

Thermostability of Vesicular Stomatitis Virus-Based Vaccines

by

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Dedication

This thesis is dedicated to my wife and children for their unwavering support towards the success of my thesis.

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Abstract

Lyophilization maximises vaccines' stability, shelf life; maintains chemical or biological functions; enables easier transportation and storage compared to a cold chain. Success of vesicular stomatitis virus (VSV) based vaccines has been highlighted by its increased adoption by pharmaceutical companies and most importantly, the recently approved vaccine for high consequence Ebola virus (EBOV). Lujo virus (LUJV) and Lassa virus (LASV), both arenaviruses, are still without licensed vaccines, however, researchers are exploring VSV-vectored vaccines. Many of these highly pathogenic viruses are endemic in tropical regions like Africa where slight breach in cold chain may affect stability of vaccines. It was established that slight changes in cold chain conditions do not affect efficacy of VSV-based vaccines, however, it is imperative to optimise thermostability of VSV-based vaccines using lyophilization. The primary hypothesis of this study is that lyophilized VSV-LASV and VSV-EBOV vaccines exposed to sub-optimal temperature will retain infectivity in cell culture and protect against LASV challenge in inbred Hartley guinea pigs. Different combinations of commercially available stabilisers were used to assess the thermostability of VSV-based vaccines at 4, 21 and 37 °C of storage temperatures. Lyophilized VSV-LASV and VSV-EBOV were recoverable in Vero cells, the recovery titer showed an inverse relationship with exposed temperature and duration of storage of 1, 7, 35 and 90 days. Furthermore, lyophilized VSV-LASV affords 100% protection and sterilising immunity against LASV infection in inbred Hartley guinea pigs.

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1.0 **Introduction**

1.1 **Arenaviruses**

1.1.1 **Taxonomy of arenavirus**

The *Arenaviridae* family consists of four genera; Antennavirus, Hartmanvirus, Mammarenavirus, and Reptarenavirus based on the animals they infect. Antennaviruses infect fish, Hartman viruses infect snakes, Mammarenaviruses infect mammals and Reptarenaviruses also infect snakes (Radoshitzky et al, 2019). Virus species in these genera were assigned based on antigenic properties and sequences of Nucleoprotein (NP) (Kuhn et. al, 2019). Antennaviruses, the newest genus, is made up of Wēnlíng frogfish 1 and 2 (WIFAV-1/2) (Shi et al, 2018), typically isolated from the actinopterygian fish. The Hartmanivirus genus is made up of Andere Heimat virus 1, Big electron-dense squares virus 1, Old-schoolhouse virus 1, and Old-schoolhouse virus 2 (Hepojoki et al, 2018). Reptarenavirus genus consist of 5 species (Boa Av NL B3 virus, CAS virus, Collierville virus, Golden Gate virus and University of Helsinki virus). (Stenglein et al, 2012) while the mammarenavirus genus has 39 species, the largest of the family. They are also divided into New World and Old-World arenaviruses based on genetic and geographical differences except for the Lymphocytic choriomeningitis virus (LCMV) that is found in multiple locations (Kuhn et. al, 2019). The Old-World arenaviruses include arenaviruses found in Africa, including ubiquitous LCMV while New World arenaviruses include viruses indigenous to the Americas (Gonzalez, Emonet, Lamballerie and Charrel, 2007; Huang et al, 2015). The Old-World arenaviruses are a monophyletic group, an attribute shared by 3 or 4 New World arenavirus groups (Albariño et al, 1996; Bowen, Peters and Nichol, 1997; Bowen et al, 2000). Lujo virus (LUJV), a virus discovered in Zambia in 2008, is more closely related to Old World viruses with few traits of New World sequences in its glycoprotein (GP) gene (Brisse et al, 2009). New World arenaviruses are sub-

categorized into three or four phylogenetic groups, A, B, C, and sometimes D (Bowen, Peters, and Nichol, 1997). Allpahuayo virus (ALLV), Flexal virus (FLEV), Parana virus (PARV), Pichinde virus (PICV), and Pirital virus (PIRV) from South America belong to Group A (Bowen et al, 2000). Group B is made up of Chapare virus (CHPV), Guanarito virus (GTOV) (Bowen et al, 2000), Junin virus (JUNV), Machupo virus (MACV), Sabia virus (SABV), non-pathogenic Tacaribe virus (TCRV), Cupixi virus (CPXV), and Amapari virus (AMAV). Latino virus (LATV) and Oliveros virus (OLVV) belong to Group C (Peters and Nichol, 1997; Bowen et al, 2000). Group D is made up of arenaviruses with divergent phylogenetic attributes such as the GP precursor and NP genes of Whitewater Arroyo virus (WWAV), Bear Canyon virus (BCNV), and Tamiami virus (TAMV) (all from North America), resulting from evolution (Charrel et al, 2002). While NP sequencing of these 3 viruses revealed a close resemblance to New-World Group A viruses, GP precursor analyses revealed a resemblance to New World Group B viruses (Charrel, de Lamballerie, and Fulhorst, 2001; Charrel et al, 2002).

1.1.2 Virion Structure

Based on the latest ICTV report (Kuhn et al, 2019), all arenaviruses share one or more of the following properties:

- a. Enveloped spherical or pleomorphic virions.
- b. Segmented, single-stranded, ambisense RNA genome without polyadenylated tracts at the 3'-termini.
- c. Nucleotide sequences that could form one or more hairpin configurations within non-coding intergenic regions (IGRs) of genomic segments.

- d. Genomic 5'- and 3'-end sequence complementarities.
- e. Capped but not polyadenylated virus mRNAs; and
- f. Induction of a persistent and frequently asymptomatic infection in reservoir hosts, in which chronic viremia and/or viruria occur.

Mammarenavirus has a bi-segmented genome; large (L), 7.3 kb, and small (S), 3.5 kb segments (Steven et al, 2018). L encodes two proteins L and Z. The L protein is the chief orchestrator of transcription and replication via RNA-dependent RNA polymerase (RdRp) and endonuclease domain (Brisse and Ly, 2019). The protein Z is known as the matrix RING-finger protein. The S segment encodes the precursor of GP that cleaves post-translationally into GP-1, GP-2, and NP. The S and L segment genes are separated by a noncoding region that can form more than one hairpin configuration. At each extremity, the 5' and 3' untranslated terminal sequences of RNA segments contain a reverse complementary sequence of 19 nucleotides. Arenaviruses have nucleocapsid antigens. Arenaviruses can be distinguished by neutralization assays, which depend on the specificity of epitopes found in the enveloped GP (Brouillette, Phillips, Ayithan, and Maury, 2013). The NP suppresses innate immunity (Meyer and Ly, 2016; Pythoud et al, 2012; Stott, Strecker, and Foster, 2020) and protects RNA by forming a polymer layer. Arenaviruses are typically spherical or pleomorphic with an average diameter between 50 and 300 nm.

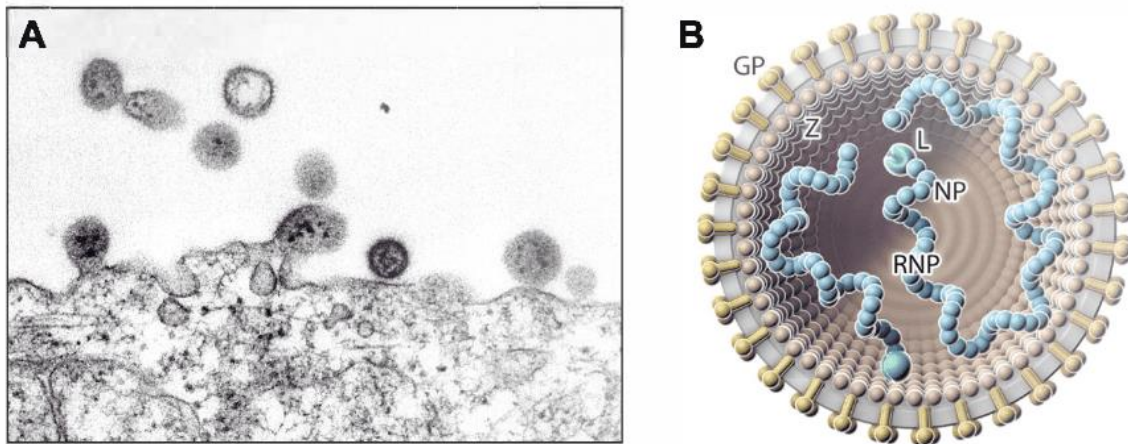


Figure 1. A is an electron image of an arenavirus. B is a schematic image of an arenavirus showing RNP, Z, L, and NP (Radoshitzky et al, 2019).

1.1.3 **Geographic Distribution of Arenaviruses**

Arenaviruses were initially thought to have originated from Asia with the earliest rodents and eventually found their way to Europe, Africa, and the Americas with the spread of rodents (Bowen, Peters, and Nichol, 1997; Brisse and Ly, 2019) but arenaviruses and rodent hosts' phylogenetic trees almost rarely share similarities (Brisse et al, 2009). Many arenaviruses have been associated with human disease with different clinical manifestations. Machupo, Lassa, Junin, Guanarito, Chapare, and Sabia viruses are known to cause severe hemorrhagic fever, in West Africa, Argentina, Bolivia, Venezuela, and Brazil (Brisse et al, 2009; Charrel and de Lamballerie, 2009). LCMV infection can result in acute neurological disease, congenital malformations, and fatal outcomes in organ transplantation recipients and immunocompromised patients (Centre For Disease Control, 2005; Schafer et al, 2014). FLEV and TCRV have caused febrile illness in laboratory workers (Centre For Disease Control, 2005). WWAV may have been associated with three fatal cases of infection in California (Lele, 2003). LCMV is the only arenavirus that has been found to circulate worldwide (Welsh, 2008). Recent studies have isolated LCMV from ticks in Ukraine and China, suggesting Tick may be a vector for LCMV (Zhang, 2018; Takagi, 2017).

1.1.4 **Incidence of Arenavirus Diseases**

In contrast to other hemorrhagic fever viruses like Ebola virus (EBOV), arenavirus transmissions to humans have occurred essentially through human interactions with rodents, a natural reservoir host of these viruses, as observed during the 2017–2018 LASV outbreak in Nigeria. However, human-to-human transmission has been noted for outbreaks, as seen in 2014, where strains across larger geographical areas were found to cluster closely together (Brisse et al, 2019). Viruses are

shed into the environment through saliva, urine, or feces of infected rodent hosts and are transmitted to humans through bites and scratches, aerosol, ingestion, inhalation, or open surface on the skin from human to human, however, some arenaviruses can be transmitted vertically to offspring during pregnancy (Vitullo and Merani, 1990). Furthermore, laboratory transmission of arenaviruses has been reported (Vitullo and Merani, 1990). Typical symptoms from arenavirus infection include headache, myalgia, sore throat, apathy, malaise, photophobia, confusion, and other mental difficulties. Severe disease may cause elevated serum glutamic oxaloacetic transaminase (SGOT), hepatitis, leukopenia, thrombocytopenia, shock syndrome, hemorrhagic manifestations, dehydration, and cardiovascular collapse. Other neurological complications have been established for arenaviral infection (Happi, 2019; Kerber, 2015).

1.1.5 **Pathogenesis of Arenavirus Disease**

Monocytic cells such as alveolar macrophages and dendritic cells, usually not activated upon arenaviral cellular entry, are the earliest targets. The lack of activation of monocytic cells suggests them to be viral “reservoirs” early on during infection, where the virus enters and spreads when these cells enter draining lymph nodes (Buchmeier, de la Torre, and Peters, 2018). Mammarenavirus infection in rodents and humans has indicated that the optimum functioning of both innate and T-cell-mediated immunity is essential to limiting viral growth, symptoms, and mortality rates (Meyer and Ly, 2016; Randall and Goodbourn, 2008). Innate immunity is activated shortly following host recognition of viral pathogen-associated molecular pattern (PAMP). Pattern recognition receptor proteins (PRRs), such as the RIG-I-like receptors (RLRs), RIG-I, and MDA5 activated by PAMPs result in cytokine expression, activation, and recruitment of innate immune

cells such as macrophages, neutrophils, and dendritic cells (Marcus and Sekellick, 1977; Marcus, Svitlik and Sekellick, 1983). RIG-I and MDA5 bind to PAMP dsRNAs in the cytosol during viral replication and alter their conformations from a closed to an open and activated state (Gack, 2014). Activated RLRs (RIG-I and MDA5) activate mitochondrial antiviral signaling protein (MAVS), which results in the translocation of transcription factors (interferon regulatory factors (IRF) IRF3, IRF7, and nuclear factor kappa B (NF- κ B)) into the nucleus of the cells to activate type 1 interferons (IFN α and IFN β) and interferon-stimulated genes (ISG) (Ikushima, Negishi and Taniguchi, 2013; Oeckinghaus and Ghosh, 2009). Old World arenavirus infections such as LASV produce reduced levels of IFN1 and proinflammatory cytokines in patients with moderate to severe symptoms. On the other hand, New World arenaviruses produce higher levels of IFN1 and cytokines with a pro-inflammatory response primarily for inhibition of viral replication (McLay, Liang and Ly, 2014; Kolokoltsova et al, 2010; Huang et al, 2014; Kolokoltsova et al, 2014; Peña et al, 2018) (Figure 5). The NP of arenaviruses has been found to inhibit the RIGI/MDA5 pathway (Zhou et al, 2010), modulated by the exoribonuclease domain of NP (Shao et al, 2018). The exoribonuclease domain of NP also modulates inhibition of the cellular protein kinase IKK ϵ (Pythoud et al, 2012) and other enzymes in the IRF pathway (Hastie et al, 2012; Rodrigo et al, 2012). Inhibition of IFN1 by NPs has been demonstrated in species that are non-pathogenic to humans such as PICV (Martínez-Sobrido et al, 2007). Additionally, the arenavirus Z protein especially those pathogenic in humans, has been recently found to inhibit the IFN1 pathway by direct interaction with RIG-I to inhibit IFN1 expression (Fan, Brieze, and Lipkin, 2010; Xing, Ly, and Liang, 2015).

1.1.6 **LASSA VIRUS**

LASV, the causative agent of the most severe arenaviral disease, was first described in 1969 (Buckley and Casals, 1970). It was named after Lassa town in Nigeria, the place of the first epidemic. It has been responsible for an estimated 300,000 cases and 5,000 mortality per year in endemic regions of West Africa (Charrel, de Lamballerie, and Emonet, 2008; Günther and Lenz, 2004). A Lassa fever outbreak in Nigeria in 2018 recorded 431 laboratory-confirmed cases involving 37 healthcare workers with a total 25% fatality rate (Nigeria Centre for Disease Control, 2018; Nigeria Relief Web, 2018; Roberts, 2018). Since its discovery, cases have been reported from Liberia, Sierra Leone, Senegal, Guinea, Mali, Burkina Faso, Côte d'Ivoire, Ghana, and Gambia (Moriyama, Loy and Robb-Smith, 2011). Cases of LASV infection have been reported in Europe, Asia, and America, from travelers returning from endemic locations (Mylne et al, 2015).

1.1.7 **LUJO VIRUS**

LUJV emerged from Lusaka in Zambia, and Johannesburg in South Africa, in 2008 (Paweska et al, 2009). The word Lujo was coined from the first two letters of Lusaka and Johannesburg, the two cities where the virus emerged (National Institute for Communicable Diseases, 2008). The index case was a travel agent in Lusaka with febrile illness accompanied by diarrhea, facial edema, severe respiratory distress, impaired renal function, severe thrombocytopenia, and elevated liver enzymes. She was flown by air from Lusaka to Johannesburg and died twelve days after the first appearance of presumed symptoms (Paweska et al, 2009). There was nosocomial transmission to four healthcare workers, three of them died giving a case fatality rate (CFR) of 80% (National Institute for Communicable Diseases, 2008). On top of the high CFR, LUJV's novelty, emergence,

high human-to-human transmission, and close resemblance to highly pathogenic arenaviruses necessitate the development of vaccines for emergency preparedness for potential future outbreaks.

1.1.8 **Arenaviral Treatments and Prophylaxis**

Numerous therapeutic options have been explored for arenaviruses to date. Ribavirin, a nucleoside analog, has been therapeutically adopted against many pathogenic viruses including arenaviruses. Ribavirin afforded 100% protection against JUNV in rhesus macaques (McKee et al, 1988). Many lead prophylactic agents failed to provide therapy in one or more arenaviral species. For example, gamma-irradiated LASV particles generated high humoral responses in non-human primates (NHP) (McCormick et al, 1992). Additionally, CD4⁺ T cell responses from some vaccinated arenaviruses provided cross-protection; however, some lacked protective efficacy against lethal viral challenge (Kotturi et al, 2010).

Immune serum treatment prevented morbidity and mortality in marmosets and guinea pigs infected with JUNV (Kenyon, Green, Eddy, and Peters, 1986). 6 days post-infection administration of serum to JUNV-infected marmosets yielded a 75% survival rate and generated neutralizing antibodies in all the treated animals but one.

Immune serum treatment also provided therapeutic efficacy against LASV (McCormick et al, 1986). Other antiviral agents have been used against arenaviruses; Favipiravir (T-705) (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a pyrazine derivative that RNA polymerases of RNA viruses that provide complete protection for guinea pigs infected with PICV (Mendenhall et al,

2011). Furthermore, T-705 has been shown to disrupt the early/intermediate stages of replication of JUNV, MACV, and GTOV (Mendenhall et al, 2011).

Replication-deficient adenovirus type 5 vector (cIFN- α) alone or with ribavirin has also shown activity against PICV in infected hamsters (Gowen et al, 2005 and 2006). cIFN- α is marketed as DEF201 for single nasal administration for pre-and post-exposure prophylaxis against PICV (Gowen et al, 2011). Phenothiazine antipsychotic drugs have shown varying degrees of inhibitory activity against arenaviruses replication. While trifluoperazine and chlorpromazine showed strong inhibitory activity against JUNV and TACV replication in cell culture. However, there was less activity against PICV suggesting a lack of broad-spectrum inhibitory activity (Candurra et al, 1996). Isoflavone has been widely used for its antioxidant properties (Andres, Donovan, and Kuhlenschmidt, 2009). They are organic compounds isolated from different plant species. Isoflavones, especially genistein, have been reported to affect viral binding, entry, replication, translation, and formation of viral envelope proteins (Vela et al, 2008; Stantchev et al, 2007). Genistein showed activity in PIRV-infected animals (Vela et al, 2008).

Vaccines are the bedrock of the prevention and management of infectious disease outbreaks. It has been used to defuse pandemic and epidemic risks. An ideal prospective vaccine for arenaviruses should provide rapid protection from a single dose, be productive if given post-exposure to arenaviruses, and be cross-protective across the filoviridae family.

For years, there have been limited options concerning the commercial availability and universality of arenaviral vaccines. JUNV vaccine (Candid #1), sourced from a human viral isolate is the only clinically used arenaviral vaccine. It is a second-generation live-attenuated Candid #1 virus vaccine that was produced and registered to offer protection to seasonal field workers against rodent-borne JUNV in Argentina. It was not approved by the US FDA partly due to the potential

reversal to pathogenic form (Parodi et al, 1958). Candid #1 vaccine was developed through traditional attenuation concepts where the pathogenic XJ strain of JUNVV was passaged in cell culture, mice, and guinea pigs (Enria and Oro, 2002). The development of vaccines with activity against other pathogenic Mammarenaviruses has proven to be a daunting task. The significance of the IFN β pathway and subsequent T-cell response in modulating arenaviral infection in mice has been established (Huang et al, 2014, Oestereich et al, 2016; Meyer and Ly, 2016; Yun et al, 2012; Rieger, Merkler and Günther, 2013; Hofer et al, 2012; Pien et al, 2012). However, arenaviruses' immunosuppression limits T-cell responses from vaccines (Schildknecht et al, 2008; Pannetier et al, 2011; Baize et al, 2009; McLay, Liang, and Ly, 2014). Arenaviruses establish infection by targeting macrophage and dendritic cells. Infected macrophage and dendritic cells are not activated and do not present increased levels of activating markers such as TNF α , IL-1 β , CD 40, CD 54, CD80, DC86, 4IL-6, IL10, or IL-12 (Yun et al, 2012 and Baize et al, 2004). Alternative ways of developing arenaviral vaccines are through recombinant viruses. LASV vaccine candidates expressing LASV GP or NP genes in vectors such as vesicular stomatitis virus (VSV) (Garbutt et al, 2004; Geisbert et al, 2005), vaccinia virus (Clegg and Lloyd, 1987; Auperin et al, 1988), MOPV (Carnec et al, 2018), Yellow fever virus 17D (Bredenbeek et al, 2006; Jiang et al, 2011), alphavirus replicons (Pushko et al, 2001; Wang et al, 2018) and inactivated rabies virus (Abreu-Mota et al, 2018) have shown promising protective efficacies against LASV infection in various animal models.

