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Reverse genetics systems as tools to overcome the genetic diversity of Lassa virus

Brett Beitzel, Christine E Hulseberg and Gustavo Palacios



Lassa virus is endemic in a large area of sub-Saharan Africa, and exhibits a large amount of genetic diversity. Of the four currently recognized lineages, lineages I–III circulate in Nigeria, and lineage IV circulates in Sierra Leone, Guinea, and Liberia. However, several newly detected lineages have been proposed. LASV genetic diversity may result in differences in pathogenicity or response to medical countermeasures, necessitating the testing of multiple lineages during the development of countermeasures and diagnostics.

Logistical and biosafety concerns can make it difficult to obtain representative collections of divergent LASV clades for comparison studies. For example, lack of a cold chain in remote areas, or shipping restrictions on live viruses can prevent the dissemination of natural virus isolates to researchers. Reverse genetics systems that have been developed for LASV can facilitate acquisition of hard-to-obtain LASV strains and enable comprehensive development of medical countermeasures.

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Introduction and taxonomic organization of the *Arenaviridae*

In the 42 years since the *Arenaviridae* were formally recognized as a family [1], the taxon has grown to recognize 41 member species [2], a quarter of which have been added within the last two years. Shortly following the discovery of arenaviruses in boas and pythons, the *Arenaviridae* monophyly was split into the *Mammarenavirus* and *Reptarenavirus* in 2014 [3], and, in 2018, the reptile-borne arenaviruses were again separated

into distinct genera, *Hartmanivirus* and *Reptarenavirus* [2]. The largest of these three current genera is the *Mammarenavirus*, which is further divided into two distinct groups: the Old World (OW) and the New World (NW) arenaviruses. In addition to the geographic basis for their separate groupings, OW and NW arenaviruses are segregated by their serological, epidemiological, and phylogenetic relationships. As are the majority of the other OW arenavirus members of the *Mammarenavirus*, Lassa virus (LASV) is a rodent-borne virus whose area of endemicity is geographically confined to Africa.

Molecular properties of arenaviruses

The genome of the arenaviruses is carried on two RNA fragments, each with two non-overlapping genes arranged in opposing orientation. The L RNA fragment encodes the RNA-dependent RNA polymerase (L) and the RING finger matrix-like protein (Z), while the S RNA fragment encodes the glycoprotein (GPC) and the nucleoprotein (NP). The ribonucleoproteins, which consist of the viral genome fully encapsidated by NPs, and the L polymerase, is replication and transcription-competent [4]. The two genes on each RNA segment are separated by a ~65–200 nucleotide intergenic region (IGR) that forms a stable hairpin structure(s). While both IGRs function in transcription termination, there is substantial difference in their predicted folded structures. The 5'-termini and 3'-termini of the RNAs have 19–25 highly conserved nucleotides that form panhandle structures on each genome segment. These noncoding regions act in *cis* with the segment's IGR to support both genome replication and transcription [5].

Established and emerging Lassa lineages

Current guidelines for the genetic distinction of arenavirus species require $\geq 12\%$ amino acid difference in the NP sequence [6]. Circulating strains of LASV in West Africa (some of which, despite exceeding the 12% allowable difference in amino acid sequence cutoff, are still recognized as LASV species) typically fall into four established lineages which are highly correlated to the geographical range of the rodent reservoir for LASV, *Mastomys natalensis*. Lineages I–III strains circulate throughout Nigeria, and lineage IV strains are endemic throughout the Mano River Union (MRU) region shared by Sierra Leone, Guinea, and Liberia, roughly 2500 km away.

As additional LASV isolates are collected and studied, an increasingly complex picture of the genetic diversity of LASV — particularly in recent years — is developing.

