multicycle analysis (Wendt et al., 2018). A similar multicistronic system might also be a viable approach for arenaviruses, where the matrix protein Z is also a potent inhibitor of viral RNA synthesis (Garcin et al., 1993), but no such system has yet been developed. In contrast, bunyaviruses are not known to encode a specific inhibitor of viral RNA synthesis; however, it would conceivably be possible to replace the second S segment encoded ORF of RVFV with the viral glycoprotein to facilitate budding using a similar approach.

Table 7
Overview of features and possible applications for reverse genetics-based HTS tools.

	recombinant reporter-expressing viruses	minigenome system	transcription and replication-competent virus-like particle system
advantages	● completely authentic infection and life cycle ● can detect inhibitors of any step in the viral life cycle	● BSL1/2 ● allows targeted selection for inhibitors of RNA synthesis	● BSL1/2 ● can model nearly all aspects of the viral life cycle (primary transcription only in naïve target cells) ● passaging of trVLPs over several passages possible with some systems
disadvantages	● BSL3/4 ● provides little information about mechanism of action ● subject to entry bias, depending on workflow setup	● non-authentic steps: initial production of non-encapsidated RNAs and illegitimate encapsidation; overexpression of RNP proteins ● cannot detect inhibitors of processes other than RNA synthesis	● non-authentic steps: initial production of non-encapsidated RNAs and illegitimate encapsidation (only in producer (p0) cells); overexpression of RNP proteins; overexpression of budding components (monocistronic trVLP systems only) ● subject to entry bias, depending on workflow setup
common applications	● mainly secondary hit validation ● primary screen for smaller libraries	● primary screening of large siRNA or drug libraries ● screens targeted toward inhibitors of viral RNA synthesis	● primary screening of large siRNA or drug libraries ● screens targeted toward inhibitors of viral RNA synthesis or entry, depending on assay workflow

4. High-throughput screening using reverse genetics

4.1. Selection and optimization of existing systems for high-throughput screening

With a variety of systems now available, the choice of the most suitable reverse genetics system for a given HTS is highly dependent on both the desired readout as well as the part(s) of the viral life cycle that should be addressed (Table 7, Fig. 2). LCM systems allow the specific analysis of individual aspects of the virus life cycle, which provides information regarding the mechanism of action of compounds identified in HTS using these systems. With classical minigenome systems only viral RNA synthesis is modelled, which allows the study of compounds/genes that specifically target this step in the viral life cycle. In contrast, the trVLP system potentially models all steps of the viral life cycle, but by comparing reporter activity in producer and target cells can also discriminate between effects on RNA synthesis and morphogenesis/budding/entry. However, it must be taken into account that both LCM systems also include non-authentic steps, such as initial transcription of non-encapsidated “naked” RNAs, and illegitimate encapsidation of this RNA, as well as the expression of viral proteins from expression plasmids, which makes it necessary to validate findings from these systems with authentic viruses (i.e. wildtype or reporter-expressing viruses). On the other hand, the use of virus in HTS allows analysis in the context of a completely authentic life cycle, but provides little information about the mechanism of action of an identified compound, and of course experiments are complicated by the need to perform the HTS in a high containment laboratory. As such many studies limit their use of infectious virus systems to the validation of primary screen hits obtained from LCM systems, rather than using them for the primary screening.

Before an existing reverse genetics-based platform can be scaled down to the small well format needed for HTS, several things have to be optimized and taken into account. Probably the most important factor is the performance of the assay with respect not only to its sensitivity, but also robustness, reproducibility, and accuracy. Such suitability for miniaturization for HTS is typically quantified based on the dynamic range (difference between the means of the positive and negative controls) and the separation band (difference between the positive controls minus three standard deviations and the negative controls plus three standard deviations) of the assay, which can then also be used to calculate a Z′ factor (separation band divided by dynamic range) (Zhang et al., 1999). This approach has proven to be a reliable means of evaluating assay suitability for HTS, with Z′ values > 0.5 indicating an excellent assay. The other major consideration is the optimization of the assay workflow itself to eliminate any unnecessary handling and to reduce reagent/supply costs, which multiply rapidly in larger screens.

