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Faculty of Health Sciences

School of Medicine

Development and evaluation of a recombinant antigen-based indirect immunofluorescence assay for the diagnosis of Lassa fever

The proposal is submitted in partial fulfilment of the requirements for the degree M.Sc. in Medical Virology, Centre for Viral Zoonoses, Department of Medical Virology, Faculty of Health Sciences, University of Pretoria.

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DECLARATION

I, Jason Gregory Bell

, declare that this dissertation, which I submit for the degree M.Sc. Medical Virology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Jason Gregory Bell

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ABSTRACT

Lassa virus (LASV) forms part of the *mammarenavirus* genus from the *Arenaviridae* family and is the arenavirus with the most significant public health impact. It is endemic to West Africa as one of seven mammarenaviruses capable of causing viral haemorrhagic fever (VHF). Outbreaks of LASV in West Africa appear annually, infecting 300 000 people with a case fatality rate of <2%. Clinically, LASV causes diverse symptoms, reasoning it indistinguishable from other febrile diseases, complicating clinical diagnosis and reliability. LASV is classified as a biosafety level 4 (BSL-4) pathogen since it causes VHF. The live virus can be handled only in a high-containment laboratory, of which there are few facilities globally, encumbering diagnostic work. LASV diagnostics are hampered because of unaffordable rapid, validated diagnostic serological tests. A need exists to develop improved serological tests for use outside of containment facilities. This would allow accessible and quicker diagnosis, with the speedy implementation of preventive measures, such as the quarantine of infected patients, limiting the spread of the virus. An immunofluorescence assay (IFA) is a diagnostic device using fixed virus-infected mammalian or insect cells expressing a specific antigen to detect antibodies by microscopy. IFAs offer quick results, crucial for suspected VHF cases as the spread of the virus can be prevented. Recombinant technology can be employed in developing diagnostic assays for VHFs as a safer alternative for assays reliant on live virus culture. These recombinant diagnostic assays could be designed and implemented outside high-containment facilities once infectious samples were inactivated. During this study, indirect IFA tests were developed, using recombinant LASV nucleoprotein (NP) and LASV glycoprotein (GP) as individual antigens. We then evaluated their utility to approach the need for a quick, standardised serological test for LASV diagnosis. Expression cassettes for recombinant expression of the LASV NP and LASV GP in mammalian cells were designed and developed. The LASV NP and LASV GP were chosen for this project, forming the major structural proteins for LASV, as most antibodies target these proteins. The study data disclose the successful expression of these recombinant proteins. A LASV NP stable cell line was also generated. This stable cell line would allow the IFA to be standardised, removing the batch-to-batch variation with recombinant antigen IFA slides prepared by transiently transfected cells. With transiently expressed LASV GP mammalian cells, and the stable LASV NP mammalian cells we developed IFA slides.

Indirect IFAs based on these recombinant antigens were examined for their ability to detect Lassa-specific antibodies in patient serum. The results were compared to a LASV IFA using virus-infected cells. The study results present a compelling correlation among the assays regarding Lassa-specific antibody detection in a panel of known Lassa antibody-negative samples, such as detection accuracy. When the LASV NP and LASV GP IFAs were used in combination on the panel of known Lassa negatives, 90% of the samples tested negative using recombinant LASV antigen-based IFAs. The virus-infected cell-based IFA accurately identified negative samples in 96.67% of the cases. The recombinant antigen-based assays were established as less sensitive (five-fold less) than the virus-infected cell-based IFA. This is anticipated since whole-virus antigen extracts allow antibody detection against any viral protein and are not restricted to specific antigens, accounting for better sensitivity. Conjointly, although limited, our initial evaluation of the recombinant LASV antigen-based indirect IFA displays it is a method that can detect LASV antibodies; it is comparable in its reliability and accuracy to the whole virus-based IFA. Based on this, the recombinant protein-based LASV IFA warrants further development and evaluation as a diagnostic device for Lassa fever diagnostics.

Keywords: Lassa virus, Immunofluorescence assay, recombinant technology, stable cell line, nucleoprotein, glycoprotein, serological assays, accuracy, Western blot, diagnosis

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LIST OF ABBREVIATIONS

µL	microlitre
ADB	agarose dissolving buffer
AMP	ampicillin
APC	antigen-presenting cells
APES	Academic and Professional Editing Services
APS	ammonia persulfate
BHK-21	baby hamster kidney cells
BSA	bovine serum albumin
BSL-4	Biosafety level 4
CCHF	Crimean-Congo haemorrhagic fever
CDC	Center for Disease Control
cDNA	complementary DNA
CHO	Chinese hamster ovary
CI	confidence interval
CMV	cytomegalovirus
CSF	cerebral spinal fluid
CVZ	Centre for Viral Zoonosis
DC	dendritic cells
DeDD	death effector domain, containing protein

DG	dystroglycan
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
EBOV	Ebola virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FP	forward primer
G-418	geneticin
GFP	green fluorescent protein
GP	glycoprotein
GPC	glycoprotein precursor
HBG	Henneberg-Sander GmbH
HEK293	Human Embryonic Kidney cells
His	histidine

HRP	horseradish peroxidase
HUDSON	heating unextracted diagnostic samples to obliterate nucleases
ICTV	International Committee on Taxonomy of Viruses
IFA	immunofluorescence antibody assay
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IGR	intergenic noncoding region
IRF-3	INF regulatory factor 3
kb	kilobases
L protein	RNA-dependant RNA polymerase
L segment	large segment
LASV	Lassa virus
LB	Luria-Bertani
LOD	limit of detection
M. natalensis	*Mastomys natalensis*
MBS	membrane binding solution
MCS	multiple cloning site
MDA-5	melanoma differentiation-associated gene-5
MDCK	Madin-Darby canine kidney
MP	macrophages

mRNA	messenger ribonucleic acid
NaOAc	sodium acetate
NB	neutralisation buffer
NCBI	National Center for Biotechnology Information
ng	nanogram
NHLS	National Health Laboratory Services
NICD	National Institute for Communicable Diseases
NK	natural killer
NP	nucleoprotein
NPV	negative predictive value
NRF	National Research Foundation
ORF	open reading frames
PCR	polymerase chain reaction
PML	promyelocyte leukaemia
PPV	positive predictive value
RDT	rapid diagnostic tests
RE	restriction enzyme
ReLASV	recombinant Lassa virus
RIG-1	retinoic acid-inducible gene-I
RNA	ribonucleic acid
RNP	ribonucleoprotein

