cross-reactivity, these methods do not necessarily distinguish lineage I from lineage II strains. Neutralization assays are required for confirmation; molecular detection and sequence determination, as performed in our investigation, allowed for definitive classification of the virus.

In this study, we detected deer tick virus by both molecular and immunohistochemical methods in the central nervous system of a patient with encephalitis. The neurotropism seen in this case, with involvement of both gray and white matter, matches the pattern of central nervous system infection for arboviruses, which may be highly neuroinvasive.[23]

The patient was known to have frequented wooded areas, although no specific contact with ticks had been reported. He presented in late spring, which suggested that transmission was probably from nymphal deer ticks, which are most active during spring and summer months. In addition, since nymphal deer ticks are small in size (1.5 mm in diameter), it is not uncommon for their bites to remain undetected. It is possible that the patient's underlying condition (CLL–SLL) predisposed him to particularly serious disease. Reports of elderly and immunocompromised patients being at a greater risk for severe encephalitis caused by West Nile virus are well documented.[24,25]

Our immunohistochemical studies with newly generated deer tick virus antibodies demonstrated prominent labeling of neuronal-cell bodies and their processes; a focus of apparent oligodendroglial infection was also identified (Fig. 4). In addition, some neurons contained rounded granular-to-tubular profiles. A segmental distribution of immunolabeling was evident in the hippocampus, as was seen in cerebellum infected by central European tickborne encephalitis virus, as described previously.[26] The parenchymal lymphocytic infiltrates in this case and in previous pathological studies of tickborne encephalitis virus[26,27] were predominantly CD8+ cytotoxic T cells, which were also seen in close apposition to surviving neurons, further indicating that immunologic mechanisms may have contributed to nerve-cell destruction in tickborne encephalitides.

Diagnostic testing for Powassan virus is not routinely performed in patients with encephalitis. More extensive testing for arboviruses, including Powassan virus, might reveal that arboviral infections are more widespread than previously reported. For Powassan virus, testing is especially important during the summer months and in regions where infected ticks are prevalent. Deer ticks transmit several tickborne diseases, including Lyme disease, human babesiosis, and human granulocytic anaplasmosis.[28] This report of deer tick virus resulting in a fatal case of encephalitis emphasizes the significance of deer ticks in transmitting a variety of infections. There are limited data on the prevalence of infection with deer tick virus among adult deer ticks, although a rate of 0.6 to 1.3% in limited geographic areas in the United States has been reported.[9] Because no specific antiviral therapy is available for Powassan infection, the best strategy remains prevention (i.e., avoidance of contact with the arthropod vector). Studies to elucidate the prevalence and relative pathogenic features of Powassan lineages I and II are warranted.

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別紙様式第２－１

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 平成 21 年 6 月 8 日	新医薬品等の区分 該当なし	機構処理欄
一般的名称	ﾃｸﾈﾁｳﾑ大凝集人血清ｱﾙﾌﾞﾐﾝ（^{99m}Tc）		研究報告の公表状況	Plos pathogens May 2009, vol.4, Issue.5,e100455	公表国 ザンビア、南アフリカ	
販売名（企業名）	テクネ MAA キット（富士フイルムＲＩファーマ株式会社）					

研究報告の概要	使用上の注意記載状況・その他参考事項等
要約：南アフリカでのアレナウイルス関連の新規の出血熱である Lujo ウイルスの遺伝子検出及び特徴づけ 2008 年に南アフリカで発生した致死性出血熱のアウトブレークにおいて、新規の旧世界アレナウイルスが分離された。旧世界の出血熱関連のアレナウイルスとしては 30 年ぶりの発見である。Unbiased pyrosequencing により、アウトブレークの犠牲者からの検体を受領してから 72 時間以内の識別と系統発生学的な特徴づけが可能であった。遺伝子解析により、他の旧世界アレナウイルスと明らかに異なる、固有のものであること、旧世界アレナウイルスと新世界アレナウイルスとのおよそ等距離にあること等が判明した。このウイルスが確認された場所の地名（Lusaka、Johannesburg）より Lujo virus（以下、LUJV）と命名した。この発見は、LUJV の宿主や地理的な分布、病原性の調査に使用される試薬の開発を可能にするとともに、病原体の発見や公衆衛生にとっての unbiased high throughput pyrosequencing の有用性を確認することができた。	特になし