1.2 **Filoviruses**

1.2.1 **Taxonomy**

Filoviruses, members of the Filoviridae, have eight established genera. Half of the sixteen described filoviruses cause pathogenic diseases in humans (Kuhn et. al, 2019). Filoviruses are negative-stranded, non-segmented RNA viruses of the order *Mononegavirales*, with distinct genetics, morphology, and biology between members (Feldmann et al, 2003). Currently, the members of the *Filoviridae* family are further classified into the genera: *Thamnovirus*, *Orthomarburgvirus* (MARV), *Tapjoevirus*, *Oblavirus*, *Dianlovirus*, *Cuevavirus*, *Striavirus*, and *Orthoebolavirus* (EBOV). *Loebvirus*, the newest member proposed in July 2023, is awaiting ICTV evaluation (Biedenkopf et al, 2023). The EBOV genus comprises six virus species (*Zaire ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus*, *Reston ebolavirus*, *Bundibugyo ebolavirus*, and *Bombali ebolavirus*), MARV genus is made up of MARV and Ravn virus (RAVV); *Cuevavirus*, *Striavirus* and *Thamnovirus* genera each has one virus specie, *Lloviu cuevavirus*, *Xīlǎng striavirus*, *Huangjiāo*, and *thamnovirus*, respectively (Kuhn et al, 2019).

The disease caused by the family has been given an umbrella name of Filovirus disease (FVD) by The International Classification of Diseases (ICD-11) (Moriyama, Loy, and Robb-Smith, 2011). There was a need for subcategories to accommodate filovirus diseases that have not yet been associated with a particular filovirus or are caused by yet-to-be-discovered novel filoviruses. The panel recommended Ebola disease (EBOD) and Marburg disease (MARD) subcategories to the filovirus parent entry. There was further subcategorization into Ebola virus disease (EVD) and Marburg virus disease (MVD) (Moriyama, Loy and Robb-Smith, 2011). EVD and MVD were restricted to diseases caused by agents belonging to a species, for example, EBOV of Zaire ebolavirus; MARV and RAVV, both of Marburg virus. Bundibugyo virus disease (BVD) and

Sudan virus disease (SVD) were additional EBOD subcategories introduced to include the remaining pathogenic filoviruses that have caused more than one registered human infection (Kuhn et al, 2019). Subcategories for EBOD or MARD without typical clinical manifestations such as atypical Ebola/Marburg disease have been proposed. Other subcategories are EVD or MVD of EBOD or MARD patients infected with EBOV or Marburg viruses not covered by BDV, EVD, SVD, or MVD (such as FVD caused by Tai Forest virus infection); and unspecified viruses suspected to cause EVD or MVD in the absence virus distinction (Kuhn et al, 2019).

1.2.2 **Virion Structure**

Filoviruses are enveloped virions with linear, non-segmented, negative-sense RNA genomes (15–19 kb). Filovirus genomes are organized in the order 3'-N-P-M-(G)-L-5' with additional genes (Negredo et. al, 2011; Shi et. al, 2018). Filovirus genomes have conserved extragenic sequences at the extreme 3'- (leader) and 5'- (trailer) ends, and short sections of these extragenic sequences are complementary. Genomes of non-fish filoviruses are flanked by conserved transcriptional initiation and termination (polyadenylation) sites bearing the highly conserved pentamer 3'-UAAUU-5'. Genes are often divided by non-conserved intergenic sequences or overlaps. The bulk of filovirus genes possess relatively long 3'- and 5'-noncoding regions (Kuhn et al, 2008; Brauburger, Deflubé and Mühlberger, 2015).

1.2.3 **Geographic Distribution of Filoviruses**

EBOV, TAFV, and MARV are endemic in Western Africa. BDBV, EBOV, MARV are endemic in Middle Africa, BDBV, MARV, SUDV, RAVV are endemic in Eastern Africa, MARV is endemic in Southern Africa, Huangjiao virus (HUJV), Reston virus (RESTV), Xilang virus (XILV) are endemic to Eastern Asia, and RESTV is endemic to South-eastern Asia. LLOV is endemic in Eastern and Southern Europe. Bats are host reservoirs of EBOV, MARV, RAVV, and likely Lioviu virus (LLOV). Actinopterygian fish is possibly the reservoir host of HUJV and XILV, and domestic pigs for RESTV (Shi et al, 2018; Kemenesi et al, 2016; Amman et al, 2017).

1.2.4 **Incidence of Disease**

The EVD outbreak of 2013-2016 that began in West Africa and later spread to other continents remains the deadliest filovirus disease outbreak to date, with 11,310 mortalities out of 28, 616 cases (World Health Organization, 2016). During these two and half years, there was a simultaneous outbreak of EVD in the E'quateur Province of the Democratic Republic of the Congo (DRC), with 49 deaths out of 69 cases (World Health Organization, 2018). A Series of cases and deaths were subsequently reported in different regions of DRC (Kobinger et al, 2009; World Health Organization, 2018). A total of 12 cases with 6 deaths were reported during the twelfth EVD outbreak in North Kivu, DRC in February 2021 (World Health Organization, 2021).

Marburg virus was first detected in 1967 during concurrent outbreaks in Marburg and Frankfurt in Germany; and in Belgrade, Serbia (Kobinger et al, 2009). Most deadly outbreaks were reported in DRC (1998 – 2000), with 128 deaths recorded from 153 cases, and in Angola (2004 – 2005), 227 deaths were reported from 252 cases (Languon and Quaye, 2019).

1.2.5 General Overview of Filovirus

The risk of filovirus disease outbreaks is high in developing countries due to low levels of disease outbreak detection, emergency preparedness, and response systems, detection of disease, availability of basic healthcare, contact tracing, quarantine, and isolation procedures (Madhav et al, 2018). Since 1967, the majority of filovirus disease outbreaks have originated or been reported in Africa. Hemorrhagic fever is a common symptom of filovirus disease with a fatality of about 90% depending on the virus (Martina et al, 2009). Filovirus disease transmissions are primarily zoonotic, from reservoir host to human, however, human-to-human transmission has been reported to have led to a new outbreak (Mbala-Kingebeni *et al.* (2021). Six of the 12 filoviruses have been known to cause disease in humans, with EBOV and MARV considered to be the most virulent members of the *Filoviridae* family (Languon and Quaye, 2021). Filovirus disease outbreaks have impacted the health and economic budgets of affected countries. Due to poor health infrastructures, African countries affected by filovirus disease outbreaks have usually struggled with disease containment, as funds (plus donor funds), resources, and personnel for regular healthcare are either misappropriated or depleted (Piot, 2014; Madhav et al, 2018). During the 2013–2016 EVD outbreak, the misappropriation of resources in affected regions was thought to increase mortality from regular health problems such as diarrhea, HIV/AIDS, and tuberculosis (Piot, 2014). In Sierra Leone, the EVD epidemic led to a reversal in previous advancements in the health sector (child mortality, maternal mortality, HIV/AIDS, malaria) due to the cessation in operation of some health facilities and the diversion of resources to combatting the EVD epidemic (Rohan and McKay, 2020).

Filovirus disease outbreaks had negative implications on education through school closures or students' reluctance to attend school to prevent exposure. In Guinea, Liberia, and Sierra Leone,

approximately 5 million children missed school during the 2013–2016 EVD from school closures (Amara, Tommy, and Kamara, 2015), thus increasing the number of out-of-school children in these regions (Brahmbhatt and Dutta, 2008). Education budgets were repeatedly diverted to contain outbreaks. The filovirus pandemic remains a public health, social, economic, and political threat.

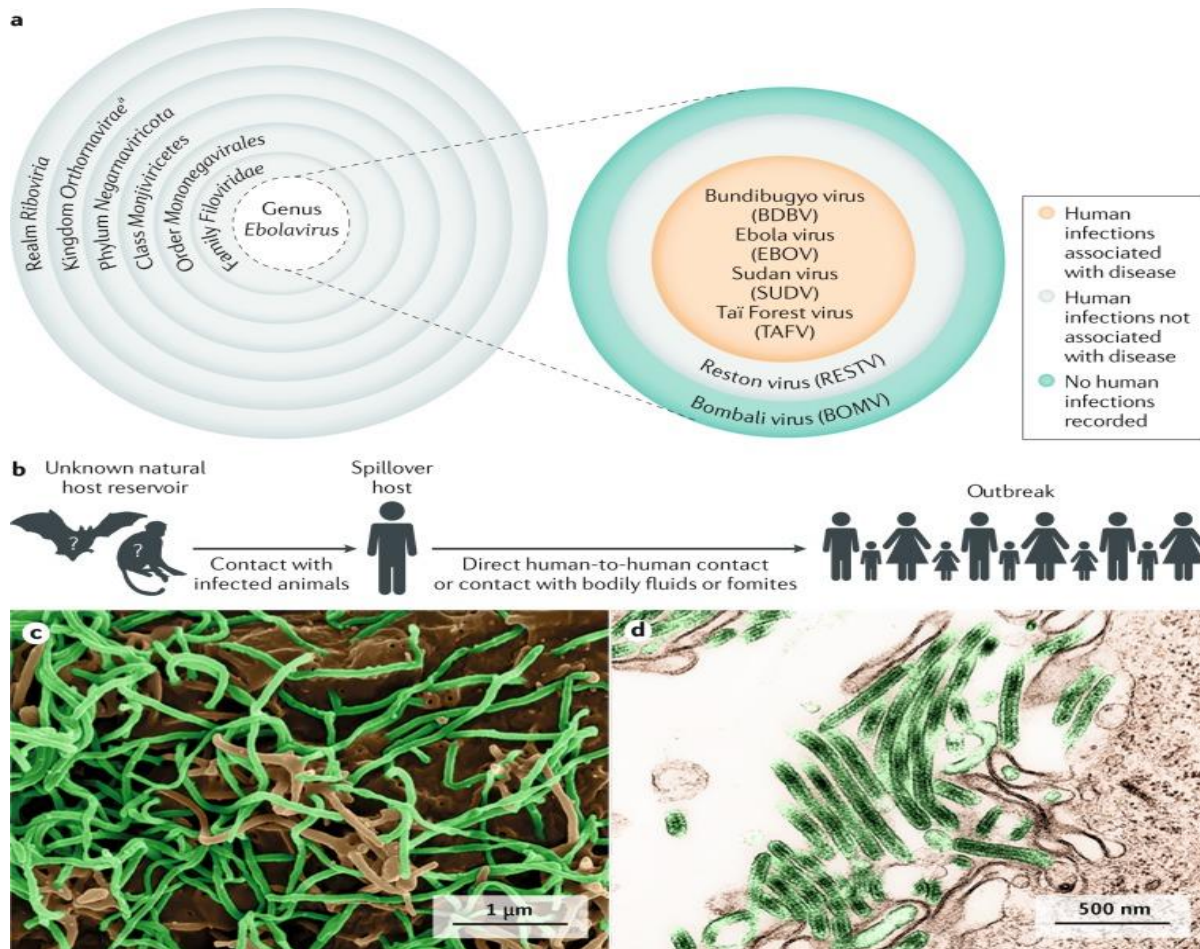


Figure 2. Filovirus taxonomy and EBOV transmission.

a. Taxonomy of EBOV genus showing those found to be human pathogens and those not yet related to human infections. b. Reservoir host(s) of EBOV have not been identified yet. It is generally speculated to be a zoonotic infection, often linked to Bats. c. Scanning electron microscope (SEM) image of budding EBOV particles (green, from givet cells). d. Transmission electron microscope (TEM) image of budding EBOV particles (green, from givet cells). (Jacob, Crozier and Fischer, et al, 2020).

1.2.6

EBOLA VIRUS

EBOV is the causative agent of EVD. EVD is usually a severe and fatal disease with outbreaks culminating from probable zoonotic transmission, leading to human-to-human transmission through direct contact or infected bodily fluids or fomites. Early symptoms (within 3 days of disease onset) of EVD are nonspecific febrile illness (such as headache, myalgia, anorexia, arthralgia, malaise, and rash) progressing to severe gastrointestinal outcomes (nausea, vomiting, and severe diarrhea). Abdominal discomfort, fatigue, diarrhea, anorexia, fever, vomiting, and myalgia dominated the most common clinical manifestations during the 2013–2016 Western African EVD outbreak (Xu et al, 2016; Fowler et al, 2014; Barry et al, 2015; Qin et al, 2015; Chertow et al, 2014; Dietz et al, 2015). As viral load surges, EVD severity rises accordingly. As the day passes by, hypotension (often from dehydration), vascular leakage, and rhabdomyolysis may present (Cournac, et al, 2016). During the late phase of EVD (second week of symptom onset), tissue hypoperfusion, encephalopathy, vascular leakage, and dysregulated inflammation culminate in multiple organ dysfunction syndrome and/or damage. Anuria, Oliguria, and abnormal electrolyte levels are evidence of renal injury (Cournac et al, 2016). In children, the symptoms, presentation, and timeline vary from adults, with shorter onset of symptoms and higher CFR. Also, children have a higher tendency to present with fever and a lower likelihood of arthritis, abdominal pain, dyspnoea, myalgia, and hiccups during the early phase of disease manifestation.

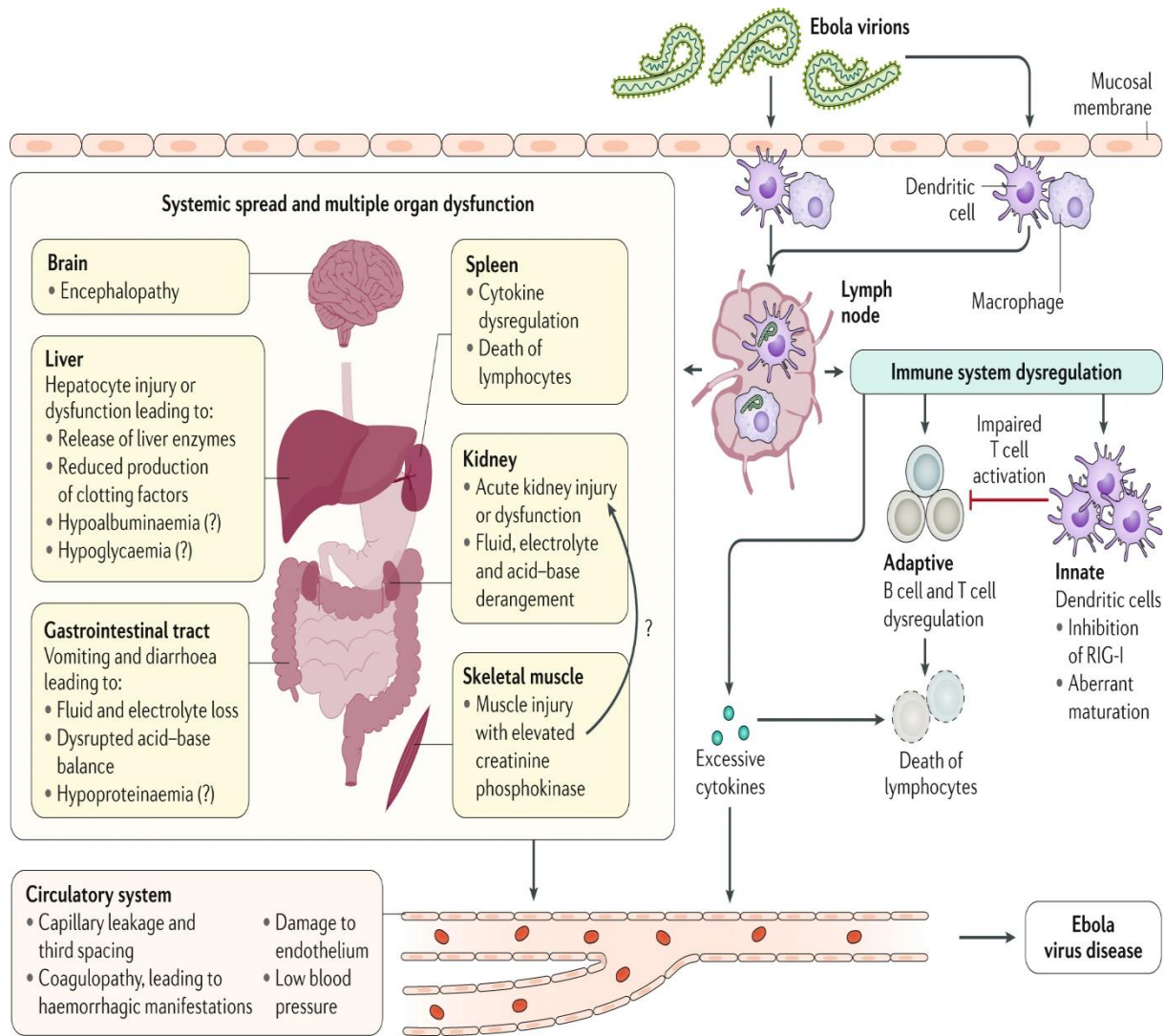


Figure 3. Pathogenesis of EVD. EBOV causes multiple bodily organ dysfunction including the immune system. Circulatory system dysfunction often results in fatality (Jacob, Crozier, and Fischer et al, 2020).

1.2.7 **Licensed Vaccines for EBOV**

EBOV GP 1 and 2 remain the most targeted immunogen for vaccine design and development against EBOV. In 2018, live-attenuated and recombinant vesicular stomatitis virus (rVSVΔG-ZEBOV-GP) was used for ring vaccination in the EVD outbreak in DRC. It was approved by the US Food and Drug Administration and the European Commission for vaccination of contacts infected with EBOV (primary ring) and contacts of infected individuals (secondary ring). As of 26 January 2020, 276,520 people in the eastern DRC had already received this vaccine candidate (World Health Organization, 2020). Other vaccine candidates were evaluated during the West Africa EVD outbreak as shown in Table 1 (Restrepo, 2018; European Commission, 2019). WHO reported the efficacy of the rVSVΔG-ZEBOV-GP as 97.5% (95% CI 95.8 - 98.5%) (World Health Organization, 2019). Although, the estimation was performed in the absence of a placebo-controlled group. Furthermore, in the Équateur Province of DRC (2018), ring vaccination with the rVSVΔG-ZEBOV-GP vaccine resulted in a 70.4% decline in geographical area risk and 70.1% in EBOV infection risk. Many healthcare workers received the rVSVΔG-ZEBOV-GP following exposure to patients infected with EBOV. In August 2019, healthcare workers were enrolled in a clinical trial evaluating the safety and immunogenicity of the rAd26 ZEBOV-GP–MVA-BN-Filo vaccine in Uganda (European Commission, 2019). It was equally employed in the pre-exposure vaccination in the Goma area of DRC on the 14th day of November 2019. This pre-exposure immunization serves as a complement to the rVSVΔG-ZEBOV-GP ring vaccination approach currently in place and has been administered to 1300 people as of December 10, 2019 (World Health Organization, 2020). In October 2019, conditional marketing authorization was approved for rVSVΔG-ZEBOV-GP by the European Medicines Agency granted the candidate vaccine (European Commission, 2019). Two months later, the European Commission and the US Food

and Drug Administration approved the rVSVΔG-ZEBOV-GP for prophylaxis of EVD (US Food and Drug Administration, 2019).

S/N	VACCINE CANDIDATES	DESIGN	CLINICAL TRIAL PHASE
1	rAd26 ZEBOV-GP and MVA-BN-Filo	Replication defective human Ad 26 vector expressing EBOV GP1,2; replication incompetent MVA-BN expressing EBOV, SUDV, MARV and TAFV GP1,2	3
2	rVSVΔG-ZEBOV-GP	Replication competent rVSIV expressing EBOV GP1,2 in place of VSIV G	3

Table 1. EBOV vaccine candidates in clinical trials. Multiple EBOV vaccine candidates with different designs are currently undergoing clinical trials.

1.3 Vesicular Stomatitis Virus

1.3.1 VSV as Agricultural Pathogen

Vesicular stomatitis (VS) is a disease of livestock caused by VSV, otherwise known as Vesiculoviruses, including vesicular stomatitis New Jersey virus (VSNJV) and vesicular stomatitis Indiana virus (VSIV). The disease is endemic in Central America, Mexico, the United States (US), and northern regions of South America (Rodríguez, 2002). In the US, the frequently affected animals are mules, horses, donkeys, cattle, and camelids (Vesicular Stomatitis Outbreak Situation Reports, 2006). Animals infected with VSV may develop vesicular (blister appearance) lesions typically found on the nostrils, lips, muzzle, oral mucosa, tongue, ears, teats, udder, sheath, ventral abdomen, and coronary bands (Pelzel-McCluskey and Winter, 2020). The symptoms are self-limiting in most affected animals, disappearing within a few weeks except for animals with underlying health conditions (Pelzel-McCluskey and Winter, 2020).

VSV can also be transmitted to humans via direct contact with infectious lesions in livestock, and typically manifests in headache, fever, fatigue, and myalgia, resolving within a week (Pelzel-McCluskey and Winter, 2020). Transmission of VSV to livestock may be through direct contact with virus-carrying fluids from lesions, saliva, and insect bites or indirectly from virus-contaminated water, feed, feeders, tack, lick tubs, or veterinary supplies (Duarte et al, 2008; Pelzel-McCluskey, 2023; Hanson, 1952).

Species from Simuliidae (black flies), Psychodidae (sand flies), and Culicoides (biting midges) are suspected vectors of VSV (Schmidtman et al, 1999; Tesh et al, 1987; Schnitzlein and Reichmann, 1985).

Management of a VS pandemic can be achieved by prompt isolation of livestock presenting with symptoms of VS from clinically healthy animals to reduce the spread of the virus via direct contact, and aggressive vector control.