The possible emergence of a fifth lineage was suggested by Manning *et al.* in 2015 [7^{*}]. Bayesian analysis of L, NP, and GPC genes of isolates from Mali and the Ivory Coast suggested that these strains may have split from lineage IV strains some 250 years ago [7^{*}]. In 2016, LASV was isolated from hitherto unrecognized rodent carriers in Nigeria (*Mastomys erythroleucus* and *Hylomyscus pamfi*) and Guinea (*M. erythroleucus*) [8]. Phylogenetic analysis of both the nucleotide and amino acid sequences on a newly identified strain isolated from *H. pamfi* in Kako, Nigeria indicated that, while clustering nearer the other Nigerian lineages, this isolate did not belong to any of them [8]. Yet another possible lineage emerged later in 2016, when a cluster of cases originating in Togo was sequenced and found to have poorly supported phylogenies with other LASV strains. The resulting trees, generated from the L, NP, and GPC sequences, showed considerable topological variability depending upon which gene(s) were used in the analysis, and, in all cases, long branches suggest distant relationships with the other lineages of LASV [9].

Sources and implications of genetic diversification in Lassa virus

As with other riboviruses, LASV has an error-prone polymerase. Accordingly, the main driving force for the evolution and diversification of arenaviruses is largely ascribed to the high mutation rates that occur during replication [5,10,11^{*}]. Examination of the genetic variability of transmission chains during multiple outbreaks consistently supports the predominance of rodent-to-human rather than human-to-human spread [12^{*},13,14]; however, human-to-human transmission may still account for nearly 20% of human infections [12^{*},13–16].

Reassortment in segmented viruses is another potential source of diversity, particularly when closely related strains coinfect a susceptible host. Comparison of phylograms constructed from full length sequences of the arenaviral genes suggests that it is unlikely that natural reassortment has contributed significantly to the evolutionary history of LASV [17,18]). Nonetheless, when the L and S segments of 162 LASV isolates from Nigeria and Sierra Leone were sequenced, three (~1.8%) of these isolates were found to be reassortants [12^{*}].

The many challenges associated with developing medical countermeasures (MCM) against a heterogeneous viral target such as LASV have long been appreciated. For one, some evidence suggests that strain pathogenicity may track with geographical distance. Strains circulating in the easternmost ranges of the Lassa Belt, that is, lineages I–III, reportedly have increased pathogenicity relative to lineage IV; accordingly, case fatality rates are typically higher in Nigeria than in the MRU [19,20]. By extension, noted differences in pathogenicity may call into question whether conclusions based on work using the ‘classic’

prototypical strain, Josiah — the lineage IV strain that serves as the workhorse for the vast majority of basic and translational LASV work [21^{**}] — will necessarily hold true for other strains.

An example of potentially critical differences in strain selection is given by an early LASV vaccine study, in which the GA391 strain from northern Nigeria (lineage III) was used to challenge a group of vaccinated outbred Hartley guinea pigs [22]. While typically only 20% or less of Hartley guinea pigs infected with Josiah strain LASV die [23], in this particular study, all of the control guinea pigs infected with GA391 strain died. Another example of lineage-dependent responses to an MCM is a 2018 study evaluating the efficacy of two arenavirus entry inhibitors, LHF-535 and ST-193 [24]. The inhibitory concentrations for LHF-535 generally clustered within a 10-fold, subnanomolar range with the exception of the lineage I strain LP, which was ~100-fold less sensitive than all other strains. Lineage-dependent and even strain-dependent variability in response to ST-193 was more pronounced, although, as with LHF-535, the sensitivity of LP strain LASV to ST-193 was reduced compared to other lineages. Given the tremendous effort, time, and expense needed to advance MCMs through clinical trials, tractable experimental systems are needed to account for LASV’s formidable genetic diversity.

Reverse genetics

The breadth of LASV diversity can make it difficult to obtain virus stocks representing a comprehensive collection of Lassa lineages. Additionally, the relatively recent development of field-deployable high-throughput sequencing (e.g. Oxford Nanopore MinION and Illumina iSEQ) will likely increase the number of viruses for which genome sequence is available in the absence of natural isolates. In cases where natural isolates are difficult to obtain or are nonexistent, reverse genetics can be used to generate virus stocks for study [25^{*}].

Virus reverse genetics systems allow the generation or rescue of replication-competent viruses from plasmid DNA, facilitating detailed study of the virus lifecycle. Precise changes to the virus genome can be made in the plasmid clones using standard molecular biology techniques, and viruses can then be rescued from those clones to examine the resulting changes to the virus phenotype.