RP	reverse primer
RPA	recombinase polymerase amplification
RT	reverse transcription
RT-LAMP	reverse transcription loop-mediated isothermal amplification assay
RT-PCR	reverse transcription-polymerase chain reaction
S segment	small segment
S1P	site 1 protease
SDS	sodium dodecyl sulphate
SKI-1	subtilisin kexin isozyme-1
SOC	super optimal broth with catabolite repression
ssDNA	single-stranded DNA
SSP	stable signal peptide
SVPL	Special Viral Pathogens Laboratory
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
UTR	untranslated region
UV	ultraviolet
VHF	viral haemorrhagic fever
WB	Western blot
Z protein	RING-finger zinc-binding protein

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction and taxonomy

According to the International Committee on Taxonomy of Viruses (ICTV), the *Arenaviridae* family belongs to the order, *Bunyavirales*, comprising 50 virus species. It is divided into four genera, indicating the *antennavirus*, *hartmanivirus*, *mammarenavirus*, and *reptarenavirus* (Figure 1.1) (Radoshitzky et al., 2015, Maes et al., 2018, Radoshitzky et al., 2019a). The *antennavirus* genus is the newest to be described, comprising two viral species, and is established in fish (Shi et al., 2018, Radoshitzky et al., 2019a). The *hartmanivirus* genus comprises four viral species found in captive snakes (Radoshitzky et al., 2019a). The *reptarenavirus* genus is found in reptiles and includes five species (Radoshitzky et al., 2015, Radoshitzky et al., 2019a). The *mammarenavirus* genus is the largest of the four and contains viruses established in mammals, representing 39 species (Radoshitzky et al., 2015, Radoshitzky et al., 2019a).

Seven of the known arenaviruses in this genus cause viral haemorrhagic fever (VHF) (Charrel et al., 2003, Bausch et al., 2014), such as Lujo, Chapare, Machupo, Junin, Sabia, Guanarito, and Lassa virus (LASV); these viruses are classified as biosafety level 4 (BSL-4) pathogens (Hummel et al., 1992, Charrel et al., 2003, Bausch et al., 2014). The *mammarenavirus* genus is grouped geographically, phylogenetically, and serologically into New World (Tacaribe serocomplex) and Old World (Lassa-lymphocytic choriomeningitis serocomplex) complexes. Members of the Old World group are predominantly established in Africa but can also be found in Europe, Asia, and Oceania (Bausch et al., 2014). Those belonging to the New World group are primarily found in America (Bausch et al., 2014).

Mammarenaviruses are zoonotic and are most often maintained in the environment through persistent infections of rodents, which can be established globally (Charrel et al., 2003, Bausch et al., 2014). In the past 10 to 15 years, novel arenaviruses–pathogenicity unknown, were identified in multiple rodent species and other non-volant mammals in East and West Africa (Bausch et al., 2014, Radoshitzky et al., 2015). In 2008, a novel and highly pathogenic *mammarenavirus* was diagnosed in Johannesburg, South Africa, called the Lujo virus (Briese et al., 2009, Paweska et al., 2009, Kerber et al., 2015). The first infection with the virus was found near Lusaka, Zambia. The patient was evacuated to Johannesburg, leading to four other

infections, with only one patient surviving (Briese et al., 2009, Paweska et al., 2009). The reservoir species for Lujo is still unknown (Paweska et al., 2009, Bausch et al., 2014, Grobbelaar et al., 2021).

The mammarenavirus with the most significant public health impact (300 000 cases annually) is LASV. It is endemic to Western Africa, indicating Guinea, Sierra Leone, Mali, Liberia, and Nigeria (Frame et al., 1970, Ogbu et al., 2007, Fischer et al., 2021, Yaro et al., 2021). LASV was first recovered from a patient living in Lassa, Nigeria, infected with an unknown viral disease in 1969 (Frame et al., 1970). According to anecdotal records, cases described in Nigeria following Lassa fever date back to 1952 (Bausch et al., 2014). Sporadic LASV cases have been reported in Benin and Togo Phylogenetic LASV analysis revealed great genetic diversity presented by at least seven LASV lineages, with three (Lineage I, II, III) present in Nigeria (Bowen et al., 2000, Whitmer et al., 2018, Olayemi et al., 2020).

Lineage IV lead to Lassa fever in Sierra Leone, Liberia, Guinea, and Mali (Bowen et al., 2000). Strains isolated in Mali and Côte d'Ivoire were assigned to lineage V (John Tyler et al., 2015, Whitmer et al., 2018, Fischer et al., 2021). The new Kako strain of LASV isolated from *Hylomyscus pamfi (*African wood mouse) rodents in Nigeria makes up Lineage VI, and Lineage VII, comprising a virus identified during a 2016 outbreak in Togo (Olayemi et al., 2016, Whitmer et al., 2018, Fischer et al., 2021). Figure 1.1 also demonstrates the diversity of the seven LASV lineages.