報告企業の意見	今後の対応
Lujo ウイルスの新規性については、従来確認されていた他のアレナウイルスとはかなり異なる固有のものであること、また、患者５人中４人が死亡していることから、高病原性であることが判明しており、新規・重大な感染症に関する報告と評価する。	ヒト血液を原料とする血漿分画製剤とは直接関連しないことから、現時点で当該生物由来製品に関し、措置等を行う必要はないと判断する。

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Genetic Detection and Characterization of Lujo Virus, a New Hemorrhagic Fever–Associated Arenavirus from Southern Africa

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Abstract

Lujo virus (LUJV), a new member of the family *Arenaviridae* and the first hemorrhagic fever–associated arenavirus from the Old World discovered in three decades, was isolated in South Africa during an outbreak of human disease characterized by nosocomial transmission and an unprecedented high case fatality rate of 80% (4/5 cases). Unbiased pyrosequencing of RNA extracts from serum and tissues of outbreak victims enabled identification and detailed phylogenetic characterization within 72 hours of sample receipt. Full genome analyses of LUJV showed it to be unique and branching off the ancestral node of the Old World arenaviruses. The virus G1 glycoprotein sequence was highly diverse and almost equidistant from that of other Old World and New World arenaviruses, consistent with a potential distinctive receptor tropism. LUJV is a novel, genetically distinct, highly pathogenic arenavirus.

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Introduction

Members of the genus *Arenavirus*, comprising currently 22 recognized species (http://www.ictvonline.org/virusTaxonomy.asp?version=2008), are divided into two complexes based on serologic, genetic, and geographic relationships [1,2]: the New World (NW) or Tacaribe complex, and the Old World (OW) or Lassa-Lymphocytic choriomeningitis complex that includes the ubiquitous arenavirus type-species *Lymphocytic choriomeningitis virus* (LCMV; [3]). The RNA genome of arenaviruses is bi-segmented, comprising a large (L) and a small (S) segment that each codes for two proteins in ambisense coding strategy [4,5]. Despite this coding strategy, the *Arenaviridae* are classified together with the families *Orthomyxoviridae* and *Bunyaviridae* as segmented single-strand, negative sense RNA viruses.

The South American hemorrhagic fever viruses Junin (JUNV; [6,7]), Machupo (MACV; [8]), Guanarito (GTOV; [9]) and Sabia virus (SABV, [10]), and the African Lassa virus (LASV [11]), are restricted to biosafety level 4 (BSL-4) containment due to their associated aerosol infectivity and rapid onset of severe disease. With the possible exception of NW Tacaribe virus (TCRV; [12]), which has been isolated from bats (*Artibeus* spp.), individual arenavirus species are commonly transmitted by specific rodent species wherein the capacity for persistent infection without overt disease suggests long evolutionary adaptation between the agent and its host [1,13–16]. Whereas NW arenaviruses are associated with rodents in the *Sigmodontinae* subfamily of the family *Cricetidae*, OW arenaviruses are associated with rodents in the *Murinae* subfamily of the family *Muridae*.