1.3.2 Vesicular Stomatitis Virus as Vaccine Backbone

Recombinant viral platforms have been increasingly employed for heterologous antigen expression *in vivo* and present propitious vectors for novel vaccines against pathogens ravaging humans such as EBOV, Tuberculosis, Human immunodeficiency virus (HIV), and Malaria (Johnson, 2013; Excler, 2010; Parks, Picker and King, 2013; Limbach and Richie, 2019; Xing and Lichty, 2006; Mullard, 2014). VSV is a negative sense RNA virus (Family; Rhabdoviridae). VSV has garnered increased relevance when it comes to designing vaccine vectors against microbial pathogens (John and Rose, 2015) due to its desirable properties such as its growth to high titer in mammalian cell lines (Lawson et al, 1995), an inherent ability to tolerate additional transcriptional units, and strong cellular and humoral responses. On top of these, VSV has low seroprevalence in humans (possibly due to its limited ability to infect humans) and the attenuated competent forms of VSV are safe and highly immunogenic in many clinical trials (Fuchs et al, 2015). VSV glycoprotein (VSV G) has been strongly linked to the immunogenicity and pathogenicity of VSV (Martinez et al, 2003; Feldmann et al, 2007). VSV devoid of VSV G demonstrated reduced or no pathogenicity (Publicover, 2004). Pathogenicity is dependent on the ability of VSV to replicate in its host. VSV's host entry and fusion with endocytic vesicles is mediated by the ability of VSV G to bind cellular receptors (Jeetendra, 2003). This vital role of VSV G in mediating cellular receptor binding and membrane fusion makes it a prime attenuation target for recombinant VSV (rVSV)

vaccines. Substitution of the VSV G gene with a foreign gene generates rVSVΔG pseudotypes with altered cell tropism and target for cell-mediated and humoral immunity (Jones et al, 2005; Marzi et al, 2011).

1.3.3 **VSV-EBOV (rVSVΔG-ZEBOV-GP)**

The EVD became a public health concern due to its unpredictable high CFR. The efficacy of numerous vaccine platforms has been substantiated by their prophylactic efficacy in human and non-human primates. ERVEBO® (rVSVΔG-ZEBOV-GP, produced by Merck), ZABDENO® (Ad26.ZEBOV from Johnson and Johnson), and cAd3-EBOZ (from GlaxoSmithKline) are vaccines used for prophylaxis against the EVD. ERVEBO®, the only licensed VSV-based vaccine as of today, is effective for the prevention of EVD caused by Zaire ebolavirus in individuals 18 years of age and older only (Centre for Disease Control, 2021). ERVEBO® is a replication-competent, live attenuated rVSV vaccine manufactured by Merck Serono. ERVEBO® is potent until the expiration indicated on the vial if stored at -80°C to -60°C (-112°F to -76°F). However, if unable to maintain -80°C to -60°C storage, thawed vaccine can be stored at 2°C to 8°C (35.6°F to 46.4°F) for up to 14 days (Centre for Disease Control, 2021). Attenuation of VSV vaccine vector by incorporating EBOV GP into a VSV vector from which the G-open reading frame (ORF) was removed (VSV-ΔG). This concept produced the rVSVΔG-ZEBOV-GP vaccine, with rhabdovirus morphology and surface expression of EBOV GP (Suder et al, 2018). Following its development, efficacy was demonstrated in cynomolgus macaques, the standard model for filovirus infection (Jones, 2005; Geisbert et al, 2008; Marzi et al, 2013). Clinical trials were carried out in Guinea, Sierra Leone, USA, Spain, and Canada (Restrepo et al, 2015 and 2017; Widdowson et al, 2016;

Halperin et al, 2017). The three vaccines currently used for prophylaxis against EVD require cold chain storage to maintain efficacy. It is important to examine the impact of break-in cold chain storage on lyophilized EBOV vaccines.

1.3.4 **VSV-LASV (rVSVΔG-LASV-GP)**

Vaccine against LASV is long overdue due to its high incidence, clade heterogeneity, and geographical distribution. Recently, the Coalition for Epidemic Preparedness Innovations (CEPI) earmarked considerable research funds to develop, manufacture, and eventually stockpile rVSVΔG-LASV-GP (Profectus Biosciences CEPI awards, 2018). A lot of research efforts geared toward the development of rVSVΔG-LASV-GP have yielded fascinating results but there is still no clinically approved vaccine against LASV. The possible versatility of rVSVΔG-LASV-GP has been boosted by the full protection afforded inbred guinea pigs and NHPs from homologous and heterologous isolates challenge; Pinneo (Nigeria), LASV Z-132 (Liberia), and Soromba-R (from Mali), 28 days post-immunization (dpi). The vaccine was thought to induce both cellular and humoral immunity, without significant differences in the blood chemistry or hematology of the animals' pre-and post-LASV challenge. This shows that a single vaccination may afford protection against all strains of LASV during a pandemic. Furthermore, there was protection for inbred guinea pigs challenged with a highly genetically divergent Pinneo strain while control animals developed clinical disease (Safronetz et al, 2015). A single dose of rVSVΔG-LASV-GP induced rapid and long-term protection from LASV infection in an outbred guinea pig (Stein et al, 2019). rVSVΔG-LASV-GP vaccine stored under sub-optimal temperature for 1 week retained protective efficacy in female Hartley guinea pigs (Stein et al, 2019).

1.4 Lyophilization of Vaccines

1.4.1 Overview

Vaccines have been estimated to prevent approximately 3 million deaths annually (World Health Organization, 2020). However, a sizable proportion of the global population dies from vaccine-preventable diseases annually (World Health Organization, 2023). Vaccines often require transportation from manufacturing plants to storage sites or countries where they are administered to the masses to prevent diseases. One of the methods of ensuring the thermostability of vaccines is lyophilization often referred to as freeze-drying. Lyophilization is the process of removal of water from the vaccine samples at low temperatures and under a vacuum to improve its stability, and shelf life while retaining potency, in addition to ease of transportation and storage when compared to a cold chain (Lale *et al*, 2011). Lyophilization is achieved when there is stability and co-existence of the three phases of water (solid, liquid, and gas), a phenomenon known as the triple point of water. Lyophilization is the most frequently devised means of drying vaccines, with lyophilized vaccines making one-fourth of all vaccines approved by the United States Food and Drug Administration (Preston and Randolph, 2021). About 75% of licensed bacterial and viral live attenuated vaccines (LAV) are lyophilized (Chen and Kristensen, 2009). Overall, unlike liquid vaccines, lyophilized LAV maintains potency and viability at freezing temperatures (World Health Organization, 2006). Licensed lyophilized vaccines retain potency for weeks to months when stored above room temperature. However, they are to be refrigerated (Path, 2012; GlaxoSmithKline, 2020).

1.4.2 **Lyophilization Process**

The process of lyophilization usually involves three steps: freezing, primary drying, and secondary drying. The freezing steps involve the removal of water from vaccines in the form of ice. Slow freezing of samples can generate large ice crystals that are destructive to cells and other sensitive materials. However, rapid cooling steps during the freezing step reduce the formation of ice crystals. Primary drying converts ice to gas in a process that removes approximately 95% of the moisture content and turns the vaccine into powder (sublimation). The pressure of the surrounding environment is maintained below that of the triple point of water during this step. Secondary drying removes residual water by desorption. Increased temperature disrupts the interaction between ice and water molecules, facilitating the removal of any residual water. Optimal glass transition temperature is an important factor in the stability of lyophilized vaccines. On top of lyophilization, the addition of excipients like nonreducing disaccharides (trehalose or sucrose) influences the potency of vaccines by direct stabilization of antigens through the replacement of hydrogen bonds previously provided by water molecules. These disaccharides further trap antigens in glassy and shiny states to enhance stability (Ohtake and Wang, 2011; Crowe, Reid, and Crowe, 1996).

1.4.3 **Advantages and Disadvantages of Lyophilization**

Lyophilization is essentially an aseptic process, avoiding the terminal sterilization procedure (Hansen et al, 2015). Lyophilization of vaccines results in a final product in a powdery form, lower in weight and volume compared to the original vaccine and readily reconstituted with sterile water or an appropriate buffer before use.

There is a possibility of antigenic degradation during lyophilization from ice crystal formation (from rapid freezing), cold denaturation, and cryoconcentration that can cause a change in sample phase, pH (promotes pH-dependent deamination), and hydrogen bonds within water molecules (Rexroad et al, 2002; Authelin et al, 2020; Bhatnagar, Pikal and Bogner, 2008). Cryoprotectants can be added to vaccines before initiating lyophilization to mitigate this and maintain stability during lyophilization. Once reconstituted, vaccines must be used promptly or preserved under cold chains to mitigate water-mediated degradation (World Health Organization, 2006). There is continued adoption of lyophilization for the preservation of the efficacy of antigens based on the number of currently marketed lyophilized vaccines (Table 2).



Figure 4. Image of Labconco Lyophilizer used for lyophilization of vaccine samples in CL2 of NML, Winnipeg (<https://labconco.com>).

S/N	Vaccines	Properties	Reference
1	Dengue (Dengvaxia)	Stable for 1 month at 25 °C, 7 days at 37 °C	Guy et al, 2011
2	<i>Haemophilus influenzae</i> (Hiberix)	Stable for 7 days at 37 °C	GlaxoSmithKline, 2020.
3	<i>Japanese encephalitis</i> (JE-Vax)	Stable for 28 weeks at 22 °C, 1 month at 37 °C	Path, 2012.
4	Measles, Mumps, and Rubella	Stable for 7 days at 37 °C	WHO, 2006.
5	Rabies (RabAvert)	Stable for 3 months at 37 °C	Path, 2012.
6	Meningococcal (MenAfrVac)	Stable for 6 months at 25 °C, >4 weeks at 40 °C	Path, 2012.
7	Rotavirus (Rotasiil)	Stable for 36 months at 25 °C, 8 months at 37–40 °C	Naik et al, 2017.
8	Typhoid	Stable for 7 days at 25 °C, 24 h at 37 °C	Cryz, O. et al, 1996.
9	Varicella (Varivax)	Stable for 2 years at –15 °C, ~4 months at +15 °C	Liska et al, 2007.

Table 2. Licensed lyophilized vaccines. The table shows the stability properties of available lyophilized vaccines currently licensed for use.

1.4 **Study Rationale, hypotheses, and objectives.**

Pathogens with high a CFR like LUJV (80%) are a public health concern. Although LUJV has not re-emerged since the index case in 2008, it is pertinent to develop a licensed vaccine that can be readily used for mass vaccination when there is re-emergence. This emergency preparedness will save a lot of lives compared to commencing vaccine development during the pathogen pandemic as seen with COVID-19. Following the development of the LUJV vaccine, the study will extend to the preservation of the LUJV vaccine and other similar pathogens.

Liquid formulations of vaccines are subject to physical and chemical degradation processes during storage (Preston and Randolph, 2021). Many lyophilized vaccines are marketed for global use as illustrated in Table 2. However, several thermolabile vaccines are often required to be transported across long distances over hours to days. This is frequently within and outside the borders of developing countries that have limited budgets available for storage facilities and maintenance. The prohibitive cost of maintaining the potency of vaccines from manufacture to administration remains a major obstacle to the extension of global vaccination programs according to the World Health Organization (WHO) (Wolfson et al, 2008). Transportation of thermolabile vaccines in cold chain storage (at low temperatures) is one of the means of maintaining the potency of vaccines.

Even with appropriate cold chain storage facilities, there are still challenges to overcome during storage; the aqueous environment can destabilize vaccine antigens through physical (aggregation, denaturation, unfolding, and precipitation) and chemical (deamidation, hydrolysis, and oxidation) processes (Carpenter, Arakawa and Crowe, 1991). Design and development of thermostable formulations for vaccines could alleviate challenges encountered in the vaccine supply chain (Lee et al, 2012).

Lassa virus, LUJV, and EBOV are pathogens of public health concern in Africa. African countries have low health sector budgets due to limited resources and often require cost-effective means of providing prophylaxis and therapy for emerging and re-emerging pathogens. Targeted lyophilization of vaccines against pathogens endemic in temperate, developing countries may be an effective means of reducing vaccine wastage. This study will assess the impact of lyophilization on some vaccines against pathogens endemic in West African countries where cold chain storage may be difficult to maintain.

Hypothesis

The central hypothesis of this study is that lyophilized VSV-based vaccines will retain thermal stability and infectivity at elevated temperatures and maintain efficacy to protect against LASV infection in guinea pigs.

Objectives

1. To develop a rVSVΔG-LUJV-GP vaccine.
2. To determine the thermostability of lyophilized rVSVΔG-LASV-GP, V rVSVΔG-EBOV-GP, and rVSVΔG-LUJV-GP vaccines by assessing the median tissue culture infectious dose (TCID₅₀) in Vero cells.
3. To evaluate the protective efficacy of lyophilized rVSVΔG-LASV-GP vaccine in inbred Hartley guinea pigs.

2.0 MATERIALS AND METHOD

2.1 Animal cell lines

Baby hamster kidney T7 (BHK-T7), Human embryonic kidney (HEK 293T), and African Green Monkey Kidney (VERO E6) cells were obtained from ATCC (Maryland, USA). These cells were used for transfection of pVSVΔG-LUJV-GP.

Vero E6 and HEK 293T cells were grown in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, Canada) containing 10% fetal bovine serum (FBS) (Wisent Inc, Canada) and 2 mM L-glutamine (Gibco, USA). BHK-T7 cells were grown in minimum essential media (MEM, Gibco, USA).

2.2 Development of rVSVΔG-LUJV-GP vaccine

2.2.1 Design and preparation of rVSVΔG-LUJV-GP

Plasmids expressing wild-type (pLUJV-GPWT) or codon-optimized Lujo virus GP (pLUJV-GPCO) were obtained from Genscript, USA. The sequences of ordered pLUJV-GPCO and pLUJV-GPWT are in Appendix A. Support plasmids expressing LUJV NP, Polymerase (L), Phosphoprotein (P), and GP; T7 polymerase and VSV vector backbone with green fluorescence protein (VSVΔG-GFP) were sourced from Darwyn Kobasa.

2.2.2 Cell transformation

E. coli cells (ATCC, Maryland, USA) were thawed on ice for 30 minutes and diluted into three portions (1:50, 1:100, and 1:1000). 2µl of pLUJV-GPWT or pLUJV-GPCO was added to each of

the *E. coli* dilutions in 1.5ml microcentrifuge tubes and left on ice for 30 mins. The tubes were incubated for 45s to create pores in the cell wall of *E. coli* cells for the uptake of plasmids. The tubes were frozen on ice for 2 minutes to seal the cell wall after the uptake of plasmids. 100µl of the content of each tube was inoculated on LB Miller + 100 µg/mL ampicillin agar plates (Appendix B) and incubated at 37°C for 24 hours to promote bacteria growth.

2.2.3 **Purification, propagation, and confirmation of pLUJV-GP and support plasmids**

A pipette tip was used to streak a big and well-isolated colony from the overnight culture colonies of pLUJV-GPWT or pLUJV-GPCO and used to inoculate 3mL of LB Miller + 100µg/mL ampicillin broth in 12ml tube (Appendix B) to make a starter culture. The starter culture was incubated at 37°C for 8 hours at 250 rpm. A similar procedure was performed for N, L, P, T7, and VSVΔG-GFP support plasmids. 1 ml of the starter cultures was used to inoculate 100 ml of LB Miller + 100µg/mL Ampicillin broth for overnight bacterial growth. The liquid cultures were subjected to centrifugation to obtain bacterial cell pellets. Purification of plasmids was achieved with Qiagen mini-prep kits and protocol (catalog 27106, Toronto, Canada). This protocol is used for the purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB broth. Isolated plasmids were eluted in 20µl Ambion™ Nuclease-Free Water (Fisher Scientific, Ottawa, Canada), and the ssRNA concentration was measured using a Nanodrop™ One Microvolume UV-Vis spectrophotometer (Fisher Scientific, Ottawa, Canada).

Purified plasmids were propagated using a Qiagen maxiprep kit and protocol (catalog 27106, Toronto, Canada). Propagated plasmids were aliquoted and preserved at -200 C. 50µl of plasmids were used for sequencing at a concentration of 150 ng/µl, using LUJV-specific primers. The

concentrations of propagated pLUJV-GPCO and pLUJV-GPWT were 378.60 and 856.6, respectively.

2.2.4 Confirmation of pLUJV-GP Plasmids

2.2.4.1 RT-PCR of pLUJV-GP

Dilutions of pLUJV-GP samples were prepared in 1.5ml tubes, the components were mixed as detailed in Appendix C, in a 96-well plate. The plate was covered with adhesive film and transferred to a spinner for 20s to eliminate bubbles in the well. The plate was transferred to Quant Studio 3 (Applied Biosystems, Thermofisher, Ottawa, Canada) and ran according to protocol conditions as illustrated in Appendix C. The PCR products were prepared for agarose gel electrophoresis. Briefly, 1% agar gel was prepared by dissolving 0.5g of Agarose powder (Difco™, USA) in a 50 ml Tris-acetate-EDTA (TAE) buffer. The bottle was heated in a microwave for a few minutes with intermittent swirling until a clear solution was formed. The bottle was cooled by running tap water on it. 5µl SYBR safe (Invitrogen™, Ontario, Canada, lot 2361834) was added to stain the DNA samples. The gel was poured into the agarose chamber and left for 30 minutes for the gel to set. 4µl of loading dye (6X) was added to the samples in 1.5ml tubes to allow visual tracking of DNA migration during electrophoresis. 5µl of DNA ladder was added to two wells in the gel while samples were spaced in between the wells. The gel was run on an electrophoresis machine (Enduro, USA) at 120V for 60 minutes. The gel was transferred to the UV chamber (AlphaImager HP, USA) for visualization of the expected band size. The whole amplification process lasted for two hours; reverse transcription was for 30 minutes at 500 C and 15 minutes at 95° C. RT PCR (40X) ran for 15s at 940 C and 15 minutes at 95° C.

2.2.5 **Infusion Cloning**

2.2.5.1 **PCR amplification and purification of pLUJV-GP**

PCR amplification was achieved using the Takara Bio kit and protocol. Briefly, the PCR components were prepared and mixed according to the manufacturer's manual. The tubes were transferred to Quant Studio 3 (Applied Biosystems, Thermofisher, Ottawa, Canada) and ran as illustrated in Appendix C. Purification of PCR products started with the preparation of 1% agar gel by dissolving 0.5g of Agarose powder (Difco™, USA) in a 50 ml Tris-acetate-EDTA (TAE) buffer. The bottle was subjected to heat in a microwave for a few minutes with intermittent swirling until a clear solution was formed. The bottle was cooled by running tap water on it. 5µl SYBR safe (Invitrogen™, Ontario, Canada, lot 2361834) was added to stain the DNA samples. The gel was poured into the agarose chamber and left for 30 minutes for the gel to set. 4µl of loading dye (6X) was added to the samples in 1.5ml tubes to allow visual tracking of DNA migration during electrophoresis. 5µl of DNA ladder was added to two of the wells on the gel while samples were spaced in between the wells. The gel was run on an electrophoresis machine (Enduro, USA) at 120V for 60 minutes. The gel was transferred to the UV chamber (Alpha Imager HP, USA) for the visualization of bands. The gel was transferred into a safe imager and covered with an orange lid of the imager. 70% alcohol was used to clean the scalpel before cutting a rectangular band out of the gel. Trimmed gels were transferred to a 1.5ml tube. The weight of the trimmed gels was determined to ascertain the volume of buffer needed for gel purification. Qiagen gel extraction kit and protocol (catalog No. 28706) was used for the purification of amplified PCR products. The purified DNA samples were eluted in 30µl Ambion™ Nuclease-Free Water (Fisher Scientific, Ottawa, Canada), and the plasmid concentration was measured with Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Fisher Scientific, Ottawa, Canada).

2.2.5.2 **Linearization and purification of pLUJV-GP**

The plasmids were mixed according to the manufacturer's specification, in a 1.5ml tube. The tube was incubated for 1 hour at 37°C. It was immediately placed on ice to stop further digestion until ready for purification. For the purification procedure, 1% agar gel was prepared by dissolving 0.5g of Agarose powder (Difco™, USA) in a 50 ml Tris-acetate-EDTA (TAE) buffer. The bottle was subjected to heat in a microwave for a few minutes with intermittent swirling until a clear solution was formed. The bottle was cooled by running tap water on it. 5µl SYBR safe (Invitrogen™, Ontario, Canada, lot 2361834) was added to stain the vector samples. The gel was poured into the agarose chamber and left for 30 minutes for the gel to set. 10µl of loading dye (6X) was added to the samples in 1.5ml tubes to allow visual tracking of DNA migration during electrophoresis. 5µl of DNA ladder was added to the first or last well on the gel while 20 µl of pLUJV-GP samples were added to the remaining wells. The gel was run on a gel electrophoresis system (Enduro, USA) at 120V for 60 minutes. The gel was transferred to the UV chamber (Alpha Imager HP, USA) for the visualization of bands. Qiagen QIAEX gel extraction kit and protocol (catalog No. 20051) was used for the purification of vector products. The plasmid DNA samples were eluted in 30µl Ambion™ Nuclease-Free Water (Fisher Scientific, Ottawa, Canada), and the concentration was measured with Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Fisher Scientific, Ottawa, Canada).