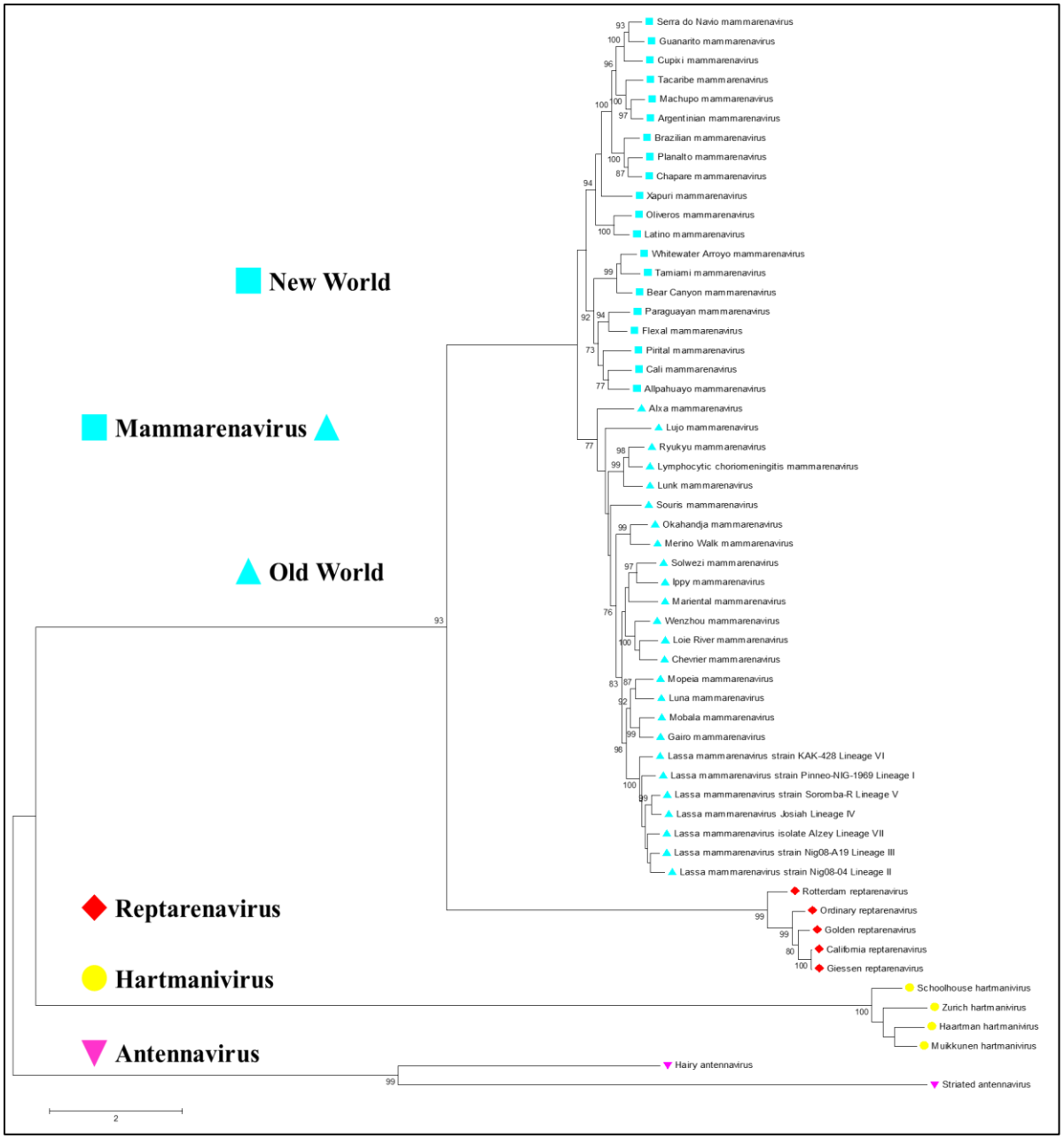

Figure 1.1: A maximum likelihood phylogenetic tree showing the diversity within the *Arenaviridae* family and LASV lineages.

The maximum likelihood phylogenetic tree was generated using the nucleoprotein sequences for all 50 virus species within the family using the Mega 6 program (Tamura et al., 2013) with a General Time Reversibility model and a discreet Gamma distribution. Bootstrap values above 70% are shown. The *mammarenavirus* (blue square and triangle) family is displayed at the top of the tree, with the Old (Triangle) and New (Square) World members being split. The LASV lineages (V-VII) are shown at the bottom of the Old World mammarenavirus branch (blue triangles). The *reptarenavirus* is displayed with the red diamonds, and the *hartmanivirus* is presented with the yellow circles. The *antennavirus* is indicated with the purple reverse triangle. All the sequences used were obtained from GenBank. The accession numbers can be corroborated in Appendix A.

1.2 Genome structure, viral replication, transmission, and epidemiology

1.2.1 Genome structure

Members of the *mammarenavirus* genus are spherical or pleomorphic with a diameter ranging from 50 nm to 300 nm, surrounded by a lipid membrane (Buckley et al., 1970, Speir et al., 1970, Charrel et al., 2003). The total genome size of the virus is 11.6 kb. These genomes comprise two single-stranded, ambisense ribonucleic acid (RNA) molecules, signifying one small (S) and one large (L) segment, each coding for two gene products (Figure 1.2) (Barber et al., 1990). The ambisense coding strategy used by each segment directs the synthesis of two proteins from non-overlapping open reading frames (ORF) in opposite directions (ambisense coding arrangement) (Cai et al., 2020, Cubitt et al., 2020).

Coding regions are separated by intergenic noncoding regions (IGR) (Perez et al., 2003, Hass et al., 2004). The IGR, found in both segments of the virus, can form hairpin structures (Zaza et al., 2018). The IGR also functions in both virion assembly and budding, and acts as a structure-dependent transcription terminator (Meyer et al., 1993, Pinschewer et al., 2005). The small (S) and large (L) segments 3' and 5' ends contain noncoding untranslated regions (UTRs), comprising conserved reverse complementary sequences of about 19 to 30 nucleotides (Auperin et al., 1982). These complementary sequences form panhandle structures (Salvato et al., 1989). The 3' and 5' UTR on both the L and S segment contains the genomic promoters, directing RNA replication and transcription (Perez et al., 2003, Hass et al., 2006).

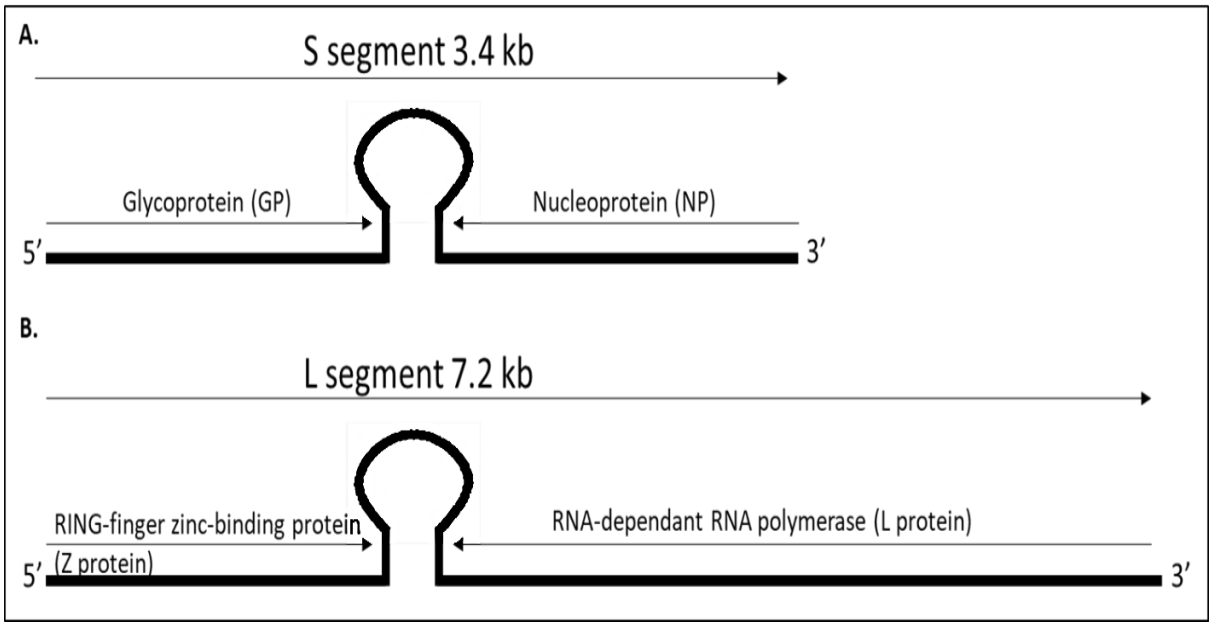

Figure 1.2: Mammarenavirus RNA genome structure.