The first description of reverse genetics involved the cloning of the full genome of the RNA bacteriophage Q β [26]. After cloning a complete copy of the phage genome cDNA into a plasmid, Taniguchi *et al.* were able to rescue viable Q β phage by transforming the plasmid into susceptible *Escherichia coli*. This was followed several years later by the first eukaryotic virus reverse genetics system for poliovirus [27]. Racaniello *et al.* cloned the complete poliovirus genome into a plasmid, and

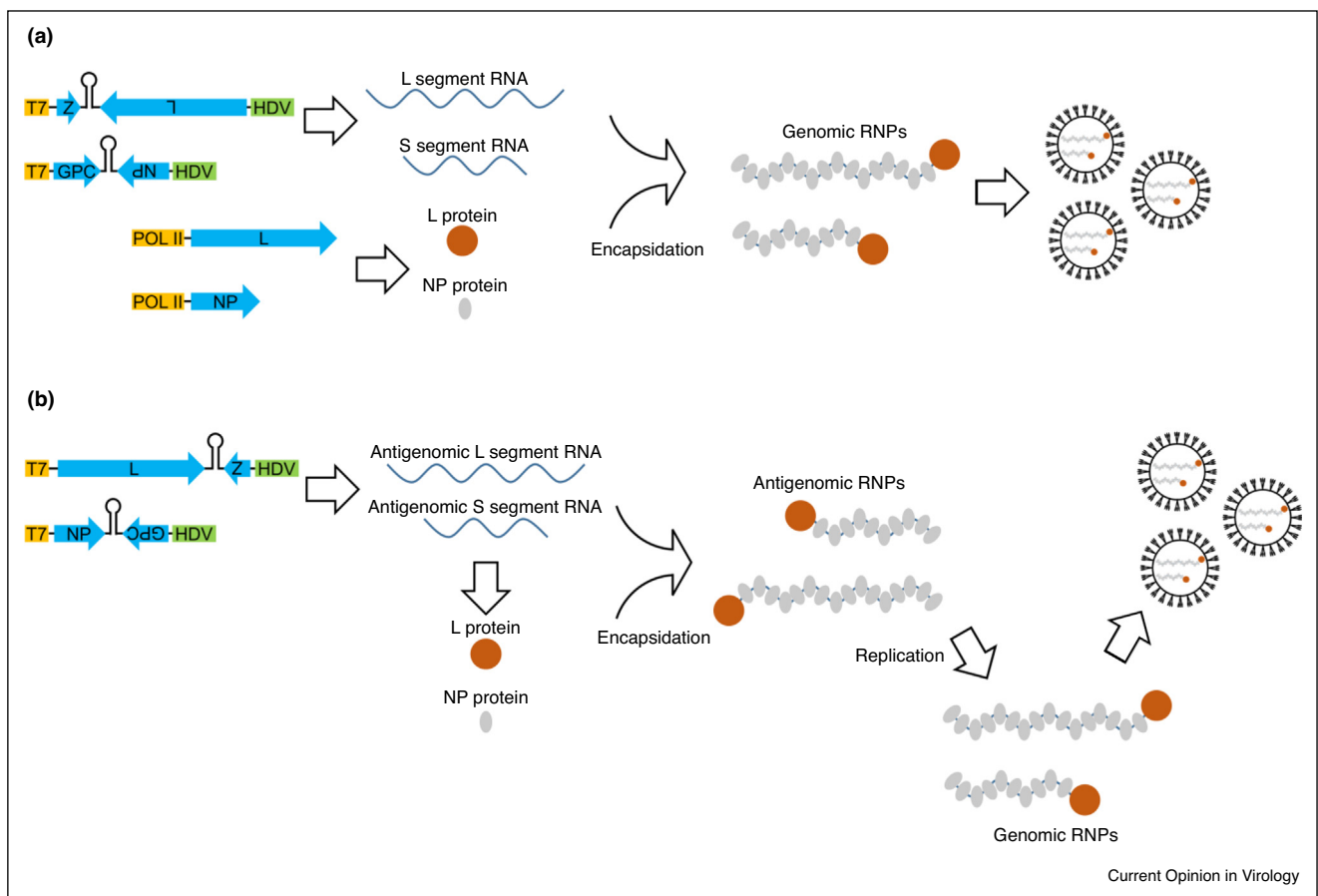
generated infectious poliovirus upon transfection of the full genome plasmid into susceptible cells. Both Q β and poliovirus are positive-stranded RNA viruses, meaning that introduction of the viral genomic RNA into a host cell can lead to virus production since the viral proteins can be translated directly from the genomic RNA by the host cell machinery. Reverse genetics systems for negative-stranded RNA viruses are not as straight-forward, since the deproteinated RNA genome cannot be directly translated by the host machinery. In order to be transcribed and replicated, the genome segments of negative-stranded RNA viruses (NSVs) must be in a ribonucleoprotein (RNP) complex with the viral nucleoprotein (N) and the RNA-dependent RNA polymerase (L). Rescuing NSVs by reverse genetics requires the polymerase and nucleoproteins to be provided in *trans* as 'helper' proteins. The helper proteins can either be provided via a