Humans are most frequently infected through contact with infected rodent excreta, commonly via inhalation of dust or aerosolized virus-containing materials, or ingestion of contaminated foods [13]; however, transmission may also occur by inoculation with infected body fluids and tissue transplantation [17–19]. LCMV, which is spread by the ubiquitous *Mus musculus* as host species and hence found world-wide, causes symptoms in humans that range from asymptomatic infection or mild febrile illness to meningitis and encephalitis [13]. LCMV infection is only rarely fatal in immunocompetent adults; however, infection during pregnancy bears serious risks for mother and child and frequently results in congenital abnormalities. The African LASV, which has its reservoir in rodent species of the *Mastomys* genus, causes an estimated 100,000–500,000 human infections per year in West African countries (Figure 1). Although Lassa fever is typically subclinical or associated with mild febrile illness, up to 20% of cases may have severe systemic disease culminating in fatal outcome [20,21]. Three other African arenaviruses are not known to cause human disease: Ippy virus (IPPYV; [22,23]), isolated from

Author Summary

In September and October 2008, five cases of undiagnosed hemorrhagic fever, four of them fatal, were recognized in South Africa after air transfer of a critically ill index case from Zambia. Serum and tissue samples from victims were subjected to unbiased pyrosequencing, yielding, within 72 hours of sample receipt, multiple discrete sequence fragments that represented approximately 50% of a prototypic arenavirus genome. Thereafter, full genome sequence was generated by PCR amplification of intervening fragments using specific primers complementary to sequence obtained by pyrosequencing and a universal primer targeting the conserved arenaviral termini. Phylogenetic analyses confirmed the presence of a new member of the family *Arenaviridae*, provisionally named Lujo virus (LUJV) in recognition of its geographic origin (Lusaka, Zambia, and Johannesburg, South Africa). Our findings enable the development of specific reagents to further investigate the reservoir, geographic distribution, and unusual pathogenicity of LUJV and confirm the utility of unbiased high throughput pyrosequencing for pathogen discovery and public health.

Arvicanthis spp. and Mobala virus (MOBV; [24]) isolated from *Praomys* spp. in the Central African Republic (CAR); and Mopeia virus (MOPV) that like LASV is associated with members of the genus *Mastomys*, and was reported from Mozambique [25] and Zimbabwe [26], although antibody studies suggest that MOPV and LASV may also circulate in CAR [27] where the geographies of these viruses appear to overlap (Figure 1). Up to present, there have been no published reports of severe human disease associated with arenaviruses isolated from southern Africa.

In September 2008 an outbreak of unexplained hemorrhagic fever was reported in South Africa [28]. The index patient was airlifted in critical condition from Zambia on September 12 to a clinic in Sandton, South Africa, after infection from an unidentified source. Secondary infections were recognized in a paramedic (case 2) who attended the index case during air transfer from Zambia, in a nurse (case 3) who attended the index case in the intensive care unit in South Africa, and in a member of the hospital staff (case 4) who cleaned the room after the index case died on September 14. One case of tertiary infection was recorded in a nurse (case 5) who attended case 2 after his transfer from Zambia to Sandton on September 26, one day before barrier nursing was implemented. The course of disease in cases 1 through 4 was fatal; case 5 received ribavirin treatment and recovered. A detailed description of clinical and epidemiologic data, as well as immunohistological and PCR analyses that indicated the presence of an arenavirus, are reported in a parallel communication (Paweska et al., Emerg. Inf. Dis., submitted). Here we report detailed genetic analysis of this novel arenavirus.

Results/Discussion

Rapid identification of a novel pathogen through unbiased pyrosequencing

RNA extracts from two post-mortem liver biopsies (cases 2 and 3) and one serum sample (case 2) were independently submitted for unbiased high-throughput pyrosequencing. The libraries yielded between 87,500 and 106,500 sequence reads. Alignment of unique singleton and assembled contiguous sequences to the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) using the Basic Local Alignment Search Tool (blastn and blastx; [29]) indicated coverage of approximately 5.6 kilobases (kb) of sequence distributed along arenavirus genome scaffolds: 2 kb of S segment sequence in two fragments, and 3.6 kb of L segment sequence in 7 fragments (Figure 2). The majority of arenavirus sequences were obtained from serum rather than tissue, potentially reflecting lower levels of competing cellular RNA in random amplification reactions.