A) The S segment of the mammarenavirus is 3.4 kb and codes for the nucleoprotein (NP) in the antisense direction and glycoprotein (GP) in the sense direction (Bowen et al., 2000, Günther et al., 2004). The GP is post-translationally cleaved into viral envelope proteins, GP1, GP2 and the stable signal peptide (SSP) (Bowen et al., 2000, Charrel et al., 2011). B) The L segment is 7.2 kb and codes for the RING-finger zinc-binding protein (Z protein) (sense direction) and the RNA-dependant RNA polymerase (L protein) (antisense direction) (Günther et al., 2004). Figure adapted from Günther et al.(Günther et al., 2004).

The S segment codes for the two structural proteins, indicating the GP in the genome sense sequence and nucleoprotein (NP) in the complementary genome sequence (Bowen et al., 2000, Günther et al., 2004). The GP forms the virion spikes and is responsible for receptor binding and cell entry (Speir et al., 1970, Bowen et al., 2000, Fedeli et al., 2018). The spike structures are typically between 8 nm to 10 nm, regularly spaced on the viral surface (Charrel et al., 2003, Radoshitzky et al., 2019a, Radoshitzky et al., 2019b). The GP gene produces the GP precursor (GPC) molecule–post-translationally, cleaved by the signal peptidase enzyme, creating the SSP and an additional precursor protein (Loureiro et al., 2019, Radoshitzky et al., 2019a, Radoshitzky et al., 2019b).

The cellular protease enzymes, subtilisin kexin isozyme-1 (SKI-1) or site-1 protease (S1P), process this to produce the mature surface GPs, GP1 and GP2 (Loureiro et al., 2019, Radoshitzky et al., 2019a, Radoshitzky et al., 2019b). The GP1, GP2 and SSP jointly form the mature trimeric GP complex (Loureiro et al., 2019, Radoshitzky et al., 2019b). GP1 interacts

with the surface receptor of the host cell and is the attachment protein (Günther et al., 2004, Radoshitzky et al., 2019b). GP2 facilitates viral fusion with the host cell membrane and is the transmembrane protein (Günther et al., 2004, Radoshitzky et al., 2019b). The SSP contributes to processing GPC, assuming a function in the GP2 pH-dependant fusion process (Radoshitzky et al., 2019b).

NP is the most abundant protein produced by LASV, comprising a major structural protein by forming the nucleocapsid (Pinschewer et al., 2003, Günther et al., 2004). The NP is a multifunctional protein binding to the viral RNA, forming the ribonucleoprotein (RNP) complex (Loureiro et al., 2019, Papageorgiou et al., 2020). The NP also creates a bead-like structure with the viral RNA and is essential for viral replication and transcription (Pinschewer et al., 2003, Günther et al., 2004). Most antibodies produced by the host immune system have been shown to target the structural proteins of the virus (Günther et al., 2004, Russier et al., 2012).

The L protein, encoded by the L segment in the complementary sequence (antisense), is a multi-domain protein responsible for conducting the replication and transcription of the virus (Leung et al., 1979, Fuller-Pace et al., 1989, Garcin et al., 1992, Peng et al., 2020). The Z protein is coded for in the sense direction on the L segment and is the smallest protein, produced by LASV, belonging to the RING-finger protein family; it acts as a bridge between the surface GP, RNP and host cell budding machinery (Hastie et al., 2016, Shao et al., 2018). The Z protein drives arenavirus budding, and through interactions with the L protein, inhibits RNA synthesis (Perez et al., 2003, Strecker et al., 2003, Shao et al., 2018).

1.2.2 Viral replication

During an infection, Old World mammarenaviruses (and some clade C New World mammarenaviruses) enter the host cells by binding to the cell-surface receptor, α-dystroglycan (α-DG) and are absorbed into the host cells by endocytosis (Borrow et al., 1994, Kunz et al., 2005, Martinez et al., 2007). α-DG is a peripheral membrane protein ubiquitously expressed on several cells and highly expressed on dendritic cells (DC) and macrophages (MP). It functions as an anchor between the subcutaneous cytoskeleton and the extracellular matrix (Rita et al., 2006, Oldstone et al., 2011). The pH-dependant fusion with the endosome releases the RNP inside the host cell, containing the NP, L protein and viral RNA, into the cytoplasm

(Pedersen, 1979, Radoshitzky et al., 2015). The RNP complex drives the RNA genome replication and transcription inside the cytoplasm (Hass et al., 2004).

During replication, the L protein generates full-length uncapped copies of the genomic and antigenomic RNA (Leung et al., 1977, Bausch et al., 2014). The ambisense nature of the genome allows the antigenomic and genomic RNAs to be used for transcription of the viral mRNA by acting as templates, capped but not polyadenylated (Hass et al., 2004, Bausch et al., 2014, Radoshitzky et al., 2015). Transcription only occurs after one round of replication–the transcription of each gene is terminated when the L protein reaches the IGR (Meyer et al., 1993, Radoshitzky et al., 2015). Genomic RNA is packaged with small amounts of antigenomic RNA, Z protein mRNA, and NP (Bausch et al., 2014). The virus particles leave the target cell by budding from the plasma membrane, forming the viral envelope (Radoshitzky et al., 2015).

1.2.3 Epidemiology and transmission

The Lassa virus is endemic to West Africa, sustained through persistent infections of the reservoir host, *Mastomys natalensis (*Natal mastomys) (Monath et al., 1974, Grobbelaar et al., 2021). The virus was first isolated from the reservoir host after an outbreak of Lassa fever in Sierra Leone in 1972 (Monath et al., 1974). The reservoir host is the Natal multimammate mouse–one of the most common and widespread mammals in sub-Saharan Africa, inhabiting most habitats except dense forests, desserts, and high mountainous areas (Colangelo et al., 2013, Gryseels et al., 2017). Upon analysis of the cytochrome b sequences of the species throughout Africa, it is observed that the *M. natalensis* species is divided into six subtaxa (Figure 1.3) (Colangelo et al., 2013, Gryseels et al., 2017, Olayemi et al., 2020, Grobbelaar et al., 2021). These subtaxa are separated into two clades–clade A and B, with clade A (A-I to A-III) mostly present in West and Central Africa (Colangelo et al., 2013). Clade B (B-IV to B-VI) is primarily in East and South Africa (Colangelo et al., 2013, Grobbelaar et al., 2021).