With respect to the development and optimization of reverse genetics-based assays for HTS, several modifications have been shown to improve assay performance, including the use of different polymerases and reporters as well as optimization of support plasmid expression. Specifically, while T7-driven transcription is the most frequently used basis for LCM systems, PolI or PolII driven systems have been shown in several contexts to yield more robust assays with higher reporter activities (Groseth et al., 2005; Nelson et al., 2017b). High background levels can pose another challenge and can result from viral RNA synthesis-independent transcription of the minigenome by cellular polymerases, e.g. from cryptic promotor elements. In such cases it may be possible to reduce background signals by the insertion of decoy ORFs (i.e. promoter-less ORFs that are inserted into one or more positions flanking the minigenome cassette in order to separate it from such cryptic promoter elements and thereby reduce viral RNA synthesis-independent reporter mRNA expression) (Dunham et al., 2018). Finally, the use of codon optimization has the potential to increase reporter activity in some systems. In particular, this approach has proven useful for several systems that use BHK-21 or BSR-T5/7 cells, which show

increased levels of reporter activity after codon optimization of the viral RNP genes for rodents (Albarino et al., 2013; Bergeron et al., 2015; Uebelhoer et al., 2014).

4.2. Applications of reverse genetics to high-throughput screening

In addition to the technical optimization of assay platform(s) intended for use as part of an HTS, it is important to consider the overall screening approach. Early HTS for VHF viruses and proof-of-concept studies often only reported results from a single primary screen without follow-up in secondary assays. The first such HTS was performed already in 2010 using an EBOV minigenome system expressing Firefly luciferase (Jasenosky et al., 2010). In this screen, 960 compounds from the Prestwick Chemical library, which contains primarily approved off-patent small molecules, were tested for their influence on viral RNA synthesis, as well as for cytotoxicity using a commercial cell viability assay. Using this approach, six compounds (cephaeline dihydrochloride, doxorubicin hydrochloride, ellipticine, emetine dihydrochloride, patulin and salinomycin) were identified as having an inhibitory effect on EBOV RNA synthesis (Jasenosky et al., 2010). More recently, a similar but much more extensive screen of 200,000 compounds using a Renilla-expressing EBOV minigenome could identify 56 inhibitors that reduced reporter activity by more than 70%, while still showing low cytotoxicity (Luthra et al., 2018a). Several of these inhibitors share common structural characteristics, such as containing an amino-tetrahydrocabazole scaffold, which was described before for another compound (GSK983) that is known to show broad antiviral activity by inhibiting *de novo* pyrimidine biosynthesis. Follow-up investigations with the racemate of GSK983 (SW835) and another inhibitor of *de novo* pyrimidine synthesis (brequinar) revealed an inhibitory effect of these compounds on EBOV minigenome activity as well as on the viral growth of EBOV, Vesicular Stomatitis virus and Zika virus (Luthra et al., 2018b). This clearly shows that despite their limitations, even single screens can generate valuable findings of biological relevance, but also reinforces the extent to which they benefit from and are strengthened by validation, in this case in the form of cross-analysis of hits between unrelated experiments.

Consequently, most HTS workflows consist of primary and secondary screens, followed by more detailed characterization of the most prominent hit(s). In the primary screen, the whole compound/RNAi library is used and the positive hits from this screen are then followed up in the secondary screen, which is either performed with the same system, but in a more thorough fashion (i.e. in the case of siRNA screening using alternate siRNAs for candidate genes, or in the case of compound screening using more concentrations and/or replicates), or using another, ideally more authentic assay. Following this approach, several HTS have already been performed for VHF-causing negative-stranded RNA viruses, using a classical minigenome system in the primary screen followed by a secondary screen using reporter-expressing or wildtype viruses (Tables 4 and 6). Of course such a workflow is targeted towards assessing only the influence of the tested libraries on viral RNA synthesis. It is also important to note that, despite having several artificial steps, many of the compounds or genes identified in these primary minigenome-based screens could be subsequently verified in an infectious context, demonstrating the predictive value of data obtained using these systems.