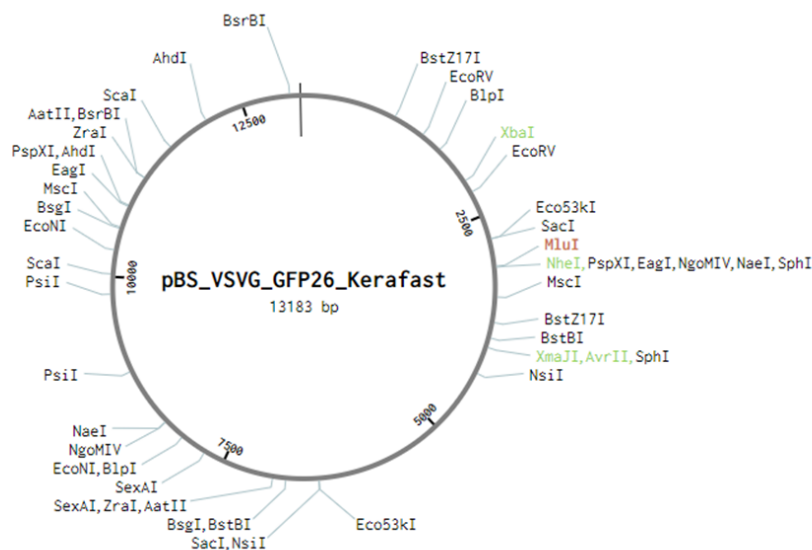


Figure 5. Map of pBSVSV-GFP showing different parts of the vector where restriction endonucleases can be cut for linearization.

2.2.5.3 Ligation

15ng of pLUJV-GP and 75ng of pBSVSVG-GFP were mixed in 2:1 ratio in 1.5ml tubes (10- μ L reactions). The tubes were incubated for 15 min at 50°C. The tubes were immediately placed on ice until ready for transformation.

2.2.5.4 Transformation, purification, and Propagation of recombinant plasmids

Tubes of *E. coli* Cells were thawed on ice. 5% v/v of recombinant plasmids were added to 100 μ L of the competent cells in a 1.5ml tube. The tubes were gently tapped to mix. The tubes were

incubated for 5 min on ice. 50µl of the contents of the tubes were spread onto pre-warmed LB Amp broth and incubated overnight at 37°C.

For the purification step, a pipette tip was used to streak a big and well-isolated colony from the overnight colonies of recombinant plasmids. The pipette was used to inoculate 3mL of LB Miller + 100µg/mL Ampicillin broth in a 12ml tube (Appendix B) to make a starter culture. The starter culture was incubated at 37°C for 8 hours at 250 rpm. 3ml of the starter cultures were used to inoculate 150ml of LB Miller + 100µg/mL ampicillin broth for overnight bacterial growth in a 500ml conical flask. The liquid cultures were subjected to centrifugation to obtain bacterial cell pellets. Purification of plasmids was achieved with the Qiagen midi-plus kit and protocol (cat. No 12943, Toronto, Canada). Isolated plasmids were eluted in 20µl Ambion™ Nuclease-Free Water (Fisher Scientific, Ottawa, Canada). The DNA concentration of recombinant plasmids was measured with the Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Fisher Scientific, Ottawa, Canada). Purified recombinant plasmids were propagated using the Qiagen maxiprep kit and protocol (catalog 27106, Toronto, Canada). Propagated plasmids were aliquoted and preserved at -200 C. 50µl of plasmids were used for sequencing at a concentration of 150 ng/µl, using LUJV-specific primers.

2.2.6 Transfection of recombinant plasmids

Barely 48 hours before transfection, BHK cells were split 1:5 into four flasks and incubated overnight at 37°C. Cells were grown to 70-80% confluence, 24 hours before transfection when BHK cells were split 1:4 were seeded into 11, 6-well plates and incubated overnight at 37°C. The cells were 80-90% confluent before transfection and actively dividing.

On transfection day, the LT1 reagent (Mirus Bio, USA) was warmed to room temperature and vortexed gently. 250µl of Opti-MEM was added to sterile 1.5ml tubes. 10µl of LT1 was added under the surface of Opti-MEM. The tubes were incubated for 5 minutes at room temperature. Plasmids were added sequentially as presented following the manufacturer's instruction. The content of each tube was mixed by pipetting gently before incubating at room temperature for 30 minutes. While incubating TransIT-LT1 reagent: DNA complex transfection mix. Cells were washed with 2ml/well Opti-MEM. 1mL fresh Opti-MEM/well was added to the plates. The transfection mix was added to the cells drop by drop in different areas of each well. The plates were rocked back and forth and side to side. The plates were incubated in the CL2 lab at 37°C for 48 hours.

On day two, 80-90% confluent Vero E6 cells were washed with PBS. Blind passage of supernatants from the transfection plates to washed Vero E6 cells was performed. Vero E6 plates were incubated at 37°C and monitored daily for GFP and/ or syncytia formation. The media was replaced 24 hours post-transfection with 1% FBS MEM. The supernatant was harvested approximately 120 hours post-blind passage and stored in a -80°C freezer. The negative control plate followed similar transfection steps and reagents except removal of G plasmid while mock control consisted of only LT1 reagent and Opti-MEM. Other transfection attempts were made by varying the amount of reaction mix components and the addition of new components to the reaction mix, including T7 polymerase.

Plasmids	ng/μl	ng	μl/reaction
VSV-N	534.0	400	0.75
VSV-P	498.2	350	0.70
VSV-L	490.4	250	0.51
VSV-G	239.9	1000	4.20
T7 polymerase	1067.9	1000	0.94

Table 3. Concentrations of support plasmids for transfection attempts Table.

2.2.7 **Transfection of T7 polymerase**

Barely 48 hours before T7 transfection, BHK cells were split 1:5 into four flasks and incubated overnight at 37°C. 24 hours to transfection and 70-80% confluence, 2.5ml of a 1:4 split of BHK was seeded into a 6-well plate and incubated overnight at 37°C. The cells were 80-90% confluent and actively dividing before transfection.

On transfection day, the LT1 reagent (Mirus Bio, USA) was warmed to room temperature and vortexed gently. 250µl of Opti-MEM was added to sterile 1.5ml tubes. 10µl of LT1 was added under the surface of Opti-MEM. The tubes were incubated for 5 minutes at room temperature. T7 plasmid express GFP was added. The tube was tapped gently before incubating at room temperature for 30 minutes. While incubating TransIT-LT1 reagent: DNA complex transfection mix, cells were washed with 2ml/well Opti-MEM. 1mL fresh Opti-MEM/well was added to the plates. The transfection mix was added to the cells drop by drop in different areas of each well. The plate was rocked back and forth and sideways. The plate was incubated in the VSV room at 37°C for 48 hours. On day two, 80-90% confluent Vero E6 cells were washed with PBS. The supernatant from the transfection plate was blind passaged into washed Vero E6 cells. Vero E6 plate was incubated at 37°C and monitored daily for GFP and/ or syncytia formation. The media was replaced 24 hours post-transfection with 1% FBS MEM. Mock control consisted of only LT1 reagent and Opti-MEM mix.

2.2.8 **Recombinant pPCAGGS-LUJV-GP (rPCAGGS) cloning**

2.2.8.1 **PCR amplification of pLUJV-GP**

The PCR components were sequentially mixed in 1.5ml tubes manufacturer's instruction. The tubes were transferred to Quant Studio 3 (Applied Biosystems, Thermofisher, Ottawa, Canada) and ran according to the manufacturer's instructions. The PCR products were analyzed by agarose gel electrophoresis and purified using a Qiagen purification kit and protocol (cat no. 28106).

2.2.8.2 **Fast digest of pPCAAGS**

The components were mixed gently, in a sequential manner following the manufacturer's instructions. The tubes were incubated at 37°C in a water bath for 5 min. The tubes were kept on ice until the ligation step. Purification of rPCAAGS-LUJV-GP was done using a Qiagen Kit Cat. No. 20051, eluted in 20µl nuclease-free water.

2.2.8.3 **Ligation reaction**

The reaction components were mixed in two 1.5ml tubes as illustrated in Appendix A. The tubes were incubated at 50°C for 15 minutes and placed on ice until transformation.

10ul reaction volume. pLUJV-GP:pPCAGGS, 2:1 molar ratio

2.2.8.4 Transformation, purification, propagation, and transfection of rPCAGGS-LUJV-GP plasmids

Tubes of *E. coli* Cells were thawed on ice. 5% v/v of recombinant plasmids were added to 100µl of the competent cells in a 1.5ml tube. The tubes were gently tapped to mix. The tubes were incubated for 5min on ice. 50µl of the contents of the tubes were spread onto pre-warmed LB Amp broth and incubated overnight at 37°C.

For the purification step, a pipette tip was used to streak a big and well-isolated colony from the overnight colonies of recombinant plasmids. The pipette was used to inoculate 3mL of LB Miller + 100µg/mL ampicillin broth in a 12ml tube (Appendix B) to make a starter culture. The starter culture was incubated at 37°C for 8 hours at 250 rpm. 3ml of the starter cultures were used to inoculate 150ml of LB Miller + 100µg/mL Ampicillin broth for overnight bacterial growth in a 500ml conical flask. The liquid cultures were subjected to centrifugation to obtain bacterial cell pellets. Purification of plasmids was achieved with a Qiagen midi-plus kit and protocol (cat. No 12943, Toronto, Canada). Isolated plasmids were eluted in 20µl Ambion™ Nuclease-Free Water (Fisher Scientific, Ottawa, Canada). The DNA concentration of recombinant plasmids was measured with the Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Fisher Scientific, Ottawa, Canada). Purified recombinant plasmids were propagated using a Qiagen maxiprep kit and protocol (catalog 27106, Toronto, Canada). Propagated plasmids were aliquoted and preserved at -200 C. 50µl of plasmids were used for sequencing at a concentration of 150 ng/µl, using LUJV-specific primers.

48 hours before transfection, BHK cells were split 1:5 into four flasks and incubated overnight at 37°C. 24 hours to transfection and 80% confluence, 2.5ml of 1:4 split of BHK was seeded into 3

6-well plates and incubated overnight at 37°C. The cells were 80-90% confluent before transfection and actively dividing.

On the transfection day, the LT1 reagent (USA) was warmed to room temperature and vortexed gently. A 250µl of Opti-MEM was added to sterile 1.5ml tubes. A 10µl of LT1 was added under the surface of Opti-MEM. The tubes were incubated for 5 minutes at room temperature. The content of each tube was mixed by pipetting gently before incubating at room temperature for 30 minutes. While incubating TransIT-LT1 reagent: DNA complex transfection mix, cells were washed with 2ml/well Opti-MEM. 1mL fresh Opti-MEM/well was added to the plates. The transfection mix was added to the cells drop by drop in different areas of each well. The plates were rocked back and forth and side to side. The plates were incubated in the VSV room at 37°C for 48 hours. On day two, 80-90% confluent Vero E6 cells were washed with PBS. Supernatants from transfection plates were blind passage into washed Vero E6 cells. Vero E6 plates were incubated at 37°C and monitored daily for GFP and/ or syncytia formation. The media was replaced 24 hours post-transfection with 1% FBS MEM. The negative control plate followed similar transfection steps and reagents except removal of G plasmid while mock control consisted of only LT1 reagent and Opti-MEM mix. The supernatant was collected and stored at -80°C before the western blot.

Barely 48 hours before transfection, BHK cells were split 1:5 into four flasks and incubated overnight at 37°C. 24 hours to transfection and 80% confluence, 2.5ml of 1:4 split of BHK was seeded into 3 6-well plates and incubated overnight at 37°C. The cells were 80-90% confluent before transfection and actively dividing.

On transfection day, the LT1 reagent (USA) was warmed to room temperature and vortexed gently. A 250µl of Opti-MEM was added to sterile 1.5ml tubes. 10µl of LT1 was added under the surface

of Opti-MEM. The tubes were incubated for 5 minutes at room temperature. The content of each tube was mixed by pipetting gently before incubating at room temperature for 30 minutes. While incubating TransIT-LT1 reagent: DNA complex transfection mix, cells were washed with 2ml/well Opti-MEM. 1mL fresh Opti-MEM/well was added to the plates. The transfection mix was added to the cells drop by drop in different areas of each well. The plates were rocked back and forth and side to side. The plates were incubated in the VSV room at 37°C for 48 hours. On day two, 80-90% confluent Vero E6 cells were washed with PBS. Supernatants from transfection plates were blind passage into washed Vero E6 cells. Vero E6 plates were incubated at 37°C and monitored daily for GFP and/ or syncytia formation. The media was replaced 24 hours post-transfection with 1% FBS MEM. The negative control plate followed similar transfection steps and reagents except removal of G plasmid while mock control consisted of only LT1 reagent and Opti-MEM mix. The supernatant was collected and stored at -80°C before the western blot.

2.2.8.5 Western blot

Cells were harvested for western blot analysis by removing culture supernatants and replacing them with 500uL of protein denaturing buffer (Appendix B). The cell lysate was collected and placed directly into a QIA shredder spin column and centrifuged at max speed for 2 min to homogenize the lysate. Confirmation of LUJV-GP expression was done via western blot. In summary, a 20uL reaction was prepared using 4uL of cell supernatant, Bolt™ (4X) LDS sample buffer (Fisher Scientific, Ottawa, ON, Canada), and Ambion™ nuclease-free water (Fisher Scientific, Ottawa, ON, Canada). The reaction was incubated at 95 °C for 10 minutes. Approximately 1.5uL of MagicMark™ XP western protein standard (Fisher Scientific, Ottawa,

ON, Canada) and 10uL of reaction were loaded onto a Bolt™ 4-12% Bis-Tris Plus gel (Fisher Scientific, Ottawa, ON, Canada) and ran at 140V. Protein transfer occurred using the iBlot®2 Dry Blotting System (Fisher Scientific, Ottawa, ON, Canada). The nitrocellulose membrane was incubated with a western blot blocking reagent (Appendix B) for 1 hour at room temperature. This was followed by blocking reagent replacement with 10mL of western blot antibody diluent (Appendix B) with guinea pigs' convalescent serum and incubated overnight. The membrane was washed with western blot wash buffer (Appendix B) and incubated with 10mL of western blot antibody diluent (Appendix B) with a 1:5000 dilution of IRDye 800CW Goat anti-Mouse IgM (0.5mg) (Cedarlane, Burlington, ON, Canada). The membrane was washed a final time and imaged using the Li-Cor® Odyssey Imaging System (LI-COR Inc., Lincoln, NE, USA).

2.3 Lyophilization and thermostability evaluation of rVSVΔG-LASV-GP and rVSVΔG-ZEBOV-GP vaccines

2.3.1 Vaccine virus propagation and storage

rVSVΔG-LASV-GP and rVSVΔG-ZEBOV-GP vaccines were provided by David Safronetz. Vero cells were seeded into a T-150 flask (TP-150 cm²) with an average cell density of 4.5×10^6 cells in DMEM. The seeded cells were infected with rVSVΔG-LASV-GP or rVSVΔG-ZEBOV-GP. Infected cells were incubated at 37 °C and monitored routinely. After 5 days post-infection (dpi), the supernatant was collected when 80–90% of CPE was evident. The collected supernatant was immediately aliquoted and stored in single-use vials at - 80 °C until lyophilization.

2.3.2 **Preparation of stabilizer formulations**

To improve the stability of prepared rVSVΔG-LASV-GP and rVSVΔG-ZEBOV-GP vaccines, four different stabilizer combinations were prepared from 5% Lactalbumin-hydrolysate (L) (Gibco), 10% Sucrose (S) (Sigma-Aldrich, Canada), 5% Trehalose (T) (MilliporeSigma, Canada) and 0.5% Gelatin (G) (MilliporeSigma, Canada). Stabilizer combinations were selected based on stability results from previous studies (51-58). The excipients were prepared in Hank's Balanced Salt Solution (Sigma, USA). The stabilizers were combined as LS, LST, and LSTG. DMEM was used as a control.

Formulation code	Composition	pH
A	LS	6.5-7.0
B	LST	7.0
C	LSTG	7.0
D	DMEM	7.0

Table 4. Stabiliser formulations.

2.3.3 **Lyophilization of virus preparations and reconstitution of lyophilized vaccines**

Lyophilization of vaccines was carried out in an automated triad benchtop freeze-dryer (Figure 4) as outlined in Table 5. 100 µl aliquots of vaccines and 100 µl of stabilizers were added to 4 ml sterile vials (Electron Microscopy Sciences, USA). The vials' stoppers were fitted halfway into the vials before placing the lot in the sample shelf of the automated lyophilizer. A lyophilization program of 16 hr was performed in the lyophilizer using the manufacturer's specification as summarised in Table 5.

Briefly, during the freezing stage of the lyophilization cycle which took almost 4 hr, the lyophilizer's shelf temperature decreased to -40°C and then held at -40°C for 1 hr. The lyophilizer vacuum pressure was maintained at 0.53 mbar during freezing and primary drying but decreased to 0.13 mbar during secondary drying. During the primary drying, the shelf temperature increased to 30°C at a rate of $0.1^{\circ}\text{C}/\text{min}$ and then held for another 0.5 hr for secondary drying to take place. At the end of the cycle, vials were sealed by moving the Stoppering control toward the "LOWER" position on the lyophilizer. This action allowed the diaphragm above the stoppering platen to inflate causing the platen to lower. The plate is lowered until it contacts the vials on the shelf. The rubber stoppers were pressed into the vials to move the vial stoppers from the halfway position to their fully inserted, sealed position. The vials were separated and stored according to the proposed temperature (4, 21, and 37°C) and duration of storage before lyophilization.

Cycle steps.	Duration (hr)	Shelf temperature (°C)	Vacuum pressure (mbar)
Freezing	3.83	-40	0.53
Primary drying	1	-35	0.53
	1	-30	0.53
	1	-25	0.53
	1	-20	0.53
	1	-15	0.53
	1	-10	0.53
	1	-5	0.53
	1	0	0.53
	0.67	5	0.53
	0.33	10	0.53
	0.33	15	0.53
	0.33	20	0.53
	0.33	25	0.53
	0.42	30	0.53
Secondary drying	0.5	30	0.13

Table 5. Automated lyophilization process. The three lyophilization steps of freezing, primary and secondary drying were depicted in the table. The lyophilization conditions for each step were also presented in the table.

2.3.4 **Titration of lyophilized rVSVΔG-LASV-GP and rVSVΔG-ZEBOV-GP using TCID₅₀**

Titration of stock lyophilized rVSVΔG-LASV-GP and rVSVΔG-ZEBOV-GP was performed based on predetermined data. Titration of lyophilized rVSVΔG-LASV-GP or rVSVΔG-ZEBOV-GP was done on days 1, 7, 35, and 90 for lyophilized viruses stored at 4, 21 and 37 °C. Virus infectivity was quantified by estimating the TCID₅₀ in 96-well microplates (Costar®, USA). Briefly, 100µl of Vero cells containing approximately 4x10³ cells per well were seeded into 96-well microplates, and incubated at 37 °C in a humidified atmosphere of 5% CO₂, 24 h before titration. The lyophilized vaccines were reconstituted with 200µl of 0.85% saline and gently swirled for homogenization immediately before use. Twenty-four well plate seeded with Vero E6 cells, a day before titration day, was 80-90% confluent on infection day. 50 µl of virus stock was transferred to 5 ml of DMEM (1:1000 dilution) in the first row of 96 well plates and thoroughly mixed by pipetting. 500 µl of the diluted virus was serially diluted with 4.5 ml of DMEM (1:1000) in subsequent rows. Media was removed from Vero E6 cells and 100 µl of diluted virus was per well. The plates were incubated for 1 hr at 37 °C before removing the virus inoculum. 1 ml of DMEM + 2% FBS + L-glut + P/S was added per well. The plates were incubated at 37 °C in the presence of 5% CO₂ and monitored daily for CPE under a light microscope. Wells showing the characteristic CPE were recorded as positive. Infection of cells led to their detachment and morphological changes. Titrations were read at 5 dpi and the endpoint titers were calculated using Reed and Muench formula (59) simplified for calculation in an MS Excel spreadsheet.