Figure 1.3 displays the geographical distribution of these subtaxa, emphasising that subtaxa A-I is established in the regions where Lassa fever commonly occurs (Gryseels et al., 2017). A plausible reason for LASV not spreading to other parts of Africa may be geographical barriers, such as rivers, mountains, forests and desserts, and the genetic differences among the rodent species. This may contribute to preventing the spread of LASV (Gryseels et al., 2017). LASV was also isolated from various rodent species, such as *Mastomys erythroleucus* (Guinea

multimammate mouse), *Hylomyscus pamfi* (African wood mouse), *Mus baoulei* (Pygmy mouse) (Kronmann et al., 2013, Olayemi et al., 2016, Yadouleton et al., 2019, Olayemi et al., 2020). No cases of LASV transmission from these species to humans were reported (Ibukun, 2020).

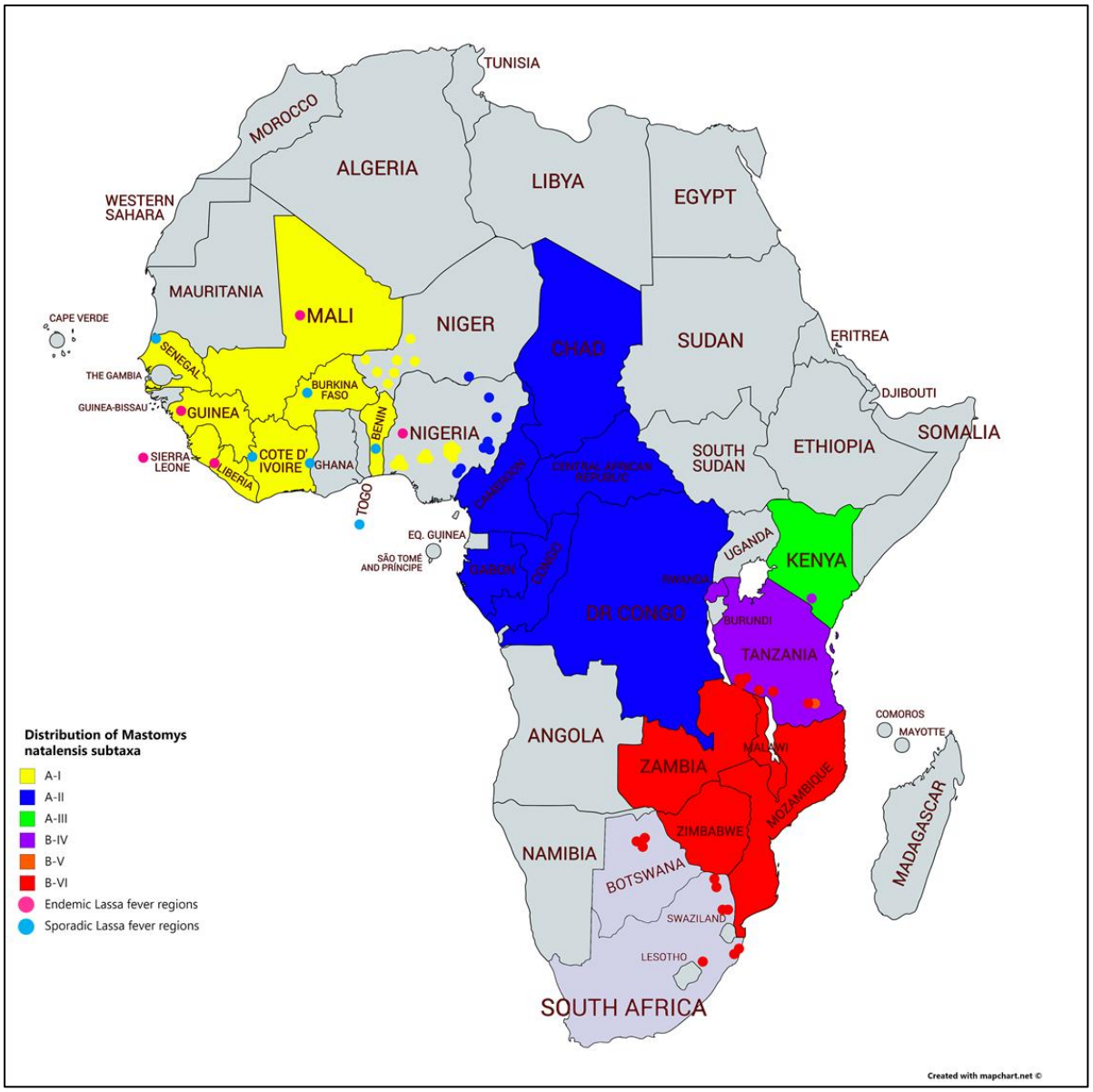

Figure 1.3: The distribution of the six Mastomys natalensis subtaxa throughout Africa and the areas affected by Lassa fever.

The pink dots show the regions where Lassa fever is endemic, and from the map, it is seen that the subtaxa A-I is dominant in these areas, showing it is the primary reservoir host of LASV. The light blue dots indicate regions where sporadic cases of Lassa fever were reported. This figure was adapted from Kerber 2015 and Gryseels 2017 (Kerber et al., 2015, Gryseels et al., 2017).

Humans are often infected with LASV when they encounter infected blood and tissue through hunting or eating rodents, or after inhaling released particles from rodent saliva, faeces, and urine (Masayuki et al., 2012). Rodents absorb the virus through the gastric mucosa, where early viral dissemination is associated with the cells through the lymphatic system (Günther et al., 2004). Rodents shed the virus in saliva, blood droplets and urine for a few days or months depending on the age of the rodents when infected (Walker et al., 1975, Olayemi et al., 2020). Studies indicate that persistent infection is established when the rodents are infected as neonates (newborn rodents), but adult rodents can clear the virus (Walker et al., 1975, Bausch et al., 2014). The domestic and peri-domestic behaviour of rodents is also a significant factor in the viral transmission to humans (Charrel et al., 2011).