Full genome characterization of a newly identified arenavirus

Sequence gaps between the aligned fragments were rapidly filled by specific PCR amplification with primers designed on the pyrosequence data at both, CU and CDC. Terminal sequences were added by PCR using a universal arenavirus primer, targeting the conserved viral termini (5′-CGC ACM GDG GAT CCT AGG C, modified from [30]) combined with 4 specific primers positioned near the ends of the 2 genome segments. Overlapping primer sets based on the draft genome were synthesized to facilitate sequence validation by conventional dideoxy sequencing. The accumulated data revealed a classical arenavirus genome structure with a bi-segmented genome encoding in an ambisense strategy two open reading frames (ORF) separated by an intergenic stem-loop region on each segment (Figure 2) (GenBank Accession numbers FJ952384 and FJ952385).

Our data represent genome sequences directly obtained from liver biopsy and serum (case 2), and from cell culture isolates obtained from blood at CDC (case 1 and 2), and from liver biopsies at NICD (case 2 and 3). No sequence differences were uncovered between virus detected in primary clinical material and virus isolated in cell culture at the two facilities. In addition, no changes were detected between each of the viruses derived from these first three cases. This lack of sequence variation is consistent with the epidemiologic data, indicating an initial natural exposure of the index case, followed by a chain of nosocomial transmission among subsequent cases.

Lujo virus (LUJV) is a novel arenavirus

Phylogenetic trees constructed from full L or S segment nucleotide sequence show LUJV branching off the root of the OW arenaviruses, and suggest it represents a highly novel genetic lineage, very distinct from previously characterized virus species and clearly separate from the LCMV lineage (Figure 3A and 3B). No evidence of genome segment reassortment is found, given the identical placement of LUJV relative to the other OW arenaviruses based on S and L segment nucleotide sequences. In addition, phylogenetic analysis of each of the individual ORFs reveals similar phylogenetic tree topologies. A phylogenetic tree constructed from deduced L-polymerase amino acid (aa) sequence also shows LUJV near the root of the OW arenaviruses, distinct from characterized species, and separate from the LCMV branch (Figure 3C). A distant relationship to OW arenaviruses may also be inferred from the analysis of Z protein sequence (Figure S1). The NP gene sequence of LUJV differs from other arenaviruses from 36% (IPPYV) to 43% (TAMV) at the nucleotide level, and from 41% (MOBV/LASV) to 55% (TAMV) at the aa level (Table S1). This degree of divergence is considerably higher than both, proposed cut-off values within (<10–12%), or between (>21.5%) OW arenavirus species [31,32], and indicates a unique phylogenitic position for LUJV (Figure 3D). Historically, phylogenetic assignments of arenaviruses have been based on portions of the NP gene [1,33], because this is the region for which most sequences are known. However, as more genomic sequences have become available, analyses of full-length GPC sequence have revealed evidence of possible relationships between OW and NW