For example, Uebelhoer et al. conducted a primary screen of 9 nucleoside analogues using EBOV and MARV minigenomes, both expressing Gaussia luciferase, with a secondary screen then based on recombinant EBOV and MARV variants expressing Gaussia luciferase from an additional ATU between the genes for NP and VP35 (Uebelhoer et al., 2014). Using these approaches, they identified 6-azauridine, an inhibitor of *de novo* pyrimidine biosynthesis, as an inhibitor of both MARV and EBOV RNA synthesis. More recently, in a truly HTS approach Martin et al. also used the EBOV minigenome system (expressing NanoLuc luciferase) in a genome-wide siRNA screen featuring 64,755 siRNAs targeting 21,566 genes to identify host factors for viral RNA synthesis (Martin et al., 2018). Hits from the primary screen were further analyzed using a Firefly luciferase-expressing EBOV and the results again highlighted the role of the *de novo* pyrimidine synthesis pathway, and in particular CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase), as an important proviral factor for the EBOV life cycle. While the findings of these studies taken together certainly highlight the importance of nucleotide (and particularly pyrimidine) biogenesis in filovirus RNA synthesis, as well as the susceptibility of these viruses to treatment with nucleoside analogues, this is certainly not a feature unique to this virus group. Indeed, for LASV, a dual reporter-expressing minigenome has recently been generated for use in HTS (Welch et al., 2016) where the genes on the S segment were exchanged for ZsGreen (ZsG) and Gaussia luciferase, so that the cells could be visualized both by fluorescent microscopy and harvested for more sensitive measurement of luciferase reporter activity. This assay was used in a proof-of-principle study to test the effect of 27 commercially available nucleoside analogues on LASV RNA synthesis, as well as on EBOV RNA synthesis (using an EBOV minigenome encoding Gaussia luciferase as a reporter). In a secondary screen, recombinant LASV and EBOV, both expressing ZsG as cleavable fusions with viral proteins (NP and VP40, respectively), as well as wildtype LASV and EBOV were used. Again this approach confirmed 6-azauridine as an inhibitor of EBOV and could show that it also inhibits LASV, indicating a broader antiviral activity for this compound. Furthermore, this screen showed that 2′-deoxy-2′-fluorocytidine (2′-dFC) also inhibits both LASV minigenome replication and viral growth. Similarly highlighting the importance of nucleosides and their synthetic pathways for many VHF-causing pathogens, a limited drug library screen using a NanoLuc luciferase-expressing JUNV minigenome also identified AVN-944 as an inhibitor of RNA synthesis (Dunham et al., 2018). Here, a combination of a NanoLuc luciferase-expressing JUNV trVLP system and infection with a related but apathogenic wildtype virus (i.e. Tacaribe virus) was used for secondary screening. Unlike ribavirin and favipiravir, which have been previously reported to inhibit arenaviruses, as well as 2′-dFC, and 6-azauridine, AVN-944 is not a nucleoside analogue but rather inhibits the rate-limiting enzyme in *de novo* GTP synthesis.

While by virtue of using minigenome systems the screens discussed above have focused exclusively on inhibitors of viral RNA synthesis, screens for entry inhibitors have also been shown to be feasible using reverse genetics-based tools, and particularly trVLP systems, as part of an HTS approach. For example, a dual-reporter trVLP assay in which GFP and Gaussia luciferase replaced the S segment genes in the vaccine strain of JUNV (Candid#1) was recently used by Rathbun et al. for an HTS with 87,321 compounds from a library that included both novel lead-like molecules as well as FDA-approved and clinically tested compounds (Molecular Screening Shared Resource) (Rathbun et al., 2015). From this primary screen, 589 hits were further validated in a secondary screen using the same approach, but in triplicates, and controlling for off-target effects using a T7-driven IRES-containing Gaussia luciferase expression plasmid. Of the remaining 120 hits, the 117 commercially available compounds were then evaluated in detail to establish CC_{50} and IC_{50} values. Based on this analysis, three undisclosed novel inhibitors were identified, and subsequent assays using JUNV Candid#1, the related LCMV and lentiviral and retroviral vectors pseudotyped with GP for several other arenaviruses (and some other human pathogenic negative-stranded RNA viruses) revealed that all three compounds block entry and appear to have a solely anti-arenaviral activity (Rathbun et al., 2015). Interestingly, this study also identified tetrandrine, which is a calcium channel blocker that had been previously shown to inhibit EBOV infection (Sakurai et al., 2015), as a JUNV inhibitor. As such this study also emphasizes the value of cross-virus platform analysis in identifying inhibitors and proviral host factors that are potentially more broadly relevant, and thus perhaps more interesting for targeted antiviral development.