co-infecting helper virus [28], or they can be provided by expression plasmids. The first described reverse genetics system for a NSV from cDNA only (i.e. no helper virus was used) was developed for rabies virus [29]. Schnell *et al.* showed that infectious rabies virus could be rescued upon expression of the viral antigenomic RNA in addition to the N, P, and L proteins in *trans* (which make up the viral replication complex).

Arenavirus reverse genetics

Reverse genetics strategies developed for arenaviruses have generally followed those for NSVs. In the genomic RNA of arenaviruses, the L and NP genes are encoded in the antisense orientation, so the proteins that they encode must be provided in *trans* in order to generate the replication and transcription-competent RNP complex.

Figure 1



Two strategies for arenavirus reverse genetics.

In the four-plasmid arenavirus reverse genetics system (a), L and NP proteins are provided in *trans* from Pol II or T7-driven expression plasmids. The genomic (or antigenomic) RNA segments are generated from a T7-driven expression plasmid. A hepatitis delta virus ribozyme sequence (HDV) generates a precise 3' end of the transcribed RNA. Encapsidation of the genomic RNAs by L and NP kickstarts virus production. In the two-plasmid system (b), antigenomic RNAs are produced from a T7 expression construct. L and NP proteins are produced by translation of these antigenomic RNAs, and in turn encapsidate the antigenomic RNAs. The antigenomic RNAs are replicated to produce genomic RNAs which can then be packaged into progeny virus.

Development of reverse genetics systems for many NSVs has usually started with the development of minigenome or replicon systems. Minigenomes are partial viral genome segments that contain all of the *cis*-acting elements required for replication and transcription, but in which many or all of the protein coding regions have been replaced with reporter proteins such as GFP, CAT, or luciferase. Viral proteins required for replication and transcription of the minigenome are provided *in trans* to drive expression of the encoded reporter protein. Minigenomes allow for elucidation of the *cis*-acting and *trans*-acting factors required for replication and transcription, and can guide the development of full-genome systems that can generate replication competent viruses. The first arenavirus minigenome system was developed for LCMV [30]. Lee *et al.* designed an S segment minigenome in which the NP gene had been replaced by CAT. In addition, the GPC coding region was removed, leaving the 5' UTR and IGR. This system showed that the 5' and 3' UTRs, along with the IGR, were sufficient *cis*-acting sequences for CAT expression, and that NP and L were the minimal viral *trans*-acting factors for CAT expression. A similar strategy was used by Hass *et al.* to generate a LASV minigenome system that showed NP and L were also the minimal *trans*-acting viral proteins required for replication and expression, and that the 5' and 3' UTRs and the IGR were sufficient *cis*-acting sequences. In addition, expression from the LASV minigenome was shown to be inhibited by ribavirin and interferon alpha similarly to replicating LASV, indicating that the minigenome system could serve as a surrogate to LASV in screens to identify antiviral compounds [31]. This LASV minigenome system was also used for fine mapping of the LASV promoter residing in a 19-nucleotide region of the 5' and 3' termini of the S segment [32].