2.3.5 **Evaluation of protective efficacy of lyophilized rVSVΔG-LASV-GP in guinea pigs**

The rVSVΔG-LASV-GP and rVSVΔG-ZEBOV-GP vaccines stabilized with LST formulation were lyophilized as described in section 2.3.3 and stored at 4 °C for one week. The lyophilized vaccines in vials were reconstituted with 200 µl of 0.85% saline in sterilized biosafety cabinets. Hartley guinea pigs were separated into 3 experimental groups (n=10). Group 1 received lyophilized rVSVΔG-LASV-GP, group 2 received lyophilized rVSVΔG-ZEBOV-GP (negative control) and Group 3 received rVSVΔG-LASV-GP (positive vaccine control). All guinea pigs received 10⁶ PFU of vaccines by the intraperitoneal route (IP) (using a 25 gauge, 1/2-inch needle with a sterile syringe, up to 1 ml depending on vaccine titer) and monitored daily. All Guinea pigs received an implanted Bio Medic Data Systems (BMDS) IPTT-300 temperature transponder chip to monitor their temperatures, using a hand-held scanner during the experiments. Before implantation, animals were sedated using inhalation isoflurane (3-5% maintained in medical grade oxygen) following disinfection of skin on the back of the animal with 70% ethanol. The needle was placed under the skin of the animals between the shoulder blades and the transponder was pushed out of the needle (18 gauge) into the subcutaneous space created. Implantation of the temperature transponder was performed at CL2. Experimental manipulations involving sharps were performed while the Guinea pigs were anesthetized with Isoflurane in oxygen at a dose rate of 5% for induction and gradually decreased to 2.5% to 3% for maintenance using a mask. Terminal signs of disease included evidence of hemorrhage, respiratory distress, decreased body temperatures, and severe weight loss of 20%. For Euthanasia, Guinea pigs were administered isoflurane (5%). Animals were held in the chamber until signs of respiration were not observed for ≥ 2 minutes followed by a thoracotomy. Cardiac blood, lung, liver, and spleen were collected for

infectious viral titers and RT-qPCR. Coded cage cards were utilized for animals' identification in cages by animal technicians to ensure that only animal technicians performing the injections knew which vaccines were used in specific cages.

At 28 days post-vaccination (dpv), all Guinea pigs were challenged with a lethal dose of 104 TCID₅₀ /10x LD₅₀ of Guinea pigs adapted-LASV (Josiah strain) by IP route (using 25G, ½ inch needle with sterile syringe, 1ml). Animal weights and body temperatures were tracked daily. Four animals were euthanized per group for sample collection 13 dpi when the negative control group (lyophilized rVSVΔG-ZEBOV-GP immunized Guinea pigs) experienced signs of disease or predetermined clinical scores. The remaining Guinea pigs per group (n=6) were monitored for disease progression and survival until all surviving animals were euthanized at 36 dpi.

2.3.6 Sample extraction from Guinea pigs' tissues

2.3.6.1 Serum extraction

Serum extraction was performed using a QIAamp viral RNA mini kit (cat no. 52906) using the manufacturer's protocol.

2.3.7 Tissue extraction

Tissue extraction (liver and lung) was performed using a QIAamp viral RNA mini kit (cat no. 74136) using the manufacturer's protocol.

2.3.8 Virus titration of Guinea pigs' tissues using TCID₅₀.

Titration of recovered viruses from serum, liver, and lung were conducted as described below: 24-well plates seeded with Vero E6 cells, 24 hr before titration day was 80-90% confluent on infection day. 50 µl of virus stock was transferred to 5 ml of DMEM (1:1000 dilution) in the first row of 96 well plates and thoroughly mixed by pipetting. 500 µl of the diluted virus was serially diluted with 4.5 ml of DMEM (1:1000) in subsequent rows. Media was removed from Vero E6 cells and added 100 µl of diluted virus per well. The plates were incubated for 1 hr at 37 °C. The virus inoculum was removed. 1 ml of DMEM + 2% FBS + L-glut + P/S was added per well. The plates were incubated at 37 °C in the presence of 5% CO₂ and monitored daily for CPE under a light microscope. Wells showing the characteristic CPE were recorded as positive. Infection of cells led to their detachment and morphological changes. Titrations were read at 5 dpi and the endpoint titers were calculated using Reed and Muench formula (Ramakrishnan, 2016), simplified for calculation in an MS Excel spreadsheet.

2.3.9 RT-qPCR of Guinea pigs' tissues.

RT-qPCR to detect the amount of GPA-LASV RNA in serum and tissues was performed as illustrated in Appendix A. The sample was run on Quant Studio 3 (Applied Biosystems) as illustrated in Appendix A. The templates for LASV tissue (LaV) and LASV serum (LaS) were serum, liver, and lung.

2.3.10 **Antibody Assay of Guinea Pigs**

ELISA was performed according to the manufacturer's protocol (Zalgen.com). Briefly, a five-point Anti-NP IgM calibration curve was prepared using 5 tubes. Dilutions of 1:50, 1:100, 1:200, and 1:400 of calibrator and samples were used for both lyophilized rVSVΔG-LASV-GP and regular rVSVΔG-LASV-GP vaccine groups. The positive control was LASV positive Guinea pigs' serum while the negative control was SARS-2 positive Guinea pig sera. 100 µl of samples were added to microplate wells. The plates were incubated for 30 minutes at 25°C. After the incubation, the plates were washed 4 times (300ul/well). They were blotted on absorbent paper to remove residual wash fluid. 100 µl of the anti-Guinea pig IgG solution was added to each well. The plates were incubated for 30 minutes at 25°C and washed 4 times with 300µl/well with a wash solution. The plates were blotted on absorbent paper to remove residual wash fluid. 100 µl of the One-Component substrate was added to each well and incubated for 10 minutes at 25°C while protected from light. 100 µl of the stop solution (2% methanesulfonic acid) was added to each well to stop the substrate color development. The optical densities of the plates were read at 450 nm.

2.4 **Statistical Analysis**

Mean difference calculations comparing the TCID₅₀ of data collected on day(s) 1, 7, 35, and 90 were performed using a two-way ANOVA on GraphPad Prism 10 (www.graphpad.com). The difference was considered statistically significant with p values were ≤ 0.05 (**P<0.001, **P<0.01, *P<0.05). The mantel-cox test was used for the survival analysis of the three groups during the protective efficacy study of the vaccines. Comparison of the optical densities and

statistical significance of humoral responses of Guinea pigs following vaccination was determined by one-way ANOVA. p values were ≤ 0.05 (***P<0.001, **P<0.01, *P<0.05).

Chapter 3. Results

3.1 Development of rVSVΔG-LUJV-GP vaccine

3.1.1 Rationale for development of rVSVΔG-LUJV-GP vaccine

There is no licensed vaccine for LUJV to date. Being a novel and emerging virus, with 80% CFR, a high rate of human-to-human transmissions, and a close resemblance to highly pathogenic arenaviruses, a vaccine is urgently needed for emergency preparedness for potential future outbreaks.

3.1.2 Objective of this study

The primary objective of this study was to develop a LUJV vaccine by modifying an existing VSV backbone using recombinant technology. This was achieved by transfecting pLUJV-GP and LUJV support plasmids in BHK cells and rescuing recombinant viruses in Vero cells.

3.1.3 Results from development of rVSVΔG-LUJV-GP vaccine

pLUJV-GPWT or pLUJV-GPCO and support plasmids were transformed in *E. coli* cells. The transformed cells were purified, propagated and sequence confirmed. RT-PCR was further conducted for pLUJV-GPWT and pLUJV-GPCO for confirmation (Figure 6). The expected bands were 1.4 kb for both pLUJV-GPWT and pLUJV-GPCO. For infusion cloning, the first step was PCR amplification of pLUJV-GPWT and pLUJV-GPCO using various combinations of primers (MluI, NheI, and AvrII). The following 5 plasmids were generated from the different combinations of forward and reverse primers: MluI- LUJV-GPCO-F, NheI- LUJV-GPWT-R, NheI- LUJV-GPCO-R, AvrII- LUJV-GPWT-R, AvrII- LUJV-GPCO-R. The PCR products were purified as described in 2.2.5.1. Double digestion of pBSVSVG-GFP (p-bluescript kerafast vector) was

achieved with restriction endonucleases. The forward primer was MluI while the two reverse primers were AvrII and NheI (Figure 7). Digestion of pBSVSVG-GFP gave two linearized plasmids: pBSVSVG and pBSVSVG-GFP. The digested vectors were purified as described in section 2.2.5.2. During purification with gel electrophoresis, aside from the expected bands for linearized vectors, a band around 900kb was observed. The impurity was GFP which was cut off by restriction endonuclease. The purified DNA samples were ligated as described in section 2.2.5.3 using a 2:1 molar ratio of insert and vector. The ligation of 4 PCR samples and two linearized vectors gave 4 different ligated products that were transformed in *E. coli* cells. The recombinant plasmids were purified, propagated, and confirmed with PCR and sequencing. Transfection was performed using the TransIT-LT1 transfection reagent (Mirus bio®) using the manufacturer's protocol. Rescue of the rVSVΔG-LUV-GP virus was unsuccessful.

3.1.4 Troubleshooting of Unsuccessful Rescue of rVSVΔG-LUV-GP virus

Transfection started with the rescuing of wild-type VSV GP to confirm that support plasmids were good and capable of rescuing recombinant viruses. Wild-type transfection was performed according to the manufacturer's manual (Mirus bio®), using BHK. The supernatant was blind passaged to Vero cells, 48 hours post-transfection. There was syncytial formation, and the Vero cells were dead after 84 hours, but GFP disappeared rapidly soon after. The next troubleshooting step was to get VSV helper plasmids (previously used to rescue rVSV-EBOV) from the Darwin Kobasa group (Special Pathogens unit, NML). Transfection was repeated using the manufacturer's protocol. No cytopathic effect (CPE) or GFP was observed before cells were dead.

The next troubleshooting step was to include wild-type VSV GP plasmid with other plasmids during transfection. The idea was that wild-type VSV GP may promote transfection of desired

plasmids since transiently transfected wild-type VSV GP is expected to be lost when BHK cells replicate. The supernatant on BHK cells was blind passaged to Vero cells after 48 hours post-transfection. No CPE or GFP was observed before cells were dead after 5 days. Mirus bio® transfection protocol was modified by extending the blind passage onto Vero cells to 72 - 84 hours post-transfection to provide more time for uptake of pLUJV-GP by BHK cells. No success was recorded.

While looking for troubleshooting techniques, I found out that BHK cells have low-expression T7 polymerase. I transfected T7 polymerase to confirm T7 polymerase expression by BHK cells. 48 hours post-transfection, there was distinct fluorescence to confirm T7 polymerase expression by BHK cells. Once confirmed, T7 polymerase was added to reagents during subsequent transfections to supplement T7 polymerase expressed by BHK cells. On top of this, there was a change of transfection cells from BHK to HEK 293 cells due to excellent rescuing records with other viruses. To ensure there was no mistake during the experimental procedures, a few of my troubleshooting steps were supervised by some members of Safronetz's group.

Lastly, it was important to confirm the expression of pLUJV-GP in mammalian cells by cloning pLUJV-GP into PCAGGS, transfecting, and performing western blot using convalescent guinea pigs' serum as primary antibody in western blot. Western blot confirmed no expression of LUJV GP. In total, there were over 180 transfection attempts with no success. Another group in the Special Pathogens unit of NML recently reported an unsuccessful rescue of the rVSVΔG-LUJV-GP vaccine. This aspect of my project which aimed at developing rVSVΔG-LUJV-GP was put on hold; I decided to continue with testing other aspects of my hypotheses without LUJV.

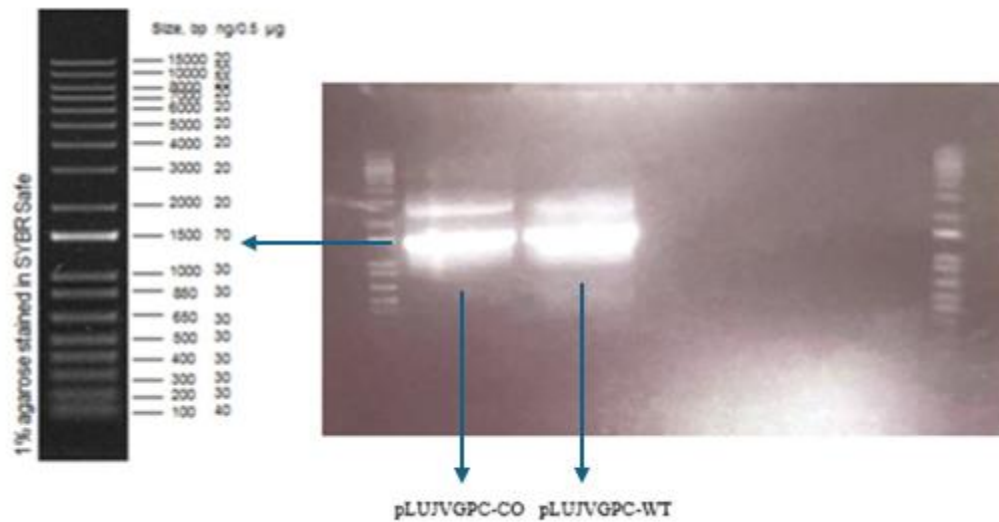


Figure 6. Image of stained gel showing PCR bands at approximately 1.4 kb for confirmation of pLUJV-GPWT and pLUJV-GPCO. These plasmids were subsequently amplified for infusion cloning.

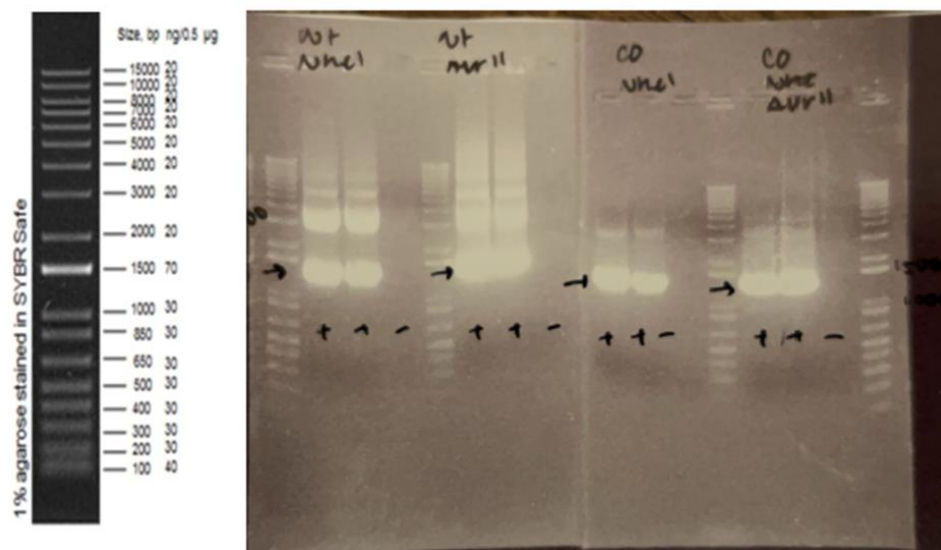


Figure 7. Image of stained gel showing PCR bands of amplified pLUJV-GPWT and pLUJV-GPCO. These PCR products were subsequently used for ligation. The ligated products were used for infusion cloning. The plus (+) denotes wells of gels with samples showing bands of amplified pLUJV-GPWT and pLUJV-GPCO. The negative (-) denotes wells that were devoid of samples.

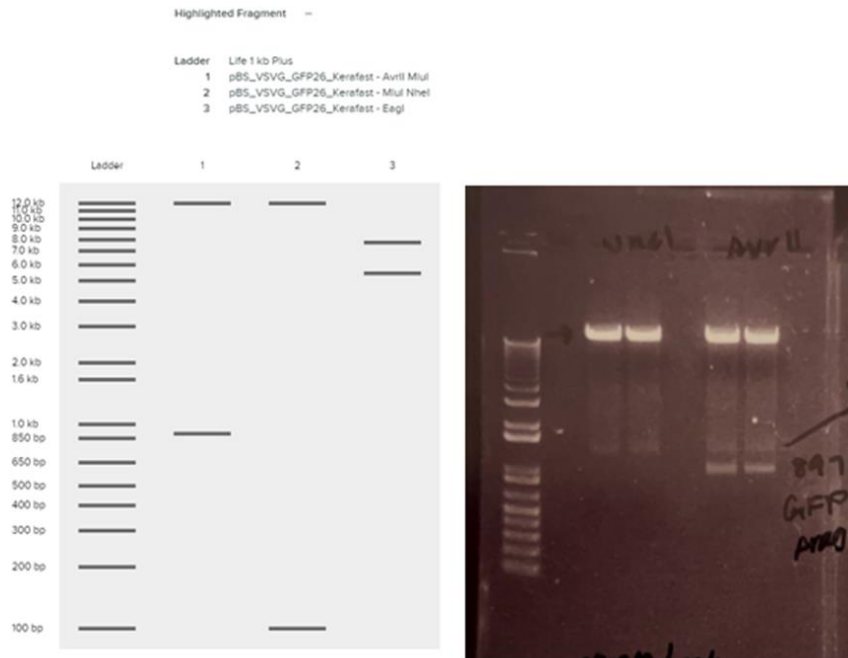


Figure 8. Image of stained gel showing PCR bands of linearized pBSVSVG-GFP for infusion cloning. The digestion of pBSVSVG-GFP was achieved with NheI and AvrII restriction endonucleases. These purified vectors were used for ligation. The ligated products were purified and used for infusion cloning.

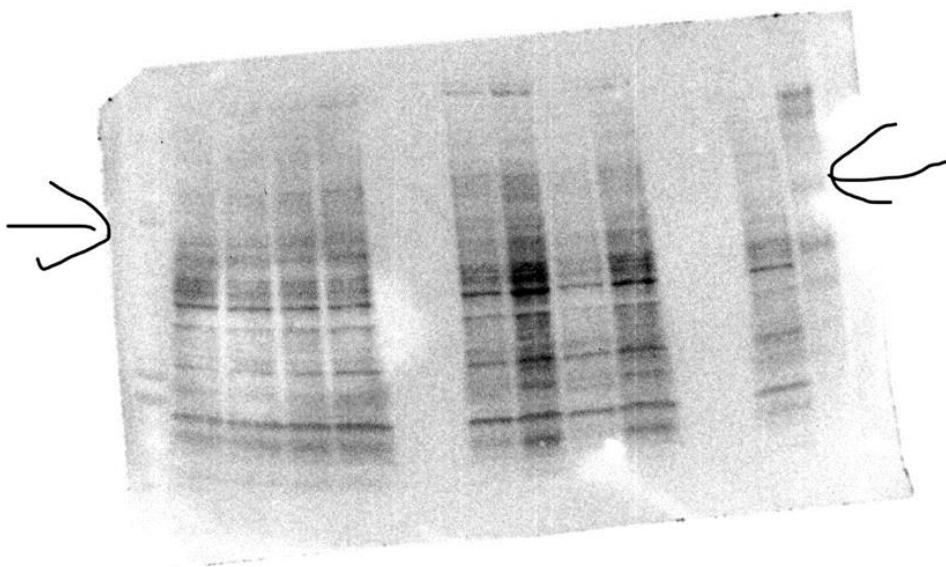


Figure 9. Image of stained gel blot from confirmation of pLUJV-GP expression in pPCAGGS. This analysis was a troubleshooting step to confirm expression of pLUJV in mammalian cells.

3.2 Evaluation of thermostability of lyophilized rVSVΔG-LASV-GP and rVSVΔG-LASV-GP

3.2.1 Rationale for evaluating thermostability of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP

Liquid formulations of vaccines are subject to instabilities that result from physical (precipitation, adsorption, unfolding) and chemical degradation (hydrolysis, oxidation, and deamidation) processes (Preston and Randolph, 2021). Vaccines are often required to be transported across long distances from hours to days to remote areas in developing countries with limited budgets for storage facilities and maintenance. This warrants the development of formulations with less stringent storage temperature requirements.

Stabilized, lyophilized PPR vaccines can withstand storage outside the cold chain and retain potency (Bora et al, 2020). We proposed the evaluation of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines stabilized with LS, LST, LSTG, and DMEM stored at 4 (fridge), 21 (room shelf), and 37 °C (incubator), and different time points of 1, 7, 35 and 90 days. Infectivity titers were measured as TCID₅₀/ml at 5 dpi. The hypothesis for this study is that lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP in the presence of stabilizers will retain infectivity at suboptimal temperature exposure.

3.2.2 Objective of this study

Evaluation of the stability of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP stabilized with LS, LST, LSTG, and DMEM by measuring infectivity using TCID₅₀/ml. The effect of stabilizers on vaccines was measured by assessing the infectivity of the vaccines in cell culture. Labconco lyophilizer will be used for lyophilization in the CL2 of the National Microbiology Lab, Winnipeg. DMEM will be used as a control (formulation D).

3.2.3 Results from thermostability evaluation of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP

The objective of the study was to see how lyophilization in the presence of stabilizers affects the stability of vaccines and how the duration of storage under suboptimal conditions affects the stability of lyophilized vaccines. Three different stabilizer formulations; LS, LST, and LSTG were prepared. The control formulation was DMEM. The stock of rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP used for lyophilization were 2.83×10^7 TCID₅₀/ml and 1.26×10^7 TCID₅₀/ml, respectively. There were 7- and 26.8-fold reductions in infectious titer from stock for rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP, respectively. 100 µl aliquots of vaccines and 100 µl of stabilizers were added to 4 ml sterile vials. Lyophilization was performed in Labconco lyophilizer for 16 hr (Table 5). The vials were separated according to the two vaccines contained, stabilizer combinations, storage temperature, and duration of storage. Titrations were tested on a predetermined duration of storage of 1, 7, 35, or 90 day(s) post-lyophilization. Titration of lyophilized vaccines was performed as described in section 2.3.4. Cytopathic effect was read at 5 dpi and the endpoint titers were calculated using Reed and Muench formula. Three lyophilized vaccine vials were misplaced during experimental storage; one rVSVΔG-EBOV-GP (stabilized with formulation B) was stored for 90 days, and two rVSVΔG-EBOV-GP (stabilized with formulation C) meant to be tittered at 35 and 90 days. The misplaced vials were excluded from the results.