Further, not only monocistronic trVLP assays have value as screening tools, but also a Renilla luciferase-expressing version of the EBOV tetracistronic trVLP system has recently been optimized for HTS. This platform was used to study the impact of a small molecule library consisting of 8354 bioactives with known targets, FDA-approved drugs, natural compounds and kinase inhibitors (Lee et al., 2018). Importantly, with this system it was possible to assess compounds that inhibit either viral RNA synthesis (effect on reporter activity in trVLP-producing cells) or budding and entry (effect on reporter activity in target cells only). This broader screening approach identified 109 new compounds, but also several known entry inhibitors (like amodiaquine, sertraline, bepridil, amiodarone, ML9), again highlighting the authenticity of these systems and their validity as screening tools.

Contrary to the situation with LCM systems, the use of reporter-expressing viruses does not easily allow a targeted screen of compounds against specific steps in the viral life cycle. Rather, the strength of this approach lies in its authenticity, owing to the lack of any artificial steps. However, the use of infectious VHF viruses (including reporter-expressing viruses) limits work to high containment (i.e. BSL 3/4) laboratories, which normally have limited access to HTS technologies. As noted, this typically means that their use is restricted to secondary follow-up analysis of limited numbers of hits. However, recently some studies have also taken the approach of using reporter-expressing viruses for primary HTS analyses (Tables 4–6). For instance, a recombinant RVFV expressing GFP instead of NSs was used for a genome-wide RNAi screen targeting 22,909 genes in HeLa cells (Harmon et al., 2016). Here the GFP expression (after cell lysis) was used as a measure of viral infection while alamarBlue was used to assess cytotoxicity of the knock-down. A secondary screen using the same approach in triplicates was then used and resulted in 381 putative proviral genes being identified, with the Wnt/β-catenin pathway being the best represented. In follow-up analyses this pathway was also shown to be important for La Crosse virus and California encephalitis virus, indicating that reliance on this pathway is a conserved feature among bunyaviruses, and making it a more attractive target for antiviral development. Another HTS used a RVFV expressing the far-red fluorescent protein Katushka instead of NSs for a primary screen of 28,437 small chemical compounds (Chemical Biology Consortium Sweden) for their effect on RVFV infection in A549 cells. Fluorescence intensity of the reporter protein was assessed by spectrophotometry, while cytotoxicity of the screened compounds was determined using a Resazurin cell viability assay. A subsequent secondary screen using the same approach to validate 641 hits from the primary screen, but testing different compound concentrations, could then identify 6 compounds that showed a strong inhibitory effect on RVFV infection (Islam et al., 2016). In contrast, for arenaviruses the only truly HTS to date that was performed using viruses as the primary screen used a GFP-expressing LCMV, which is a related but much less pathogenic relative of LASV (Ngo et al., 2015). Indeed, when they are available, the use of such reporter-expressing BSL2 surrogate viruses can offer an approach that combines the strengths of virus-based screening (i.e. authenticity), while retaining the flexibility of working at lower containment levels, as well as the convenience and sensitivity of reporter based detection. In this case GFP was expressed from the S segment as a cleavable fusion with NP (GFP-P2A-NP). Using this approach 30,400 small molecule compounds were screened, which led to the identification of compound F3406 as an inhibitor of LCMV entry. A similar approach was recently developed as a proof-of-concept using a LASV that also expressed a cleavable GFP-P2A-NP fusion (Cai et al., 2018). This platform was optimized for HTS by evaluating the stability of the reporter in different cell types and validated using two known inhibitors of LASV, favipiravir and ribavirin, which then allowed evaluation of different cell lines for their suitability for HTS based on high-content imaging. Interestingly, although all cell lines tested (Vero E6, A549, HeLa and Huh7) showed equal numbers of infected cells, treatment with ribavirin or favipiravir led to different levels of inhibition, indicating a possible need to evaluate potential antivirals in different cell lines, something that so far has not been extensively considered in the context of HTS. Similarly, a recombinant CCHFV expressing ZsG as a fusion with N (ZsG-P2A-N) was recently used in a screen of 38 nucleoside analogues and 2 IMPDH inhibitors (Welch et al., 2017). Here virus inhibition was quantified by measuring reduction in ZsG fluorescence relative to infected, but mock-treated cells. Interestingly 2′-dFC, which had already been identified in a HTS described above to inhibit LASV (Welch et al., 2016), showed inhibition that was actually more potent than the known inhibitors ribavirin and favipiravir (as reflected by the determined EC_{50} values). However, while the potential of reporter-expressing LASV and CCHFV for future HTS has been clearly demonstrated by these studies, a more extensive analysis has so far not been reported.