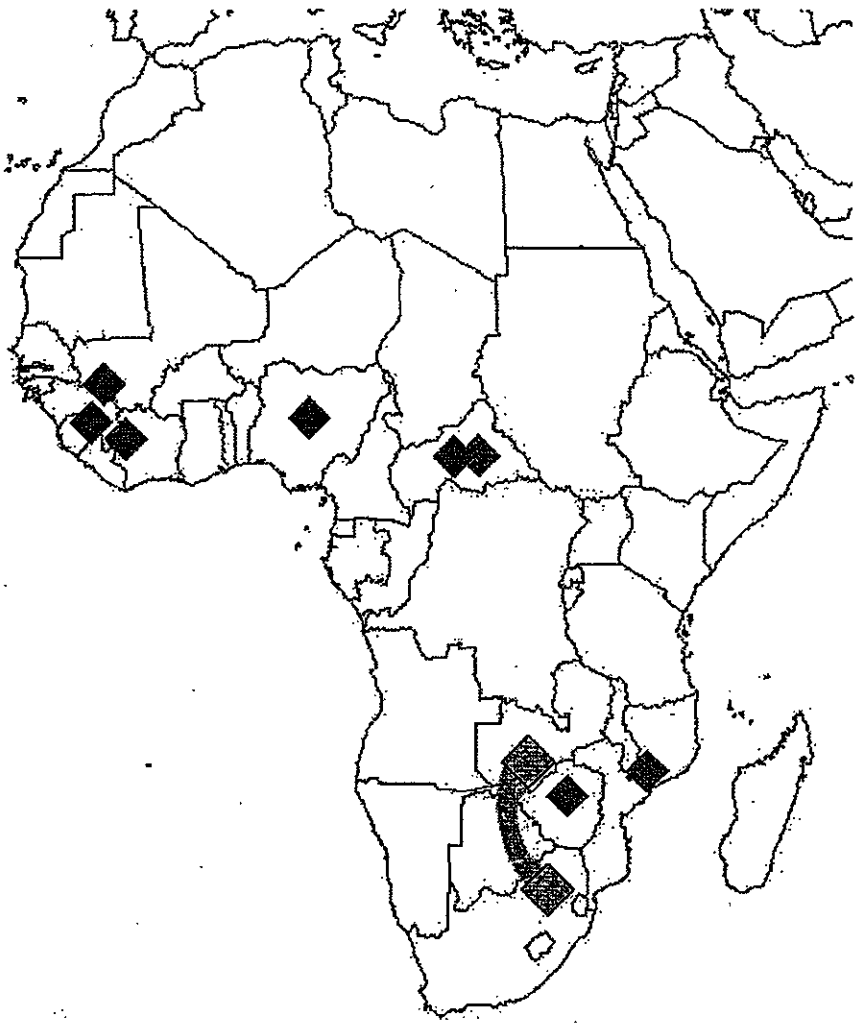

Figure 1. Geographic distribution of African arenaviruses. MOBV, MOPV, and IPPYV (blue) have not been implicated in human disease; LASV (red) can cause hemorrhagic fever. The origin of the LUJV index and secondary and tertiary cases linked in the 2008 outbreak are indicated in gold.
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arenaviruses not revealed by NP sequence alone [34]. Because G1 sequences are difficult to align some have pursued phylogenetic analyses by combining the GPC signal peptide and the G2 sequence for phylogenetic analysis [16]. We included in our analysis the chimeric signal/G2 sequence (Figure 3E) as well as the receptor binding G1 portion (Figure 3F); both analyses highlighted the novelty of LUJV, showing an almost similar distance from OW as from NW viruses.

Protein motifs potentially relevant to LUJV biology

Canonical polymerase domains pre-A, A, B, C, D, and E [35–37] are well conserved in the L ORF of LUJV (256 kDa, pI = 6.4; Figure 4). The Z ORF (10.5 kDa, pI = 9.3) contains two late domain motifs like LASV; however, in place of the PTAP motif found in LASV, that mediates recognition of the tumor susceptibility gene 101, Tsg101 [38], involved in vacuolar protein sorting [39,40], LUJV has a unique Y_{77}REL motif that matches the YXXL motif of the retrovirus equine infectious anemia virus [41], which interacts with the clathrin adaptor protein 2 (AP2) complex [42]. A Tsg101-interacting motif, P_{90}SAP, is found in LUJV in position of the second late domain of LASV, PPPY, which acts as a Nedd4-like ubiquitin ligase recognition motif [43]. The RING motif, containing conserved residue W_{44} [44], and the conserved myristoylation site G_2 are present [45–47] (Figure 4). The NP of LUJV (63.1 kDa, pI = 9.0) contains described aa motifs that resemble mostly OW arenaviruses [48], including a cytotoxic T-lymphocyte (CTL) epitope reported in LCMV (GVYMGNL; [49]), corresponding to G_{122}VYRGNL in LUJV, and a potential antigenic site reported in the N-terminal portion of LASV NP (RKSKRND; [50]), corresponding to R_{55}KDKRND in LUJV (Figure 4).