As observed in Figure 10, the infectivity of lyophilized rVSVΔG-LASV-GP varied with stabilizer combinations at 4° C and duration of exposure. LST stabilizer combinations yielded the highest titers for lyophilized rVSVΔG-LASV-GP over 90 days at 4° C ($p > 0.05$). LS had 0.25 log₁₀ while LST had a 1.25 log₁₀ decline in the recovery of the virus over the study period. Overall, LS and

LST displayed a relatively constant thermostability over 90 dpi. Surprisingly, DMEM, with 6.36 log₁₀ virus loss over the study period had higher infectivity titer over LSTG (no detectable virus) after 30 dpi.

At 21 °C, LS had the highest decline in infectivity (3 log₁₀) while LST had a 1.5 log₁₀ decline in titer, the lowest of all the stabilizer formulations at this storage condition. At no point was DMEM formulation recoverable at 21 °C. At 37 °C, all the formulations had no virus recovery, suggesting that lyophilized rVSVΔG-LASV-GP vaccine may not be stable at temperatures above 21 °C. Lyophilized PPR vaccines at 37°C exposure had a total loss of infectivity (Bora et al, 2020). Generally, LS and LSTG stabilized lyophilized rVSVΔG-LASV-GP vaccines are more stable under studied conditions. Mariner et al found similar results for lyophilized PPR vaccines (Mariner, Gachanja, Tindih, and Toye, 2017). At 2-8 °C, the superiority of LST and LSTG formulations was established (Bora et al, 2020). Another study found an extended shelf-life of 2051 days at refrigeration temperature for LST (Sarkar et al, 2003).

For lyophilized rVSVΔG-EBOV-GP, there was little variation in the infectivity of the virus over 90 days at 4 °C. LS had 0.42 log₁₀ loss in titer while LSTG had a 1.55 log₁₀ decline in titer (like DMEM). These titer results suggest that the lyophilized rVSVΔG-EBOV-GP vaccine may be stable for 90 days without the need for a stabilizer. ERVEBO®, the only licensed EBOV (Zaire) vaccine, is recommended to be used immediately after thawing, if not used immediately, the vaccine may be stored for only 4 hours at room temperature (up to 25°C; 77°F) (Centre for Disease Control, 2021).

At 21°C, the infective titer was lower than the starting titer at 90 dpi; DMEM, LS, and LST had over 4 log₁₀ decline in titer at 90 dpi. LSTG had 3.75 log₁₀ titer loss, the lowest at 21°C. DMEM maintained recovery for only 7 dpi. At 37 °C, all the formulations were not infectious after 24

hours. This suggests that lyophilized rVSVΔG-EBOV-GP vaccine maintained at 37 °C may lose immunogenicity after 24 hours. Comparatively, lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines exhibited a similar trend of infectivity loss over 90 days at studied temperatures. The major difference is that DMEM formulation for lyophilized rVSVΔG-EBOV-GP was more stable at 4, 21, and 37 °C. While all the formulations of lyophilized rVSVΔG-LASV-GP vaccine showed no infectivity at 37 °C, DMEM and other formulations of lyophilized rVSVΔG-EBOV-GP were infective within 24 hours.

Generally, from the pre-and post- lyophilization titers, rVSVΔG-EBOV-GP is more stable than rVSVΔG-LASV-GP. Also, the LS formulations of lyophilized rVSVΔG-LASV-GP and lyophilized rVSVΔG-EBOV-GP exhibited superior thermal stability over other formulations tested. However, superior thermostability property of LS formulation over others was not statistically significant ($p>0.05$).

3.2.3.1 Key Points

Lyophilization reduced the infectivity of rVSVΔG-EBOV-GP of lyophilized rVSVΔG-LASV-GP vaccines as shown by the fold reductions in infectious titer post-lyophilization when compared to the stock titer. There were 7 and 26-fold reductions in infectious titers of rVSVΔG-EBOV-GP and rVSVΔG-LASV-GP vaccines after lyophilization. Stabiliser formulations reduce the lyophilization stress on vaccines by increasing glass transition temperature (T_g), adding stabilizing hydrogen ions lost from the removal of water, maintaining antigen is a vitrified glass state, and reducing molecular mobility that promotes antigenic degradation. Overall, excipients containing stabilizers improve the stability of lyophilized vaccines as seen from the results of this study. The effectiveness of stabilizers in improving vaccine stability reduces with increasing storage

temperature. LST formulation provided the best thermostability for lyophilized rVSV Δ G-EBOV-GP and rVSV Δ G-LASV-GP vaccines.

Vials	rVSVΔG-EBOV-GP titer pre-lyophilization (log ₁₀ TCID ₅₀ /vial)	rVSVΔG-EBOV-GP titer post-lyophilization (log ₁₀ TCID ₅₀ /vial)	rVSVΔG-EBOV-GP fold decrease (log ₁₀ TCID ₅₀ /vial)	LASV titer pre-lyophilization (log ₁₀ TCID ₅₀ /Vial)	LASV titer post-lyophilization (log ₁₀ TCID ₅₀ /vial)	rVSVΔG-LASV-GP fold decrease (log ₁₀ TCID ₅₀ /vial)
1	1.26x10 ⁷	8.62x10 ⁶	3.98	2.83x10 ⁷	1.86x10 ⁶	26.4
2	1.26x10 ⁷	4.0x10 ⁶	8.60	2.83x10 ⁷	1.26x10 ⁶	27.0
3	1.26x10 ⁷	4.0x10 ⁶	8.60	2.83x10 ⁷	1.26x10 ⁶	27.0
Mean	1.26x10 ⁷	5.54x10 ⁶	7.06	2.83x10 ⁷	1.46x10 ⁶	26.8

Table 6. Preliminary TCID₅₀ titers for non lyophilized and lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP without stabilisers. The titers of rVSVΔG-EBOV-GP and rVSVΔG-LASV-GP taken before lyophilization were compared to the values after lyophilization to determine the impact of lyophilization on the stability of both vaccines. There was average 7-fold reduction in titer for rVSVΔG-EBOV-GP while rVSVΔG-LASV-GP had 26.8-fold decline in infectious titer.

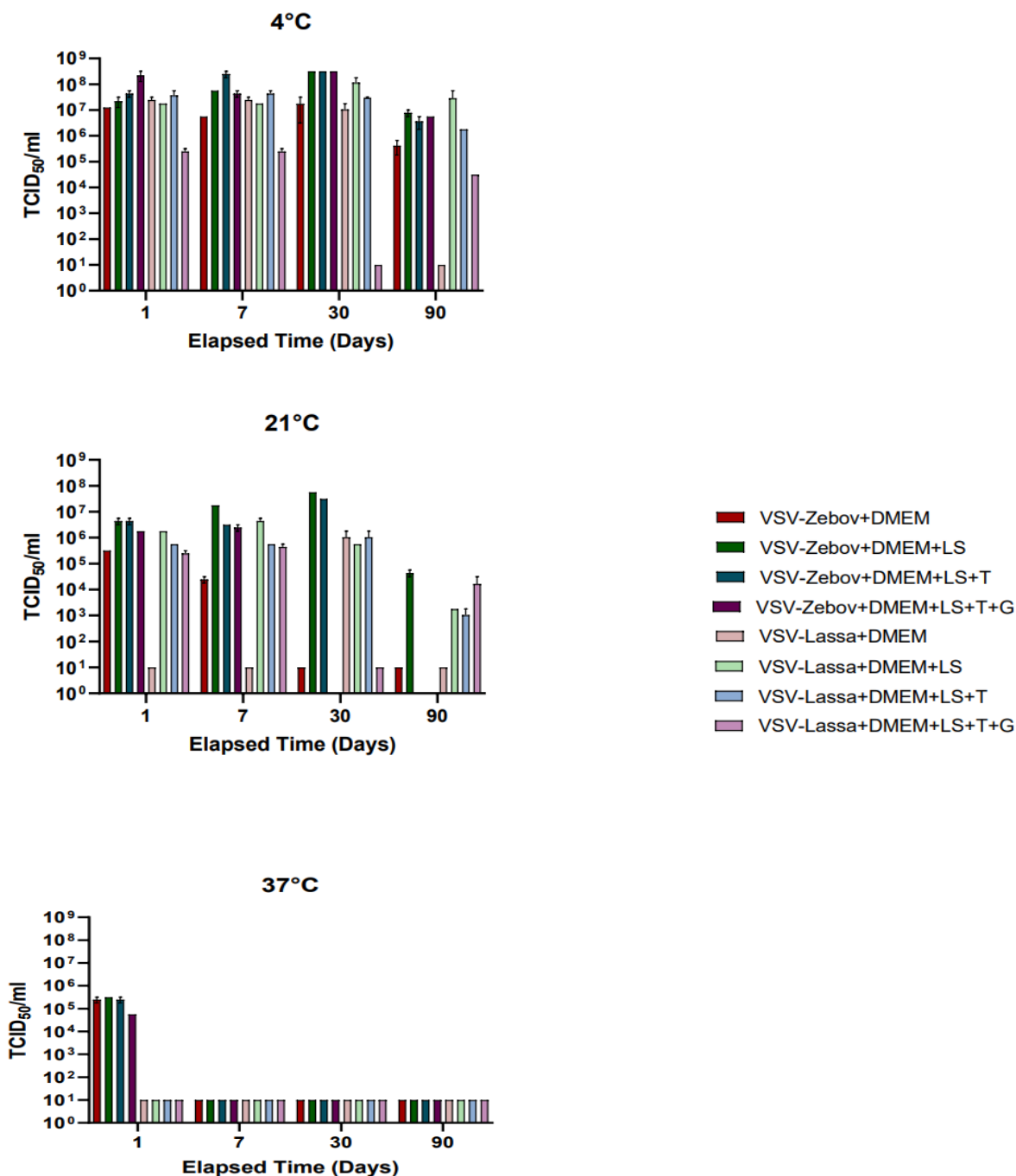


Figure 10. Virus recovery chart for lyophilized rVSVΔG-LASV-GP (N=72) and rVSVΔG-EBOV-GP (N=72) vaccines stabilised with formulation LS, LST, LSTG and DMEM stored at 4, 21 and 37 °C. Infectivity titres were measured as TCID₅₀/ml. LS and LST formulations

of the two vaccines had highest recovery titers over 90 days. Lyophilized rVSVΔG-LASV-GP vaccine formulations were not stable at 37° C.

3.3 Evaluation of protective efficacy of lyophilized vaccines in guinea pigs

3.3.1 Rationale for evaluating protective efficacy of lyophilized vaccines in guinea pigs.

The annual estimated Lassa fever cases in West Africa is 100,000-300,000 with over 5,000 (approximately 1%) annual deaths (Lassa fever fact sheet, 2012). Both Lassa fever and EVD are endemic to West Africa. Rapid and long-term protection from the rVSVΔG-LASV-GP vaccine against GPA-LASV infection in guinea pigs has been established (Stein et al, 2019). While there is no licensed Lassa fever vaccine yet, ERVEBO® and Zabdena® are the only licensed EBOV vaccines available in the market as of today. It is recommended to be used immediately after thawing. If not used immediately, ERVEBO® may be stored for only 4 hours at room temperature (up to 25°C; 77°F) (Centre For Disease Control, 2021).

Hence, we proposed to examine and compare the protective efficacy of rVSVΔG-LASV-GP and lyophilized rVSVΔG-EBOV-GP in an animal model as one of the solutions to the challenges of poor vaccine storage ravaging developing countries especially west African countries where both EVD and Lassa fever are endemic.

3.3.2 Objective of this study

The objective of this study was to evaluate and compare the protective efficacy of rVSVΔG-LASV-GP and lyophilized rVSVΔG-EBOV-GP in Guinea pigs. All animal manipulations were conducted according to approved NML guidelines. The overall hypothesis for this study was that lyophilized rVSVΔG-LASV-GP would retain potency and protect guinea pigs against lethal LASV infection.

Thirty guinea pigs were divided into three groups of 10. The first group received lyophilized rVSVΔG-LASV-GP, the second group received lyophilized rVSVΔG-EBOV-GP (negative control) and the third group received rVSVΔG-LASV-GP (positive control). At 28 days post-vaccination, all guinea pigs were challenged as described in section 2.3.5. The remaining guinea pigs per group were monitored for disease progression and survival till 36 dpi when all surviving animals were euthanized. When the control animals reached humane endpoints on 12 dpi, 4 animals per group were euthanized for viremia and tissue titer analysis.

3.3.3 Results for Objective 2

Lyophilized rVSVΔG-LASV-GP vaccinated animals achieved statistically significant 100% protection (Figure 11, $p=0.0063$, Mantel-Cox test), showing complete protection from LASV infection. Furthermore, no virus was detected in the tissues (liver, lung, and serum) examined, further suggesting that lyophilized rVSVΔG-LASV-GP offered sterilizing immunity to guinea pigs. The lack of clinical diseases like weight loss, fever, and detectable viral load in tissues afforded by lyophilized rVSVΔG-LASV-GP vaccine aligned with a similar study with rVSVΔG-LASV-GP vaccine (Bausch et al, 2001; Morrison, 1989). Although protective efficacy was evaluated with vaccines stored at 4°C for 7 days, lyophilized EBOV vaccine stored for 12 weeks at 40°C retained immunogenicity (Chisholm et al, 2020). Viral RNA load in tissues was evaluated by qRT-PCR analysis of blood, liver, and spleen. The lyophilized rVSVΔG-LASV-GP vaccinated group demonstrated considerably lower CT values for tissues than the lyophilized rVSVΔG-EBOV-GP and VSV-LASV groups (Figure 15).

Negative control animals that received lyophilized rVSVΔG-EBOV-GP displayed a gradual rise in basal body temperature from 2 dpi to a peak of 41 °C, 9 dpi in some animals before gradual

decline till the study humane endpoints were reached (Figure 12). All animals but 1 succumbed to infection (Figure 11) and exhibited significant weight loss, about 25% in some animals. This classic clinical symptom of diseases displayed by this group suggests lyophilized rVSVΔG-EBOV-GP offered no protection against LASV infection (Josiah strain). Similarly, infectious titer was recovered in tissues of guinea pigs immunized with lyophilized rVSVΔG-EBOV-GP; lung (6.8×10^5) had highest titer while serum (2.2×10^4) and liver (4.7×10^4) have considerably low viral titers. The higher viral titer recorded in the lungs is suggestive of the lung as initial tissue of high-level rVSVΔG-LASV-GP replication (2-12 dpi). There was a high Viral RNA load in the blood, liver, and spleen, as evaluated by qRT-PCR.

The group 3 animals received the rVSVΔG-LASV-GP vaccine that was not lyophilized. All animals survived but one and were exsanguinated on 13 dpi when the clinical score reached the humane endpoint. This animal had a similar onset of clinical symptoms as negative controls, with a peak temperature of 40 °C at 8 dpi and loss of weight reaching approximately 20% at 13 dpi. These animals showed a progressive increase in body weight and basal body temperature pattern like animals vaccinated with lyophilized rVSVΔG-LASV-GP. More like the group vaccinated with lyophilized rVSVΔG-LASV-GP, there was no detectable infectious virus in the liver, lung, and serum, and little to no viral RNA recovered in blood, liver, and spleen examined.

All the animals immunized with lyophilized rVSVΔG-LASV-GP survived the GPA-LASV challenge. They experienced hyperthermia that disappeared after 72 hours. While 5 of 6 animals vaccinated with rVSVΔG-LASV-GP survived till 36 dpi, all but 1 of the animals vaccinated with lyophilized rVSVΔG-EBOV-GP (control) succumbed to death at 36 dpi. Animals vaccinated with lyophilized rVSVΔG-EBOV-GP experienced significant hyperthermia approaching 41 °C that persisted for two weeks until death. They also experienced considerable weight loss more than

10% as opposed to the other groups, suggesting lyophilized rVSVΔG-EBOV-GP offered no protection against GPA-LASV challenge. As described in section 2.1.5, 4 Animals were euthanized at 12 dpi per group for sample collection early during infection. Collected blood, liver, and spleen underwent RNA extraction. The extracted RNA was evaluated using RT-qPCR. Results showed that lyophilized rVSVΔG-EBOV-GP immunized guinea pigs had higher CT values than other groups ($p>0.05$) (Fig 15). The CT value was also a reflection of the burden of disease experienced by this group. Generally, viral RNA results were consistent with the survival results obtained from all the groups. The presence of RNA in the tissue does not automatically reflect an infectious viral particle.

TCID₅₀ analysis for tissue extracts from liver, lung, and serum collected from guinea pigs immunized with lyophilized rVSVΔG-LASV-GP, lyophilized rVSVΔG-EBOV-GP, and rVSVΔG-LASV-GP vaccines and challenged with GPA-LASV showed detectable viral particles in only the tissues of the lyophilized rVSVΔG-EBOV-GP group only ($p>0.05$) (Figure 14). The lungs had the highest recoverable virus, suggesting the lungs to be the initial site of infection of LASV.

For a humoral response assay, LASV GP-specific ELISA developed for humans (Zalgen Labs) was used for antibody response in Guinea pigs immunized with both regular and lyophilized rVSVΔG-LASV-GP vaccines. This prevented a direct correlation of antibody response in Guinea pigs. However, optical density measured at 450 nm revealed a 50-fold increase in antibody production in the groups that received lyophilized rVSVΔG-LASV-GP, or rVSVΔG-LASV-GP vaccines compared to post-infection as well as the group immunized with lyophilized rVSVΔG-EBOV-GP vaccines. Furthermore, similar calculated concentration values (ng/mL) for both regular and lyophilized rVSVΔG-LASV-GP vaccines suggest that lyophilization has little or no

effect on the degradation of antigens. EBOV-GP-specific ELISA was used to evaluate IgG response. The group vaccinated with lyophilized rVSVΔG-EBOV-GP produced titers ≥ 6400 .

3.3.4 **Key Points**

Results obtained from the animal study showed that both regular and lyophilized rVSVΔG-LASV-GP retained immunogenicity at 4 °C storage for 1 week, protected against GPA-LASV infection in guinea pigs, and offered sterilizing immunity. Also, the results obtained suggested that lyophilized rVSVΔG-EBOV-GP does not offer cross-protection against the GPA-LASV challenge in guinea pigs. Both regular and lyophilized rVSVΔG-LASV-GP vaccinated groups experienced hyperthermia that was fully resolved after about 4 days. They also experienced weight gain while the control group experienced significant weight loss exceeding 15%.

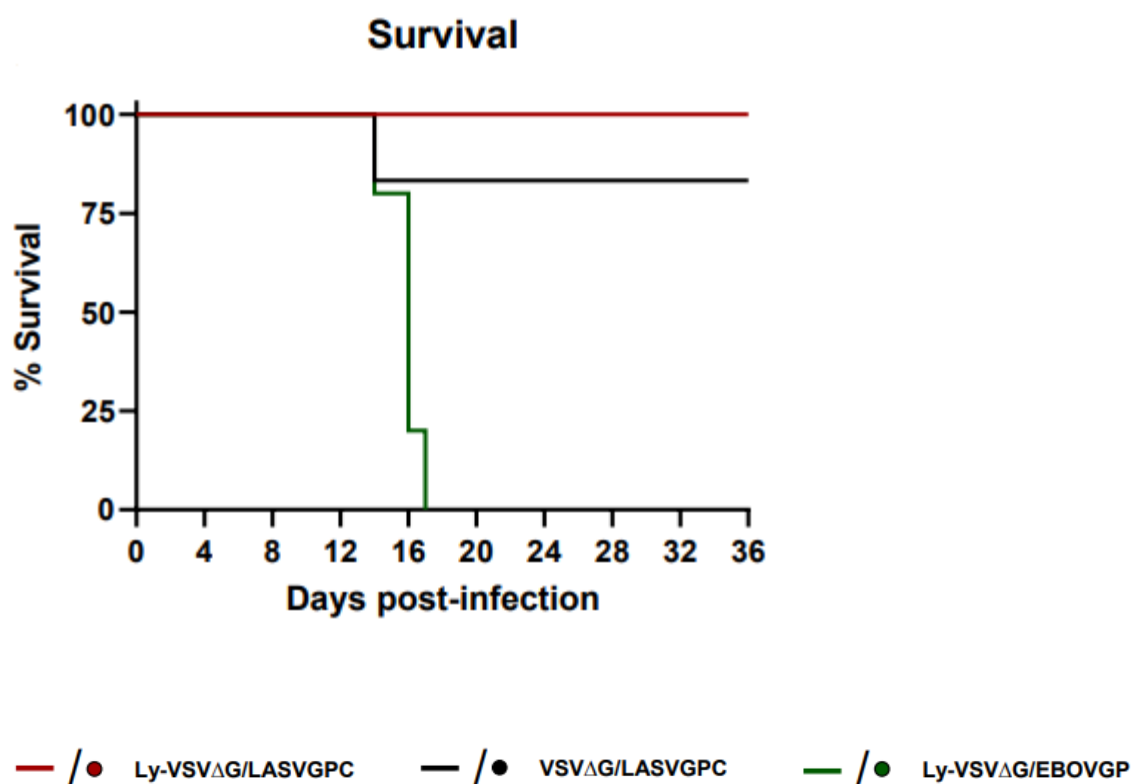


Figure 11. Survival curve of lyophilized rVSVΔG-LASV-GP (N=10), lyophilized rVSVΔG-EBOV-GP (N=10) and rVSVΔG-LASV-GP (N=10) challenged guinea pigs. Animals were treated with lyophilized rVSVΔG-LASV-GP (blue), lyophilized rVSVΔG-EBOV-GP (red), or LASV (green). Survival analysis was conducted using a log rank Mantel-Cox test with a p value cut off 0.0063.