Following development of arenavirus minigenome systems, two general strategies have been developed for rescuing replication-competent viruses. In the first (Figure 1a), the L and NP proteins are supplied *in trans* via pol II or T7 promoter-driven expression plasmids [33]. RNA analogs of the L and S segments are generated by expression from T7-driven plasmids and become encapsidated by the expressed NP and L proteins to form the functional RNP complex required for viral replication, kickstarting production of progeny virions. The second strategy for arenavirus rescue (Figure 1b) is a two-plasmid system: one plasmid expressing the antigenomic L segment, and one plasmid expressing the antigenomic S segment [34]. Antigenomic RNAs are produced via T7-driven expression, and L and NP are translated from the antigenomic RNAs. The L and NP proteins can then form RNP complexes with the antigenomic RNAs, facilitating replication into genomic RNAs and generation of progeny viruses. Both of these strategies have been used successfully to rescue recombinant LASV [35,36].

Arenavirus reverse genetics systems have been used in a wide variety of studies. As previously mentioned, minigenome systems have been used to define the minimal *cis*-acting and *trans*-acting factors required for viral replication, map critical residues of the viral promoter, and serve as virus surrogates in assays to identify antiviral compounds [30–32,37–39]. Rescue of replication-competent recombinant arenaviruses bearing engineered changes in the genome has allowed studies of virus–host interactions [36,40], mapping functionally important regions of the virus genome [34,35,41], and generating tagged viruses to simplify countermeasure development [39,42].

Rescue of replication-competent engineered arenaviruses has also introduced several intriguing strategies for the generation of attenuated vaccines. Swapping the locations of GPC and NP on the S segment of LCMV resulted in strong attenuation in a mouse model, but could protect from challenge with a virulent strain [43]. Recombinant LCMVs bearing codon-deoptimized GP or NP are also attenuated *in vivo*, and can protect from subsequent challenge [44,45]. Novel tri-segmented recombinant arenaviruses have been developed by splitting the GPC and NP genes onto two separate S segment analogs [43,46,47]. The tri-segmented recombinant viruses are only partially attenuated in a mouse model, but surviving mice develop protective immunity, warranting further study of the tri-segmented viruses as a vaccine platform [46]. A similar approach has recently been developed using non-pathogenic Pichinde virus as a vaccine vector to express heterologous antigens [48]. Finally, a hyper-attenuated chimeric Mopeia virus bearing the LASV GPC has been shown to be protective in a LASV NHP challenge study [49].

As described above, LASV lineages can potentially respond differently to medical countermeasures (MCMs), and also vary in their ability to be detected by current diagnostic assays [24,50]. These variable responses highlight the need to test new MCMs and diagnostic assays against a wide variety of LASV samples from different lineages. Reverse genetics can facilitate the acquisition of divergent strains to characterize differences in lineage response. Future directions involve the production of diverse isolates of LASV via reverse genetics to test responses to MCMs and analyze the sensitivity of diagnostic assays.

Conclusions

LASV genomic diversity reflects the large area of endemicity throughout sub-Saharan Africa. MCMs developed against one LASV lineage may not work against other lineages, highlighting the importance of testing against divergent strains. Although the advent of high-throughput sequencing has made it increasingly easy to obtain full viral genome sequences at the source of

an outbreak, dissemination of natural virus isolates to the research community is becoming increasingly difficult due to logistical, biosafety, and political concerns. Reverse genetics systems developed over the past decade can be used to acquire diverse LASV strains when natural isolates are difficult to obtain, enabling testing of MCMs and diagnostic assays against the breadth of LASV lineages.

Conflict of interest statement

Nothing declared.

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In this comprehensive review article, the authors discuss applications of reverse genetics and other surrogate systems for Lassa. The mechanisms for arenaviral genetic diversity and the implications imposed on drug and vaccine development are also presented in detail.

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