The GPC precursor (52.3 kDa, pI = 9.0) is cotranslationally cleaved into the long, stable signal peptide and the mature glycoproteins G1 and G2 [51–54]. Based on analogy to LASV [55] and LCMV [56], signalase would be predicted to cleave between D_{58} and S_{59} in LUJV. However, aspartate and arginine

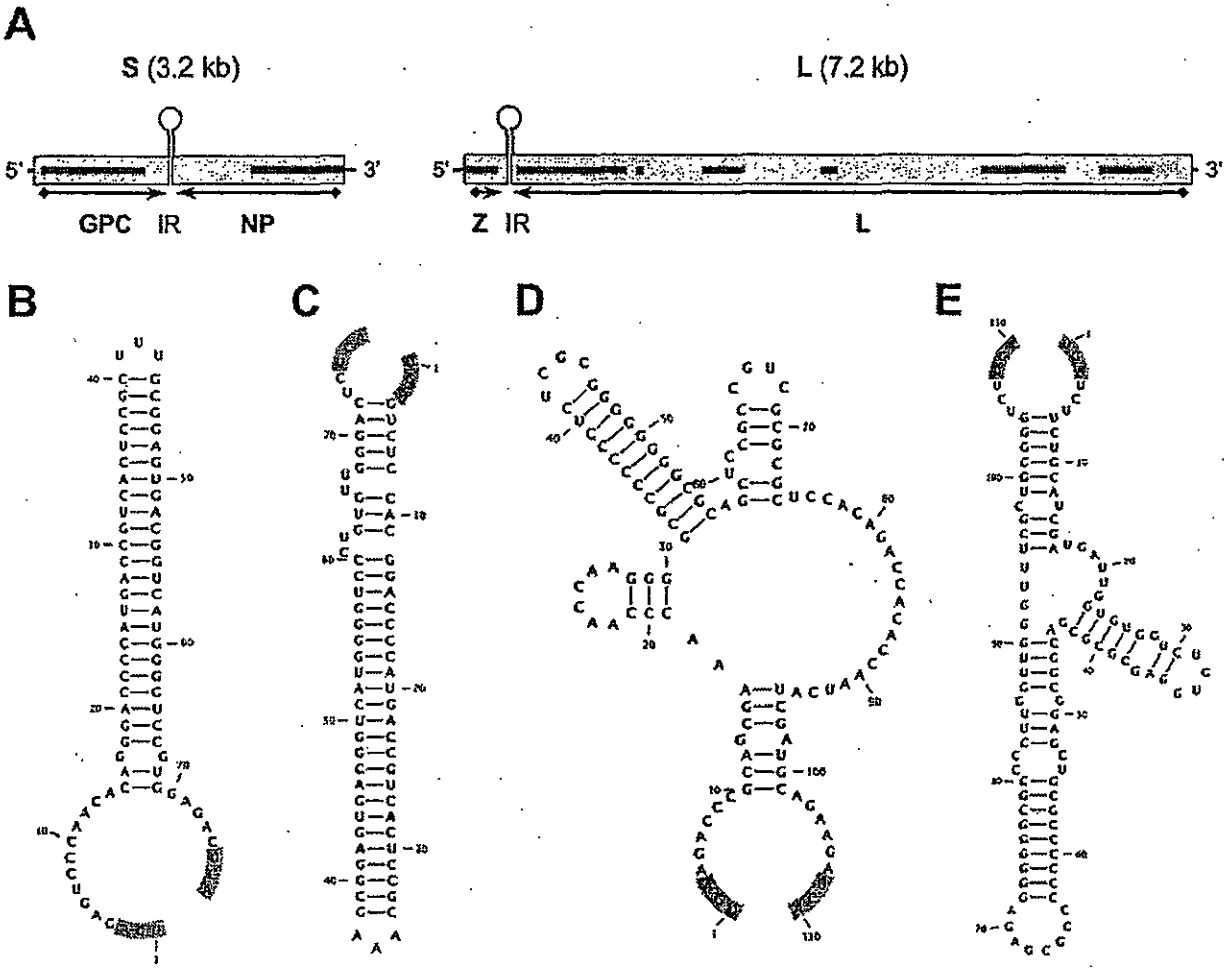

Figure 2. LUJV genome organization and potential secondary structure of intergenic regions. Open reading frames (ORF) for the glycoprotein precursor GPC, the nucleoprotein NP, the matrix protein analog Z, and the polymerase L, and their orientation are indicated (A); blue bars represent sequences obtained by pyrosequencing from clinical samples. Secondary structure predictions of intergenic regions (IR) for S (B, C) and L segment sequence (D, E) in genomic (B, D) and antigenomic orientation (C, E) were analyzed by mfold; shading indicates the respective termination codon (opal, position 1), and its reverse-complement, respectively.
doi:10.1371/journal.ppat.1000455.g002

residues in the −1 and −3 positions, respectively, violate the (−3,−1)-rule [57]; thus, cleavage may occur between S_{59} and S_{60} as predicted by the SignalP algorithm. The putative 59 aa signal peptide of LUJV displays a conserved G_2, implicated in myristoylation in JUNV [58], however, it is followed in LUJV by a non-standard valine residue in position +4, resembling non-standard glycine residues found in Oliveros virus (OLVV [59]) and Latino virus (LATV; http://www2.ncid.cdc.gov/arbocat/catalog-listing.asp?VirusID = 263&SI = 1). Conservation is also observed for aa residues P_{12} (except Amapari virus; AMAV [60]), E_{17} [61](except Pirital virus; PIRV [62]), and N_{20} in hydrophobic domain 1, as well as I_{32}KGVFNLYK$_{40}$SG, identified as a CTL epitope in LCMV WE (I_{32}KAVYNFATCG; [63]) (Figure 4).