In contrast, reporter-expressing EBOVs expressing GFP from ATUs have been particularly popular as HTS tools in recent years, and have repeatedly been used as a primary screen for truly high-throughput applications. In one screen a GFP-expressing EBOV (featuring an ATU between NP and VP35) was used for a primary high-content screening of 1990 compounds from the small molecule diversity set library from the National Cancer Institute (Panchal et al., 2012). This primary screen, which identified 45 inhibitory compounds, was followed by a dose-response study using the same platform to validate the most promising hit, compound NSC 6219. This compound possesses antioxidant properties and is known to act as scavenger of reactive oxygen species. In follow-up studies it inhibited not only EBOV-GFP, but also RVFV, LASV and Venezuelan equine encephalitis virus, again indicating a broader antiviral effect. Another screen with the same GFP-expressing EBOV but testing a library of 2600 approved drugs and molecular probes identified a further 171 compounds targeting diverse cellular mechanisms (e.g. ion transport, protein processing, cell signaling) as inhibitors of EBOV. Interestingly, many of these compounds were found to be class II cationic amphiphilic drugs, strongly indicating their relevance for the inhibition of EBOV infection (Johansen et al., 2015). Of the identified hits, 30 were confirmed by testing in an 8 dose curve in both Vero E6 and HepG2 cells, with cytotoxicity controlled using the CellTiter-Glo viability assay. These were then subsequently subjected to mechanistic analysis, which showed that inhibition for most of these antiviral drugs seems to take place at the level of viral entry (Johansen et al., 2015). In particular, detailed analysis of the estrogen receptor antagonists clomiphene and toremifene showed that they not only inhibit the growth of wildtype EBOV but also other filoviruses (Johansen et al., 2013, 2015). Follow-up mechanistic studies demonstrated that their mechanism of action is unrelated to their classical function in the estrogen pathway, but rather that they inhibit EBOV entry at a step after binding and internalization. Further, the authors could show that two other cationic amphiphilic drugs (bepridil and sertraline) could protect mice from EBOV infection *in vivo*. To date the effect of these compounds on other VHF-causing viruses has not been examined, although data using VLPs carrying LCMV GP suggest that there may be some effect of clomiphene and toremifene (Johansen et al., 2013). Regardless, this work clearly highlights not only the predictive power of these systems as screening tools, but also the value of screening existing licensed drugs for off-target antiviral effects that would otherwise be difficult to predict, particularly as they may not conform with established mechanisms of action.