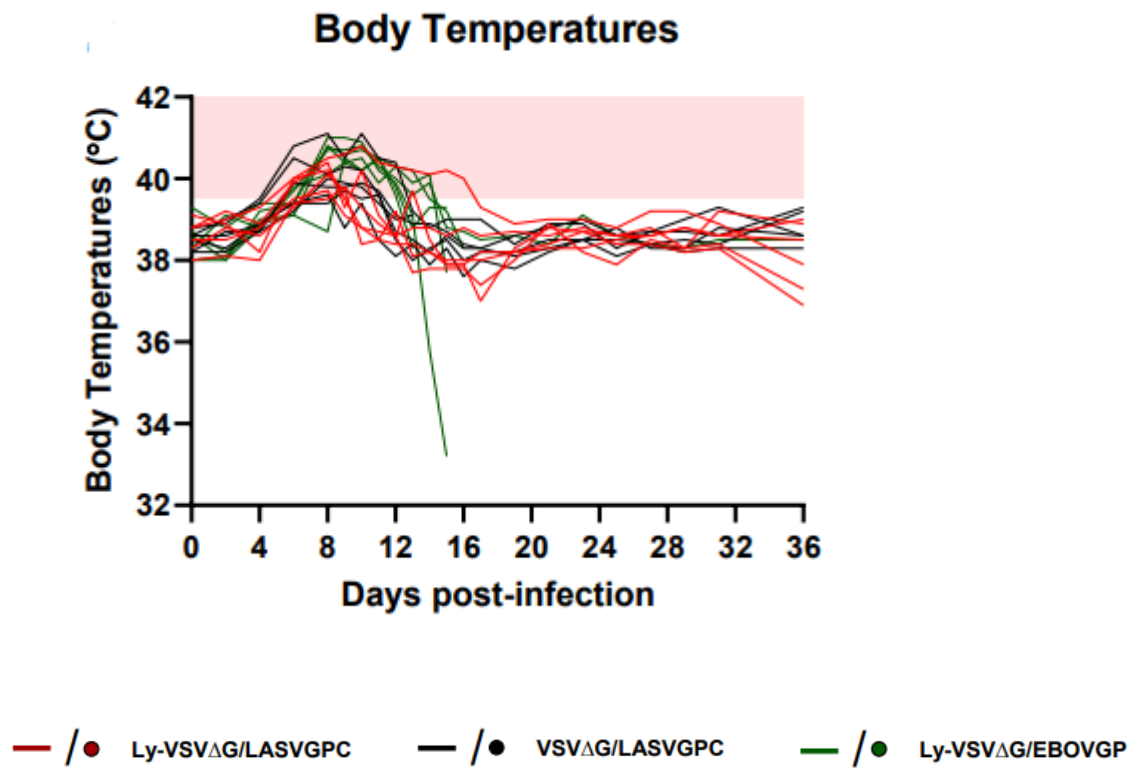


Figure 12. Temperature analysis for guinea pigs vaccinated with lyophilized rVSVΔG-LASV-GP (blue, N=10), lyophilized rVSVΔG-EBOV-GP (red, N=10), and rVSVΔG-LASV-GP (green, N=10), challenged with GPA-LASV.

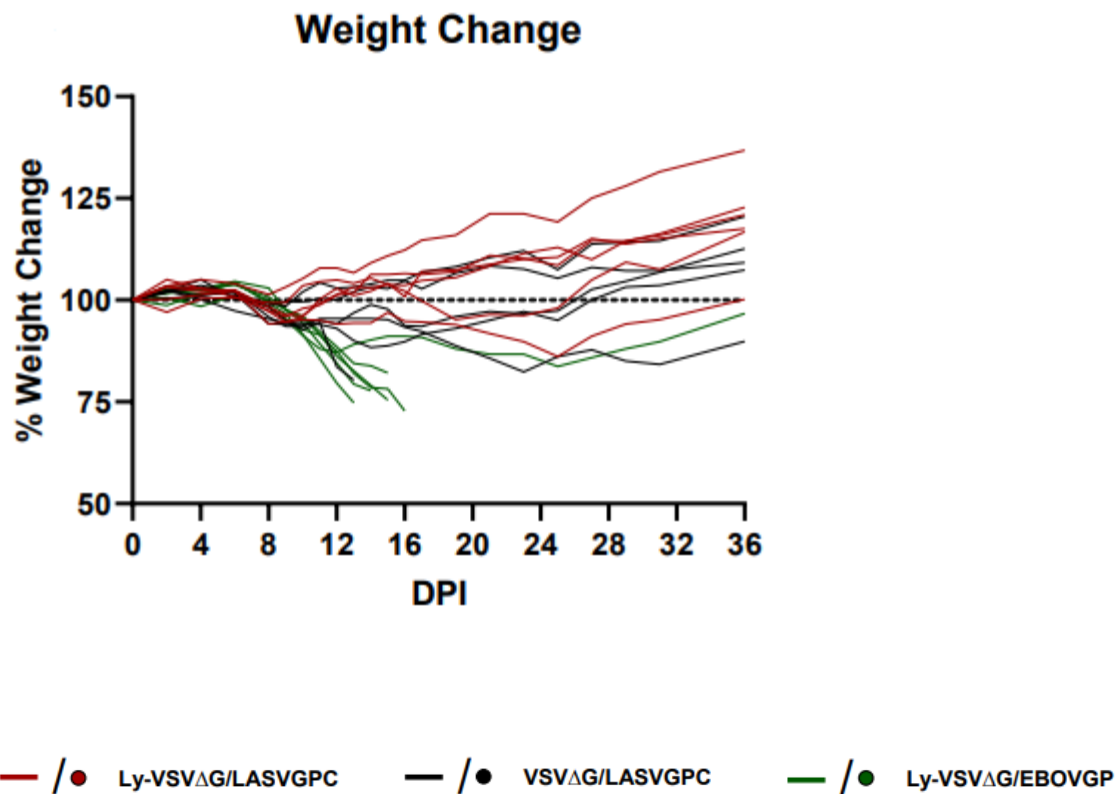


Figure 13. Percent weight change analysis for guinea pigs vaccinated with lyophilized rVSVΔG-LASV-GP (blue, N=10), lyophilized rVSVΔG-EBOV-GP (red, N=10), and rVSVΔG-LASV-GP (green, N=10), challenged with GPA-LASV.

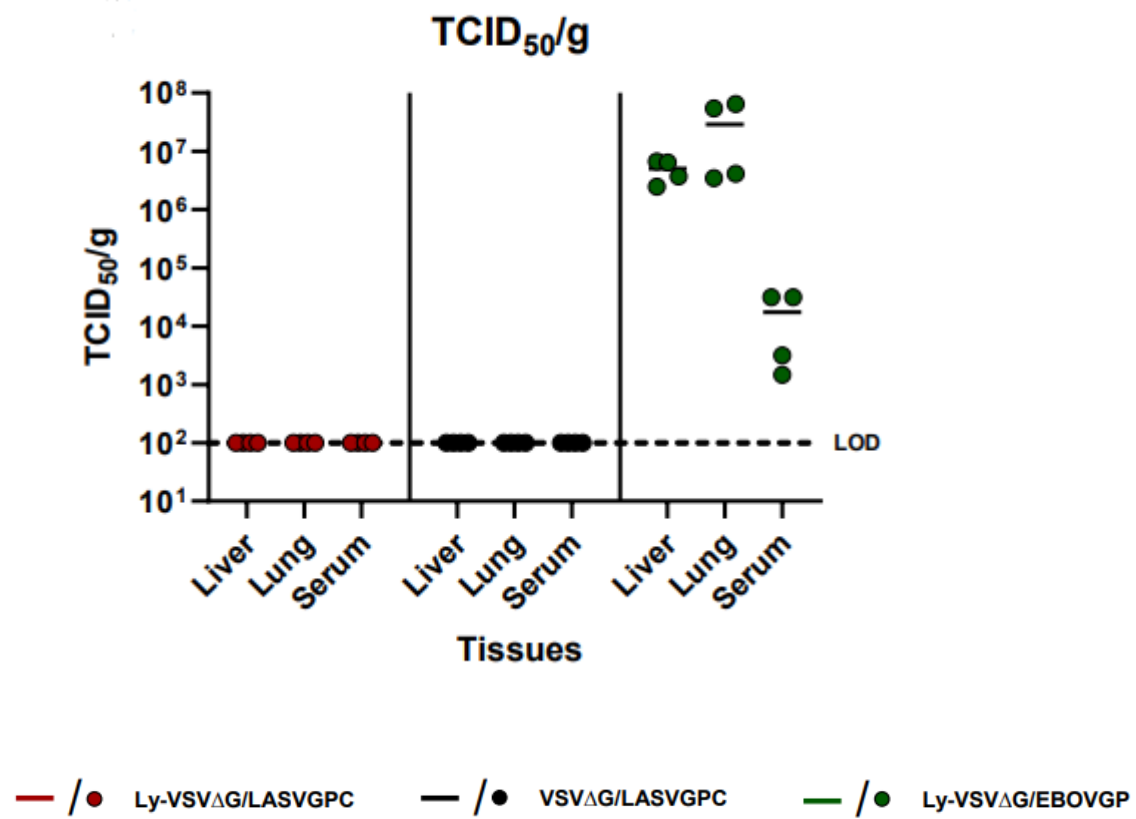


Figure 14. Tissues' virus analysis for guinea pigs immunised with lyophilized rVSVΔG-LASV-GP (blue, N=6), lyophilized rVSVΔG-EBOV-GP (red, N=6) and rVSVΔG-LASV-GP (green, N=6) vaccine, challenged with GPA-LASV.

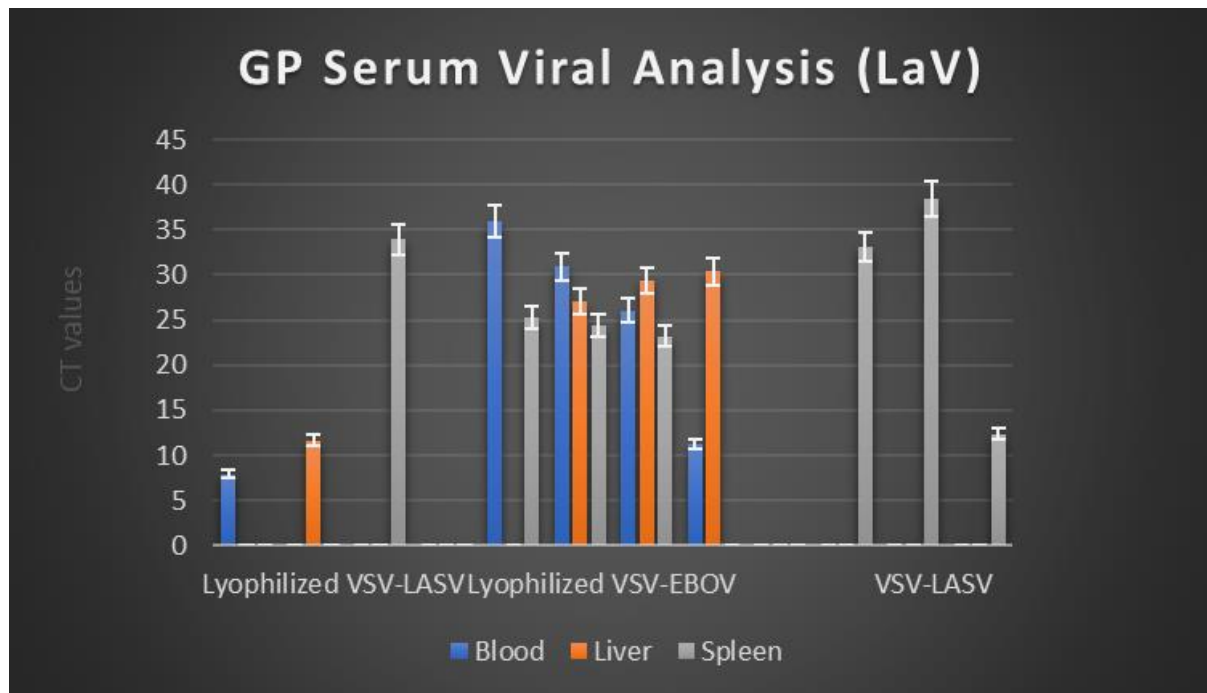


Figure 15. Real-time PCR analysis (LaV) for blood, liver and spleen collected from guinea pigs immunised with lyophilized rVSVΔG-LASV-GP (N=6), lyophilized rVSVΔG-LASV-GP (N=6) and rVSVΔG-LASV-GP (N=6) vaccines, challenged with GPA-LASV.

Lyophilized rVSVΔG-EBOV-GP immunised guinea pigs had higher CT value over other groups ($p > 0.05$). The high CT value from the tissues from the lyophilized rVSVΔG-EBOV-GP immunised group reflected the burden of disease experienced by this group.

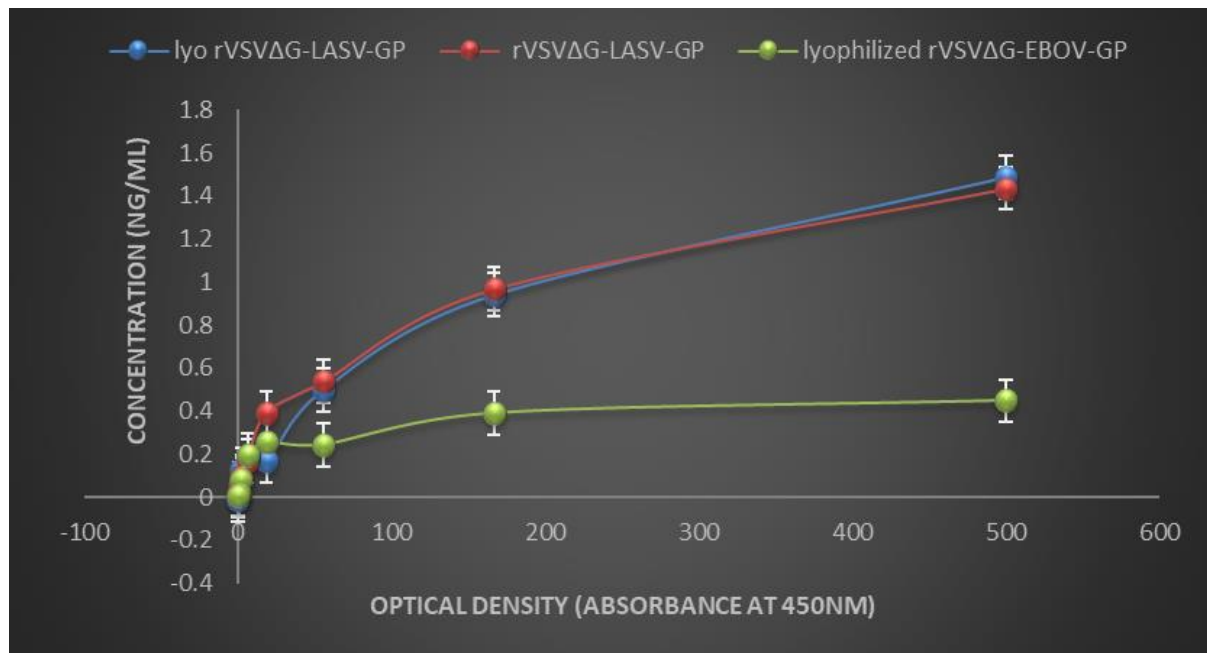


Figure 16. Antibody responses of vaccinated Guinea pigs. Guinea pigs were immunised with lyophilized rVSVΔG-LASV-GP (N=10), rVSVΔG-LASV-GP (N=10) or lyophilized rVSVΔG-EBOV-GP (N=10) vaccines. The optical density (absorbance) was read at 450nm, 5 minutes after addition of stop solution. Statistical significance was determined by one way ANOVA. *, $p = <0.05$; ***, $p = <0.0001$.

Chapter 4. Discussion

4.1 Development of rVSVΔG-LUJV-GP vaccine

Though LUJV is currently not in the news as one of the pathogens ravaging the World right now, the development of a vaccine against such a virus with high CFR warrants the immediate attention of researchers. All attempts to develop the Lujo vaccine were futile possibly due to the following reasons:

The GPC precursor of LUJV unlike other arenaviruses cleaved into the mature glycoproteins G1 and G2. However, The G1 of LUJV (essential for receptor interactions.) is shorter and very highly divergent than those of other arenaviruses (Charrel et al, 2008). This could be a reason for the difficulty in rescuing LUJV.

Also, the gene sequence of LUJV NP, a support plasmid essential for the rescue of LUJV is distinct from other arenaviruses at the amino acid and nucleotide levels. At the amino acid level, it differs by 41% from MOBV and LASV and 55% from TAMV. In the nucleotide region, it is 36% divergent from IPPYV and 43% from TAMV. This out-of-range divergence (<10-12% to >21.5% OW arenavirus species) places LUJV in a unique phylogenetic range (Bowen et al, 2000 and Emonet et al, 2006).

The unsuccessful rescue of the rVSVΔG-LUJV-GP vaccine despite many troubleshooting stagnated the progress of this aspect of my thesis. On top of the troubleshooting steps discussed in section 3.1.4, I ensured that cells were more than 90% viable and had enough time to recover following passaging, cells were 70-90% confluent before transfection, choice of lipid-based transfection procedure, confirmation that LUJV can be successfully rescued, and use of appropriate transfection reagents (Lipofectamine).

My original goal was to develop the rVSVΔG-LUV-GP vaccine, lyophilize it, evaluate its thermostability using TCID₅₀, and protective efficacy in Guinea pigs along with rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP. However, I moved on with lyophilization, thermostability testing, and protective efficacy evaluation of rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP in Guinea pigs.

4.2 Thermostability of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP

A vaccine is thermally stable if it can withstand suboptimal temperatures and retain some level of infectivity (OIE, 2018). However, for this discussion, the thermostability of vaccines is the ability of the product to remain potent at suboptimal temperature for a duration long enough to allow the immunization process in the absence of cold storage.

Lyophilized vaccines retain thermostability and shelf-life when exposed to elevated temperature (Lale *et al*, 2011). The thermostability of vaccines was initially assessed by TCID₅₀, defined as the dilution of a virus required to infect 50% of a given cell culture. Infectivity of the virus correlates to viral replication in cells to obtain a titer for a given virus stock (Karakus, Cramer, Lanz, and Yángüez, 2018). The goal of this project was to identify thermostable formulations of lyophilized VSV vaccines that will maintain potency in high-temperature regions like West Africa where adequate storage conditions for vaccines are hard to come by. Vaccination Nurses need to travel long distances to remote rural areas to vaccinate people who are at higher risk of infectious and chronic diseases than urban dwellers (Wang, 2014). The choice of stabilizers used for the lyophilization of rVSVΔG-LASV-GP used for the animal study was based on previous stability study (Bora *et al*, 2020). LS provided optimum stability for PPR vaccines (Sarkar *et al*, 2003; Mariner, Gachanja, Tindih, and Toye, 2017; Riyesh *et al*, 2011). It was, therefore, important to

find a stabilizer combination that retains the potency of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines used for prevention of Lassa fever and EVD in endemic, respectively, in hot climatic west African countries.

The stability of lyophilized rVSVΔG-LASV-GP varied with stabilizer combinations, temperature, and duration of exposure. At 4° C, there was a consistent level of virus recovery of the lyophilized rVSVΔG-LASV-GP vaccine with decreasing titers over 90 dpi for the three stabilizers. Comparatively, DMEM stabilized lyophilized rVSVΔG-LASV-GP vaccine lost recovery at 30 dpi (6.36 log₁₀). Of the three stabilizers, LS and LST displayed the best thermostability profile at 4° C over 90 dpi. However, LST and LSTG provided superior thermal stability over LS for the lyophilized PPR vaccine (Bora et al, 2020).

For rVSVΔG-EBOV-GP vaccines at 4°C, the three stabilizers and DMEM displayed a good viral recoverability profile over 90 dpi. The stability of DMEM indicates that lyophilization is enough to preserve the rVSVΔG-EBOV-GP vaccine at 4°C. ERVEBO®, the only licensed EBOV (Zaire) vaccine is recommended to be used immediately after thawing, if not used immediately, the vaccine may be stored for only 4 hours at room temperature (up to 25°C; 77°F) (Centre For Disease Control, 2021). Overall, LST had a statistically superior (p/0.0076) thermostability profile over other stabilizers. Also, the rVSVΔG-EBOV-GP vaccine is more thermodynamically stable than the rVSVΔG-LASV-GP vaccine.