Analogous to other arenaviruses, SKI-1/S1P cleavage C-terminal of $RKLM_{221}$ is predicted to separate mature G1 (162 aa, 18.9 kDa, pI = 6.4) from G2 (233 aa, 26.8 kDa, pI = 9.5) [52,53,64]. G2 appears overall well conserved, including the strictly conserved cysteine residues: 6 in the luminal domain, and 3 in the cytoplasmic tail that are included in a conserved zinc finger motif reported in JUNV [65] (Figure 4). G2 contains 6 potential glycosylation sites, including 2 strictly conserved sites, 2 semi-conserved sites N_{335} (absent in LCMVs and Dandenong virus; DANV [19]) and N_{352} (absent in LATV), and 2 unique sites in the predicted cytoplasmic tail (Figure 4). G1 is poorly conserved among arenaviruses [16], and G1 of LUJV is no exception, being highly divergent from the G1 of the other arenaviruses, and shorter than that of other arenaviruses. LUJV G1 contains 6 potential glycosylation sites in positions comparable to other arenaviruses, including a conserved site N_{93}HS (Figure 4), which is shifted by one aa in a motif that otherwise aligns well with OW arenaviruses and NW arenavirus clade A and C viruses. There is no discernable homology to other arenavirus G1 sequences that would point to usage of one of the two identified arenavirus receptors; Alpha-dystroglycan (α-DG) [66] that binds OW arenaviruses LASV and LCMV, and NW clade C viruses OLVV and LATV [67], or transferrin receptor 1 (TfR1) that binds pathogenic NW arenaviruses JUNV, MACV, GTOV, and SABV [68] (Figure S2).