Human transmission can occur after agricultural or recreational movements into the habitats of the rodent host (Charrel et al., 2011). The rodents also enter the urban areas searching for food (Charrel et al., 2011). In the endemic regions, the rodents are hunted and eaten by humans, providing another transmission source for LASV to humans (David et al., 2012). The virus spreads rapidly from one human to another through direct contact of the mucous membranes with infected secretions (blood, saliva, urine, semen, or vomit). Nosocomial infections are common during patient care when caregivers and health workers handle infected blood or secretions of a patient (Fichet-Calvet, 2014).

Those most at risk of infection reside in overcrowded rural areas, where *M. Natalensis* is established (Ogbu et al., 2007). Health workers and researchers also risk infection if the proper infection control and biosafety measures are not applied (Raabe et al., 2017). Lassa fever outbreaks can occur at any time during the year. It is possible for asymptomatic or recently infected patients (still incubating the virus) to transport Lassa fever to non-endemic areas from the endemic regions (Günther et al., 2004, Ogbu et al., 2007). Historically, LASV outbreaks in endemic countries occur during the dry seasons (November to March) possibly due to an increase in interactions between the reservoir host and the humans during this period (Olayemi et al., 2020).

LASV infects 500 000 people annually and has a case fatality rate of <2% in endemic regions (Bausch et al., 2014, Meyer et al., 2018). This fatality rate rises to 15% to 20% for those admitted to a hospital attributable to severe infection, and 30% for pregnant women. During nosocomial infections, it can be as high as 50% (Ogbu et al., 2007, Raabe et al., 2017). In

Nigeria, the most recent outbreaks had case fatality rates ranging from 20% to 25% (Sattler et al., 2020).

1.3 Clinical manifestations

The Lassa virus has an incubation period ranging from seven to 21 days (Frame et al., 1970, Walker et al., 1975). The disease is linked with a wide range of clinical symptoms, including flu-like symptoms, haemorrhaging, and neurological manifestations (Ogbu et al., 2007, Shaffer et al., 2014). The early symptoms of Lassa fever are general and flu-like, characterised by fever, malaise, severe headaches, and general weakness (Figure 1.4) (McCormick et al., 1987). Other non-specific symptoms that may develop include gastrointestinal manifestations, such as nausea, vomiting, and diarrhoea (McCormick et al., 1987, David et al., 2012). More severe symptoms that can develop during the disease, include abnormal bleeding, multi-organ failure, pulmonary oedema, respiratory distress, encephalopathy, hypotension, and temporary or permanent deafness (Figure 1.4) (Frame et al., 1970, Raabe et al., 2017).

Most LASV infections (80% of cases) remain subclinical and do not progress further than showing mild symptoms. In some cases (20%), the patient will develop more severe symptoms and may encounter death (Sogoba et al., 2012). In patients surviving the disease, symptoms usually disappear after 10 to 15 days (Ogbu et al., 2007). LASV and other pathogenic arenaviruses' symptoms are non-specific and varied, complicating clinical diagnosis and reliability (Bausch et al., 2000, Masayuki et al., 2012).

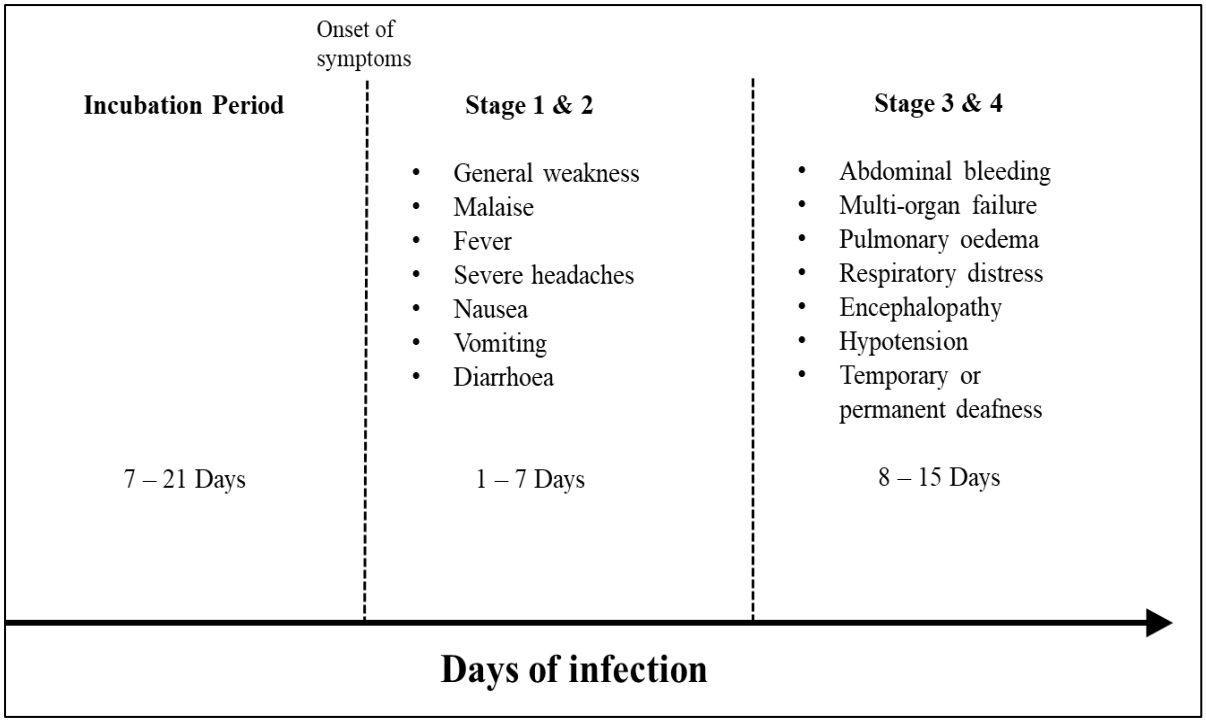

Figure 1.4: The timeline for the manifestation of symptoms during a Lassa virus infection.

The incubation period is shown along with the early non-specific symptoms (Stage 1 and 2) and more severe symptoms (Stage 3 and 4). The length of each stage is also shown. The diagram was adapted from Richmond et al., 2003 (Kay Richmond et al., 2003).

1.4 Pathogenesis

Knowledge of LASV pathogenesis is limited. The development of the disease, ultimately leading to death in severely ill patients, remains unclear (David et al., 2012). An increase in LASV viraemia links to a fatality rise (McCormick, 2008). Developing fatal Lassa fever links to the inability to develop a cellular immune response and neutralising antibodies, controlling the release and replication of the virus (David et al., 2012). During an infection, LASV initially targets antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages (MP), widely distributed in mucosal tissue, various organs and skin (Baize et al., 2004, Russier et al., 2012).