At 21 °C, LS and LST stabilized both lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines for at least 90 dpi. LS and LST had better viral recovery over 90 dpi. Similarly, Mariner et al revealed statistically significant superiority of LS and LST for lyophilized PPR vaccines stored at 25 °C (Mariner, Gachanja, Tindih, and Toye, 2017). DMEM and LSTG lost infectivity after a few days and weeks, respectively.

At 37 °C, both lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines in the presence of the three stabilizers lost infectivity after 35 dpl. This is contrary to the immediate loss of infectivity of PPR vaccines stabilized with T in the presence or absence of L at 37 °C (Mariner, Gachanja, Tindih, and Toye, 2017). The three stabilizers had a similar degradation curve unlike the superior stability of LST and LSTG reported for lyophilized PPR at similar temperatures by Bora et al (Bora et al, 2020).

Altogether, the results obtained showed that LS, LST, and LSTG improved the stability of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP.

4.3 Protective efficacy of lyophilized rVSVΔG-LASV-GP

Previous studies demonstrated the protective efficacy of VSV-based LASV vaccines against non-homologous LASV infection in guinea pigs and macaques (Stein et al, 2019 and 2021; Safronetz et al, 2015). Also, the VSV-based LASV vaccine offered long-term protection against LASV infection in guinea pigs (Stein et al, 2019). The goal of this study was to assess the impact of lyophilization on the protective efficacy of the lyophilized rVSVΔG-LASV-GP vaccine in guinea pigs.

Following immunization and challenge of Hartley guinea pigs, animals vaccinated with lyophilized rVSVΔG-LASV-GP achieved statistically significant 100% protection (Figure 11, $p=0.0063$, Mantel-Cox); 83.3% of guinea pigs immunized with rVSVΔG-LASV-GP and 16.6% of guinea pigs immunized with lyophilized rVSVΔG-EBOV-GP (control) survived challenge.

Furthermore, no virus was detected in the tissues (liver, lung, and serum) examined, further suggesting that lyophilized rVSVΔG-LASV-GP offers sterile immunity to guinea pigs. The lack of clinical diseases like weight loss, fever, and detectable viral load in tissues afforded by the

lyophilized rVSVΔG-LASV-GP vaccine aligned with a similar study with rVSVΔG-LASV-GP vaccine (Safronetz et al, 2015; Morrison, 1989). The lyophilized rVSVΔG-EBOV-GP group exhibited significant weight loss with manifestation of infection suggesting lyophilized rVSVΔG-EBOV-GP offered no protection against LASV infection (Josiah strain). Similarly, there was a high infectious titer recovered from the lung, serum, and liver of the lyophilized rVSVΔG-EBOV-GP group. The highest viral titer recorded from the lungs may indicate the lung as the site of initial LASV replication. There was a high Viral RNA load in the blood, liver, and spleen, as evaluated by qRT-PCR.

4.4 Findings, conclusion, and Future Directions

The thermostability of vaccines diminishes rapidly in solution form. Lyophilization of vaccines promotes thermostability, even at elevated temperatures. There's increasing adoption of lyophilization by pharmaceutical companies as revealed by the number of currently licensed vaccines discussed in earlier chapters.

This study found that lyophilization reduced the degradation of rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP, even better in the presence of LS, LST, and LSTG. Moreover, rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines are stable for at least 90 days at 4 °C in the presence of LS, LST, and LSTG. This stability may indicate that lyophilized forms of these vaccines can be easily maintained during mass vaccination programs without the requirement for ultralow temperature storage, likewise during ring vaccination where vaccines are stored for extended periods until needed as in the case of ERBOV® in West Africa. LS and LST formulations offered better stability than LSTG and DMEM.

This proof-of-concept study can be further upscaled to evaluate the long-term thermostability (up to 2 years) of lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP vaccines and be applied to other vaccines. It will be ideal to have thermal stability data of reconstituted and stored lyophilized rVSVΔG-LASV-GP and rVSVΔG-EBOV-GP. Since protective efficacy was evaluated for vaccines stored at 4 °C for 7 days, it is important to evaluate the protective effects of vaccines stored above this temperature. Detailed knowledge of the thermostability of vaccines will reduce vaccine shortage, knowing fully well that a slight break in the cold chain may not affect vaccine potency, and save cost from unwarranted destruction of vaccines from short cold chain breach. More study is required to validate the protective efficacy of lyophilized rVSVΔG-LASV-GP vaccine against other geographically and genetically distinct strains in the GP model. Ultimately, this study needs to be evaluated in nonhuman primates (NHP) since there's a difference in the pathogenesis of LASV infection between NHPs and Guinea pigs.

4.5 **Limitations of this study**

1. This study evaluated the thermostability of lyophilized vaccines exposed to a suboptimal temperature for 90 days. This was practically assessing how long lyophilized vaccines will remain potent when vaccination teams on the field are without cold chain storage. The study failed to account for the thermal stability of reconstituted lyophilized vaccines to determine how long they are stable after reconstitution as always stipulated by vaccine manufacturers.
2. The study applied the rinderpest lyophilization approach used for LASV and EBOV vaccines. It failed to account for the outcomes that might have resulted from using other methodologies of lyophilization like Xerovac technology.

3. It would be ideal to assess the thermal stability of lyophilized VSV-based vaccines at temperatures higher than 37 °C (up to 50 °C), as this range could be encountered in some African countries where these vaccines are commonly required. A longer duration of storage of over 1 year will provide information about the long-term thermal stability of lyophilized vaccines.

Chapter 5. Appendixes

APPENDIX A: Commercial Reagents:

Vaccine Preparation

- Qiagen Plasmid *Plus* Giga Kit (Qiagen, Toronto, ON, Canada)
- TransIT-LT1 Transfection Reagent (Sigma- Aldrich, Oakville, ON, Canada) LUJV-GPC expression reagents and equipment.
- HyClone™ Dulbecco's Modified Eagle's Medium (Fisher Scientific, Ottawa, ON, Canada).
- HyClone™ Penicillin Streptomycin (100X) Solution (Fisher Scientific, Ottawa, ON, Canada).
- HyClone™ Fetal Bovine Serum (Fisher Scientific, Ottawa, ON, Canada).
- Gibco™ Opti-MEM Reduced Serum Medium (Fisher Scientific, Ottawa, ON, Canada).
- EVOS M5000 Imaging System (Fisher Scientific, Ottawa, ON, Canada) LUJV-GPC Western Blot reagents and equipment.
- QIAshredder homogenizers (Qiagen, Toronto, ON, Canada).
- Bolt™ (4X) LDS Sample Buffer (Fisher Scientific, Ottawa, ON, Canada).
- Ambion™ Nuclease-Free Water (Fisher Scientific, Ottawa, ON, Canada).
- Bolt™ 4-12% Bis-Tris Plus Gels (Fisher Scientific, Ottawa, ON, Canada).
- NuPAGE™ MES SDS Running Buffer (20X) (Fisher Scientific, Ottawa, ON, Canada)
- MagicMark™ XP Western Protein Standard (Fisher Scientific, Ottawa, ON, Canada).
- Invitrogen Mini Gel Tank (Fisher Scientific, Ottawa, ON, Canada).
- iBlot®2 Dry Blotting System (Fisher Scientific, Ottawa, ON, Canada)
- iBlot®2 NC Mini Stacks (Fisher Scientific, Ottawa, ON, Canada).
- Tween™ 20 (Fisher Scientific, Ottawa, ON, Canada).
- Odyssey® Blocking Buffer (PBS) (LI-COR Inc., Lincoln, NE, USA).

- Li-Cor® Odyssey Imaging System (LI-COR Inc., Lincoln, NE, USA).
- Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Fisher Scientific, Ottawa, ON, Canada).
- Gibco™ Phosphate Buffered Saline pH 7.2 (1X) (Fisher Scientific, Ottawa, ON, Canada).
- Bolt™ (4X) LDS Sample Buffer (Fisher Scientific, Ottawa, ON, Canada).
- Bolt™ 4-12% Bis-Tris Plus Gels (Fisher Scientific, Ottawa, ON, Canada).
- NuPAGE™ MES SDS Running Buffer (20X) (Fisher Scientific, Ottawa, ON, Canada).

Animal Cell Culture Reagents

- HyClone™ Dulbecco's Modified Eagle's Medium (Fisher Scientific, Ottawa, ON, Canada)
- HyClone™ Penicillin Streptomycin (100X) Solution (Fisher Scientific, Ottawa, ON, Canada)
- HyClone™ Dulbecco's Modified Eagle's Medium (Fisher Scientific, Ottawa, ON, Canada)
- HyClone™ Penicillin Streptomycin (100X) Solution (Fisher Scientific, Ottawa, ON, Canada)
- HyClone™ Fetal Bovine Serum (Fisher Scientific, Ottawa, ON, Canada).
- UltraPure™ L.M.P. Agarose (Low Melting Point) (Fisher Scientific, Ottawa, ON, Canada)
- Crystal Violet Biological Stain (Sigma- Aldrich, Oakville, ON, Canada)

Reverse Transcriptase PCR Reagents and Equipment

- RLT lysis buffer (Qiagen, Hilden, Germany)
- RNeasy mini kit (Qiagen, Hilden, Germany)
- Viral RNA mini kit (Qiagen, Hilden, Germany)
- Quantstudio3 Real Time PCR System (Fisher Scientific, Ottawa, ON, Canada)

APPENDIX B: Laboratory-made Reagents:

General Laboratory Buffers

All materials were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Ottawa, ON, Canada) unless otherwise stated.

-Phosphate Buffered Saline, pH 7.4

-8g of NaCl

-0.2g of KCl

-1.44g of Na₂HPO₄

-0.24g of KH₂PO₄

-Adjust the pH to 7.4 with HCl, autoclave

-Protein Denaturing Buffer

-50mM HEPES 8.8

-100mM DTT

-Fill to 4mL with 4% SDS

-Western Blot Running Buffer

-50mL NuPAGETM MES SDS Running Buffer (20X)

-Fill to 1L with RO water

-Western Blot Wash Buffer

-1L of laboratory prepared PBS pH 7.4

-1mL of Tween 20

-Western Blot Blocking Reagent

-20mL of Odyssey® Blocking Buffer (PBS) (LI-COR Inc., Lincoln, NE, USA)

-20mL of laboratory prepared PBS pH 7.4

-Western Blot Antibody Diluent

-20mL of Odyssey® Blocking Buffer (PBS) (LI-COR Inc., Lincoln, NE, USA)

-20mL of laboratory prepared PBS pH 7.4

-0.2% Tween 20

-0.1M glycine-HCl

-Fill to 1L with ddH₂O

-Adjust pH to 2.7, filter sterilise with 0.22µM filter

-Antibody Stabilising Buffer

-1M Tris-HCl

-Fill to 1L with ddH₂O

-Adjust pH to 9.0, filter sterilise with 0.22µM filter

-20% NaCl SafeBlue Stabilising Buffer

-20g NaCl

Bacterial Cell Culture

-LB Miller + 100 µg/mL Ampicillin broth

-10 grams Bacto-tryptone

-5 grams Bacto-yeast extract

-10 grams NaCl

-Fill to 1L with ddH₂O, autoclave

-Add 0.1grams of Ampicillin

-LB Miller + 100 µg/mL Ampicillin agar plates

-Add 20 grams of agar to the above recipe

-Fill to 1L with ddH₂O

-Autoclave and let cool down to ~60°C.

-Add 0.1 grams of ampicillin

-Pour into sterile petri plates

Animal Cell Culture

-Supplemented DMEM medium

-500mL HyClone™ Dulbecco's Modified Eagle's Medium (Fisher Scientific, Ottawa, ON, Canada)

-50mL of HyClone™ Foetal Bovine Serum (Fisher Scientific, Ottawa, ON, Canada), heat inactivated.

-5mL of HyClone™ Penicillin Streptomycin (100X) Solution (Fisher Scientific, Ottawa, ON, Canada)

APPENDIX C: Plasmid Sequences and primers:

pLUJV-GPWT sequence

ATGGGGCAGATTGTTGCCGTTTTCCAAGCTATCCCTGAGATTCTAAACGAGGCAATT
AACATTGTGATAATTGTGATCATAATGTTACCCCTCATCAAGGGCGTTTTCAATCTAT
ACAAATCCGGTCTTTTCCAGCTGGTCATCTTCTCCTACTCTGTGGCAAGCGTTGTGA
CTCATCATTTGCTATCAGGATTCAATCTGGAGACAGTTCATTTCAACATGTCACTGTTG
AGCTCCATCCCTATGGTCTCTGAACAGCAGCACTGCATCCAGCACAATCACAGCTCC
ATAACCTTCAGTTTGCTGACCAACAAGTCCGACTTGGAAGAGTGCAATTTACCCGC
CTGCAAGCTGTTGACAGGGTGATCTTTGATCTGTTTCAGAGAGTTTCATCACCGTGTT
GGGGATTTTCCTGTTACTTCTGATCTGAAATGCTCTCACAACACCAGCTACAGAGTG
ATAGAATATGAAGTCACCAAAGAGAGTCTCCCTCGATTGCAGGAAGCTGTGAGCAC

CTTGTTCCCAGACCTCCATCTCTCCGAAGATAGGTTTCTGCAAATTCAGGCCCATGAT
GACAAGAACTGCACTGGTCTACACCCATTGAACTACCTGAGGCTCCTGAAAGAGAA
TTCAGAAACCCACTACAAAGTGAGGAAATTAATGAAATTGTTTCAGTGGTCTTTGAG
TGATGAGACAGGTAGCCCTCTGCCAGGTGGACACTGTCTAGAAAGATGGCTCATCTT
TGCGTCTGATATAAAGTGCTTTGATAATGCAGCGATTGCCAAATGCAATAAAGAGCA
TGATGAGGAGTTCTGTGACATGTTGAGGCTATTTGACTACAACAAAGCAAGTATTGC
CAAGCTTAGGGGCGAAGCCTCAAGCTCGATCAACTTGTTGTCGGGGAGAATCAATG
CCATCATTTTCAGACACACTTTTGATGAGATCATCCCTCAAGAGGCTGATGGGCATAC
CATACTGCAATTATACAAAGTTCTGGTATTTAAACCATACAAAACCTGGGGATACATT
CTTTACCCCGATGTTGGCTGGTTAGTAATGGGAGTTACCTCAACGAAACAAAATTTA
CTCATGATATGGAAGATGAGGCAGATAAACTGCTGACCGAGATGCTAAAGAAAGAG
TATGTTAGGAGACAAGAGAAAACACCCATTACTCTGATGGACATACTGATGTTTAGT
GTCTCTTTCTATATGTTTTCTGTAACACTATGCATATGCAACATACCCACCCATCGGC
ACATCACAGGTTTGCCGTGTCCCAAACCACACAGACTCAGAAAGAATGGCACCTGT
GCCTGTGGCTTCTTTAAGTCAATAAACAGGTCCACTGGTTGGGCCAAACACTGA

pLUJV-GPWT Sequence

ATGGGGCAAATAGTGGCGGTATTCCAAGCTATCCCAGAAATACTGAACGAGGCTAT
AAATATAGTCATCATTGTTATCATAATGTTCAACCCTCATAAAGGGAGTATTTAATCTT
TACAAATCTGGCTTGTTCCAACCTGGTTATATTCCTGCTTCTTTGCGGTAAGCGATGCG
ACTCCAGTCTTCTTTCCGGTTTCAACTTGGAGACGGTTCATTTCAACATGTCTTTGCT
TAGTAGCATTCCAATGGTAAGTGAGCAACAACACTGCATCCAGCATAATCACTCATC
AATAACTTTCTCCCTCCTCACCAATAAGTCTGATCTTGAGAAATGCAACTTCACGCG
GCTTCAAGCTGTGATAGGGTTATCTTTGACCTCTTTGAGAGTTTCATCACCGCGTT

GGAGACTTTCCTGTTACGAGTGATTTGAAATGTTACATAATACGTCATATAGGGTG
 ATAGAATATGAGGTCACCAAGGAAAGTTTGCCACGGCTGCAAGAGGCAGTCAGCAC
 TCTTTTCCAGACCTGCACCTTTCAGAAGACCGATTTCTGCAAATACAAGCGCATGA
 TGATAAGAATTGCACGGGACTTCACCCGCTTAACACTTGC GCCTGTTGAAAGAGAA
 CTCAGAACTCACTACAAAGTACGAAAACCTTATGAAATTGTTTCAGTGGTCTCTGTC
 TGATGAGACCGGCAGTCCGTTGCCGGGAGGCCACTGCCTCGAAAGGTGGCTTATATT
 TGCTTCAGACATTAAGTGCTTTGACAATGCCGCCATCGCTAAGTGCAACAAGGAGCA
 TGATGAAGAATTTTGTGATATGCTTCGACTTTTCGACTATAATAAAGCGTCAATTGC
 AAAACTGAGAGGTGAAGCAAGCAGTTCAATTAACCTTGCTTTCTGGCAGAATCAATG
 CCATTATAAGTGACACTCTCCTCATGCGCTCTTCATTGAAGCGCCTTATGGGGATTCC
 GTATTGTA ACTATACGAAGTTTTGGTACTTGAACCATACTAAGTTGGGCATACACTC
 ACTCCCTCGCTGCTGGCTCGTAAGTAATGGTAGCTATCTCAATGAGACGAAGTTTAC
 GCATGACATGGAGGATGAAGCCGATAAGTTGTTGACTGAGATGCTGAAGAAGGAGT
 ATGTACGGCGACAGGAGAAGACACCAATCACCTGATGGATATCCTTATGTTCTCTG
 TCAGTTTTTACATGTTTTCTGTGACACTTTGCATCTGCAACATAACCAACGCACAGACA
 CATAACTGGGCTGCCCTGTCCCAAGCCCCACAGACTCCGCAAAAATGGCACGTGTG
 CGTGTGGTTTTTTTTAAGAGCATAAATCGATCTACGGGATGGGCGAAGCAT.

Primers Used for Amplification of pLUJV-GP Plasmids

Primer	Lot number	T _m
MluI-pLUJV-GPWT-F	304609215	68°C
MluI-pLUJV-GPCO-F	304609216	68°C
NheI-pLUJV-GPWT-R	304609217	67°C
NheI-pLUJV-GPCO-R	304609218	67°C

AvrII-pLUJV-GPWT-R	304609219	65°C
AvrII-pLUJV-GPCO-R	304609220	65°C

Restriction Endonucleases for pLUJV-GP

Name	Cat.No.	Incubation Temp.	Heat inactivation
MluI	R0198S	37°C	65°C
NheI	R0131L	37°C	65°C
AvrII	R0174L	37°C	80°C
NEBuffer 2	B7002S		-

- TransIT-LT1 Transfection reagent (MIR2300, lot12034003)

-Opti-MEM I Reduced-Serum Medium

Primers for Cloning rPCAGGS

Lot No	Name	Tm(°C)
318811947	MluI-LUJV-GPWT-F	68.6
318811948	MluI-LUJV-GPWT-R	67.3
318811949	NheI-LUJV-GPCO-F	68.4
318811950	NheI-LUJV-GPCO-R	67.5

- PrimeSTAR Premix (2X) (AHE1012A)

PLUJV-GP PCR amplification reagents

Target	LaV	LaS
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Primer lot #	202209	201904
Probe lot #	202209	202209
Taqpath 4X	01318205	

PLUJV-GP PCR amplification cycling conditions

Cycling conditions Target = **1.4kb**

	Temp	Time	Cycle(s)
Initial denaturation	94°C	60s	25
Denature	98°C	10s	25
Anneal	62°C	10s	25
Extend	72°C	40s	25
Final extension	72°C	120s	1

-

Appendix D

Abbreviations

ICTV	International Committee on Taxonomy of Viruses
LCMV	Lymphocytic Choriomeningitis Virus
IGR	Intergenic Regions
L	Large
S	Small
Z	Matrix Protein
GP	Glycoprotein
NP	Nucleoprotein
LUJV	Lujo Virus
ALLV	Allpahuayo Virus
FLEV	Flexal Virus
PARV	Parana Virus
PICV	Pichinde Virus
PIRV	Piritital Virus

CHPV	Chapare Virus
GTOV	Guanarito Virus
JUNV	Junin Virus
MARV	Marburg Virus
MACV	Machupo Virus
SABV	Sabia Virus
TCRV	Tacaribe Virus
CPXV	Cupixi Virus
AMAV	Amapari Virus
LATV	Latino Virus
OLVV	Oliveros Virus
WWAV	Whitewater Arroyo Virus
BCNV	Bear Canyon Virus
TAMV	Tamiami Virus
NHP	Non-human Primates
CFR	Case Fatality Rate
WHO	World Health Organization

EVD	Ebola Virus Disease
DRC	Democratic Republic of Congo
VS	Vesicular Stomatitis
VS NJV	Vesicular Stomatitis New Jersey Virus
VS IV	Vesicular Stomatitis Indiana Virus
L	Lactalbumin Hydrosylate
IP	Intraperitoneal Route
TCID	Tissue Culture Infective Dose
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
qPCR	Quantitative Polymerase Chain Reaction
NML	National Microbiology Laboratory
GPA	Guinea Pig Adapted
CPE	Cytopathic Effect
DMEM	Dulbecco's Modified Eagle Medium
S	Sucrose

T	Trehalose
G	Gelatin
Tg	Glass Transition Temperature
DPV	Days Post Vaccination
DPI	Days Post Infection

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