Overall, looking at these emerging data from HTS studies it is clear that some pathways appear more frequently than others. Particularly the inhibition of *de novo* nucleoside synthesis, as well as the use of nucleoside analogues, are approaches that appear effective in various siRNA and drug screens for the inhibition of filoviruses (Luthra et al., 2018b; Martin et al., 2018; Uebelhoer et al., 2014; Welch et al., 2016), bunyaviruses (Welch et al., 2017) and arenaviruses (Dunham et al., 2018; Welch et al., 2016), although their feasibility *in vivo* remains to be shown for many of the identified compounds. However, in addition to the existing *in vivo* and/or clinical data indicating utility of nucleoside analogues in the treatment of both arenaviruses and bunyaviruses,

this clearly appears to be a promising antiviral target that needs to be further explored moving forward. However, HTS is clearly also turning up unexpected findings, such as the role of calcium channels or the Wnt/β-catenin pathway, as well as identifying off-target antiviral effects of well-known and even licensed drugs that are unrelated to their normal mechanism of action. Clearly such findings cannot be easily predicted based on our current knowledge of the viral life cycle of VHF viruses, and thus can only be identified as a part of large-scale screening approaches.

4.3. Data archiving and accessibility

The major challenge that has now arisen as a result of the often enormous quantity of data generated by such HTS approaches concerns the issues of data archiving and accessibility. Given the tremendous effort involved in performing detailed mechanistic follow-up, normally only one or a few hits from a given HTS are selected for further analyses in any given study (i.e. dose studies and mechanism of action analysis). This means that often huge parts of the generated datasets remain unused, although there might be other important findings waiting to be discovered, i.e. based on analysis of the data using alternative approaches. In particular, comparative analyses of several screens has the potential to give hints regarding important proteins/compounds/pathways that may have been overlooked simply based on not being the strongest individual hit(s) in a given screen, not being part of a particularly well-represented compound class or having no clearly relatable mechanism of action. In addition, especially the comparison between HTS for different viruses has unexploited potential to identify widely active antivirals or targetable pathways with relevance for multiple viruses. However, such analyses are only possible if the datasets from already existing HTS are made publicly available, which has unfortunately not been the case for most of them. Databases like the GenomeRNAi database, which contains many genome-wide RNAi screens, serve as an example for platforms collecting and centralizing HTS datasets publicly to aid these kinds of investigations (Schmidt et al., 2013). Needless to say, such transparent data availability is also necessary to satisfy the standards of scientific rigor in allowing the field as a whole to examine and scrutinize the results of such studies. Given that this field is still in its infancy, at least with respect to VHFs, it would appear that now would be the proper time to establish guidelines for issues such as data accessibility, although it is likely that the success of any such initiatives will depend heavily on the support of individual journals in implementing such standards as part of the peer review process.

5. Conclusion

Reverse genetics-based tools, including both LCM systems and recombinant reporter-expressing viruses, are increasingly available for a wide range of VHF-causing negative-sense RNA pathogens and offer great possibilities for conducting HTS for the identification of novel antivirals and/or host cell pathways involved in the viral life cycle. Further, systems are constantly being improved and new approaches developed to strengthen and simplify existing screening platforms. In particular, LCM systems have proven to be very potent tools for HTS because they allow the study of specific aspects of the viral life cycle, contributing important mechanistic data and allowing researchers to focus on specific processes in their analyses, all without the need for high containment laboratories. This then allows researchers to access the expertise and equipment needed for HTS analysis, which is not normally available in high containment facilities. However, despite these strengths, infectious viruses are still needed to validate the findings from LCM screens in the context of an authentic viral infection. Also here reverse genetics has made significant contributions in the form of reporter-expressing viruses, which allow detection of virus infection, as well as offering simple and sensitive quantification of viral growth. Indeed, such reporter-expressing viruses are now even being used to some extent as primary screens as a part of HTS workflows, although this remains technically challenging for BSL4 agents. In any case, the adaption of reverse genetics-based systems for HTS clearly offers a range of potent and promising platforms for both drug and host-target based screens, and this is now being demonstrated by the research coming out of this field. Indeed, in our estimation this approach represents one of the most promising options to identify viable drug candidates for VHF treatment, either through drug library screening for repurposing or the identification of novel broad-spectrum host factors, both of which rely heavily on serendipitous findings that clearly benefit from an HTS approach.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Funding

Funding was provided by the Friedrich-Loeffler-Institut, and by the Deutsche Forschungsgemeinschaft (DFG; grant numbers 389002253 and 324484564).

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