This widespread distribution, of the target cells, leads to the spread of the virus throughout the body to the organs and tissues (liver, adrenal gland, spleen, APC), helping to establish the infection (Russier et al., 2012). APCs are vital components of the innate immune response, initiating the inflammatory response and viral control mechanisms (Russier et al., 2012). They

present antigens, triggering the adaptive immune system through a humoral, cellular response (Russier et al., 2012).

A distinct feature of a LASV infection is that the APCs are not activated despite the massive virus release (Baize et al., 2006, Russier et al., 2012). When the APCs are infected with LASV, there is no change in the viability of the cells; therefore, the massive release of new viral particles can be sustained for extended periods (Russier et al., 2012). LASV infected DC's cannot produce the pro-inflammatory cytokines or express the activation molecules on their surfaces, ultimately leading to the failure of the DC to mature (Baize et al., 2004, Basler, 2017). The pro-inflammatory cytokines are the primary factors implicated in initiating the adaptive immune response. The inability to produce these cytokines leads to a failure to induce the T cell response (CD4+ and CD8+ cells) (Baize et al., 2004, Russier et al., 2012).

The failure of the DC to mature or activate in response to a LASV infection favours viral replication because immature DC can produce significantly more LASV particles than the mature DC (Baize et al., 2004). In severe infections, immature DC cannot effectively present antigens on their surfaces, resulting in immune suppression owing to tolerance and defective immunity (Russier et al., 2012). The MP also show little activation upon LASV infection apart from the production of a small amount of Type 1 interferon (IFN) (Baize et al., 2004, Baize et al., 2009). Another feature is that the functioning of the vascular endothelium is interfered with by LASV (Moolla et al., 2020). However, the mechanism where LASV disrupts the vascular endothelial cells remains unclear. The infected endothelial cells show no cytopathic effect (Moolla et al., 2020).

Lassa virus produces four proteins, where three hold an essential role in the virus's pathogenesis (McLay et al., 2013). The GP is the only antigen on the surface of LASV, making it the primary target for the host's humoral immune response (Hastie et al., 2017, Ibukun, 2020). The LASV GP can escape the host's immune system in several ways: it is highly glycosylated, resulting in a 'glycan shield' making few regions susceptible to antibody binding (Sommerstein et al., 2015, Hastie et al., 2018, Ibukun, 2020). The protein experiences conformational changes that alter the presentation of GP1 and GP2, facilitating immune escape (Hastie et al., 2018, Ibukun, 2020). As aforementioned, GP binds to a specific receptor α-DG, mediating LASV entry into the target cell and the infectivity of the virus (Kunz et al., 2005, Oldstone et al., 2011).

The widespread expression of the receptor on several cell types is thought to contribute to the pantropic nature of LASV (McLay et al., 2013). Dystroglycan (DG) is a cell-surface molecule responsible for linking the extracellular matrix proteins to the internal actin-cytoskeletal machinery (Rita et al., 2006) and is post-translationally cleaved into an α- and β-subunit(Oldstone et al., 2011). The α-DG subunit binds to the β-subunit, which spans the length of the cell membrane and binds intracellularly to dystrophin, causing linkage of the DG complex to the actin-cytoskeleton network (Oldstone et al., 2011, McLay et al., 2013). The GP has a high affinity for the receptor and was shown to out-compete the receptor's natural ligand (Oldstone et al., 2011, Rojek et al., 2012, McLay et al., 2013). When the GP binds to α-DG, it allows viral entry into the host cell, destabilising the cellular membrane (Rojek et al., 2012). This destabilisation can cause cellular signalling loss, contributing to LASV pathogenesis (Rojek et al., 2012).

The NP is the most abundant LASV protein. It inhibits IFN regulatory factor 3 (IRF-3), nuclear translocation, and Type 1 IFN production (IFNβ and INFα), leading to uninhibited virus replication and the adaptive immune system failure (Günther et al., 2004, Russier et al., 2012). This results from a double-stranded RNA (dsRNA) specific 3’ to 5’ exonuclease activity in the C-terminal of the protein, similar to the DEDDh family of enzymes (Hastie et al., 2011, Russier et al., 2012, Papageorgiou et al., 2020). The DEDDh enzyme is a subgroup of the DeDD superfamily of exonuclease enzymes (Hastie et al., 2011). The DeDD group of enzymes contains conserved catalytic residues of aspartic acid (Asp)–glutamic acid (Glu)–Asp–Asp in the active site (Hastie et al., 2011).

The DEDDh enzymes hold a histidine (His) residue close to the DeDD core group of amino acids (Hastie et al., 2011). By digesting LASV dsRNA, NP prevents the sensing of the virus by the pattern recognition receptors, melanoma differentiation-associated gene-5 (MDA-5) and retinoic acid-inducible gene-I (RIG-I), and subsequent activation of the Type I IFN response (Russier et al., 2012). NP prevents the initiation of the innate immune response (Hastie et al., 2011, Russier et al., 2012, Mantlo et al., 2019) by blocking nuclear factor-kappa B (NF-κB) activation and inhibiting IFN regulatory factor 3 (IRF-3) by binding to IκB kinase-related kinase IKKε; therefore, inhibiting the phosphorylation and autocatalytic activity of IRF-3 (Hastie et al., 2011, Russier et al., 2012, Mantlo et al., 2019).

The Z protein binds to cellular promyelocyte leukaemia protein (PML), typically established in the nucleus of cells and helps regulate the tumour suppressor p53 (McLay et al., 2013, Meyer et al., 2018). The binding of the Z protein to cellular PML redistributes the protein to the cytoplasm, inhibiting cellular apoptosis (McLay et al., 2013, Meyer et al., 2018). Preventing and deregulating the host cell's apoptosis machinery may help LASV establish persistent infections (Baize et al., 2004).

1.5 Immune response to Lassa virus infection

A feature of pathogenic Old World mammarenaviruses is they suppress the immune system–evidence suggests that robust T cell responses are critical for controlling and clearing a LASV infection (Johnson et al., 1987, Baize et al., 2009, Sakabe et al., 2020). LASV recovery was associated with a strong and early innate and adaptive immune response, such as an effective T cell response (CD4+ and CD8+ cells) caused by an activation of APC (Baize et al., 2006, Baize et al., 2009, Galan-Navarro et al., 2017, Prescott et al., 2017, Moolla et al., 2020). The importance of the T cell response to LASV is also emphasised because, in fatal infections, patients fail to activate the T cell response (Fischer et al., 2021). The antibody response observed in patients infected with LASV suggests it is slow to develop and alone is inadequate to control infection; therefore, it cannot be an indicator of patient survival (Johnson et al., 1987, Baize et al., 2006, Baize et al., 2009, Sakabe et al., 2020).

During Lassa fever infections, immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies can be produced with IgM antibodies, developing during the first few weeks, with IgG antibodies developing a few days after IgM antibodies appear (Figure 1.5) (Wulff et al., 1979, Prescott et al., 2017). The IgM antibody titre decreases rapidly after a month of infection; however, sometimes, these antibodies may persist for years (Happi et al., 2019). IgG antibodies peak after about a month and slowly decline after a year (Wulff et al., 1979). The IgG antibodies generated during infection have broad specificity–they are directed towards GP1, GP2, NP and Z protein of LASV (Baize et al., 2009, Russier et al., 2012). In severe cases, no detectable level of antibody responses is found because of immune suppression or failing to mount an effective immune response (Ftika et al., 2013).

While the IgM and IgG antibodies are produced, neutralising antibodies are not produced in substantial amounts; therefore, antibodies are ineffective at controlling an infection (Moolla et

al., 2020). Neutralising antibodies may only be produced during recovery from the virus (Russier et al., 2012, Galan-Navarro et al., 2017). A lack of effective neutralising antibodies is attributable to the conformational changes in the trimeric GP during cell entry (Hastie et al., 2018, Ibukun, 2020). The conformational changes are caused by changes in pH and receptor binding status (Hastie et al., 2018, Ibukun, 2020). Some suggested these changes because the GP binds to α-DG to enter the host cell but experiences a conformational change to recognise the LAMP1 receptor in the endosome (Hastie et al., 2017, Hastie et al., 2018, Ibukun, 2020).

A natural killer (NK) cell response is observed during a LASV infection. NK cells proliferate owing to LASV infected MP, leading to developing memory NK cells (David et al., 2012, Russier et al., 2012). This response has not been studied in detail, and its role in controlling infection is not understood (Russier et al., 2012). Studies indicate that T cells hold an essential function for the outcome of Lassa fever; severe infection links to a defective T cell response, whereas during successful control, there is a robust response (Russier et al., 2012). Early activation of T cells (Both CD4+ and CD8+) is noted during a LASV infection, and can be detected in patients following recovery from a LASV infection, even when the antibody response is low or absent (Prescott et al., 2017, Sakabe et al., 2020). A powerful memory CD4+ T cell response towards LASV NP and LASV GP was established in healthy LASV-seropositive individuals inhabiting endemic regions, indicating that mild infections are linked to the activation of T cells (Baize et al., 2006).

1.6 Diagnosis of Lassa fever

The wide range of clinical symptoms produced by Lassa fever makes it indistinguishable from other febrile diseases, such as typhoid fever, malaria, influenza, and other VHFs, complicating clinical diagnosis (Ogbu et al., 2007, Kerber et al., 2015, Raabe et al., 2017). External from the endemic countries, limited global laboratories can perform LASV diagnosis because it requires a high-containment facility (BSL-4) owing to the infectious nature of the collected samples. Laboratory diagnosis of Lassa fever includes isolating the virus by cell culture or detecting the viral nucleic acids by reverse transcription (RT) -polymerase chain reaction (PCR), and may also include detecting Lassa-specific antibodies by serological methods, such as enzyme-linked immunosorbent assay (ELISA) or immunofluorescence antibody assay (IFA) (Sogoba et al., 2012, Bausch et al., 2014). Each of the diagnostic techniques listed above can be used

separately or in combination to confirm a Lassa fever infection depending on the stage of infection.

Various diagnostic methods can diagnose LASV. The advantages and disadvantages are discussed below; however, no commercially available assays for LASV have been validated for diagnostic purposes (Mazzola et al., 2019). Most assays developed for LASV diagnosis are 'in-house' assays and are predominantly employed for research (Mazzola et al., 2019). Figure 1.5 demonstrates when each diagnostic assay can be used and the duration they can detect LASV or antibodies.

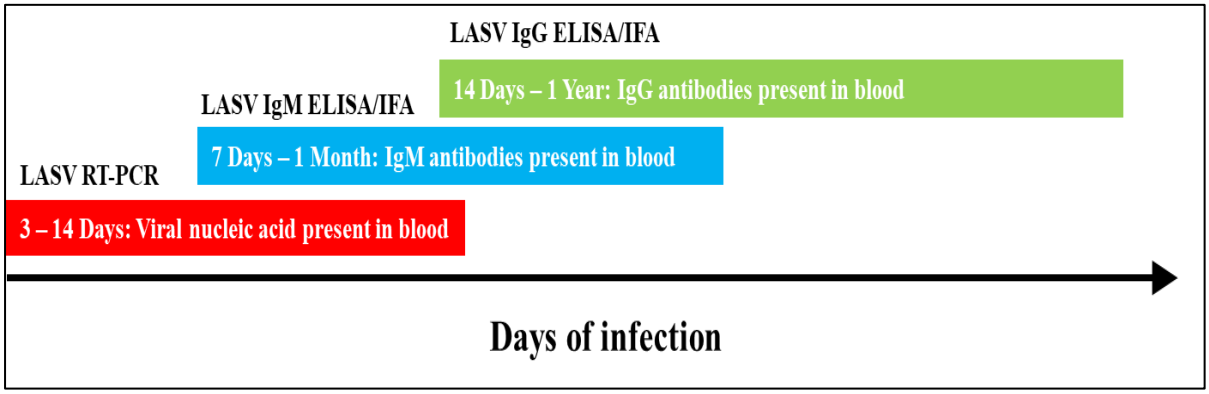

Figure 1.5: Timeline of application indicating when diagnostic assays can be used to diagnose Lassa fever.

The diagram shows when the viral nucleic acids and antibodies first appear in an infected individual and the duration of their presence. While other samples can contain viral nucleic acids, blood was used to design this diagram. This diagram was designed using the information in the pathogenesis, immune response, and diagnosis section.

1.6.1 Nucleic acid detection

Detecting LASV nucleic acids can be conducted using blood from a suspected patient. The RT-PCR is the 'gold standard' for early and rapid diagnosis of Lassa fever (Günther et al., 2004). This technique involves the reverse transcription (RT) of the viral RNA into complementary DNA (cDNA) before the PCR and may be conducted using inactivated specimens (Drosten et al., 2003). The RT-PCR can be conducted on either conventional or real-time PCR platforms (Racsa et al., 2016, Raabe et al., 2017) and is specific and sensitive, offering an accurate and rapid diagnosis when the patient is viraemic and before seroconversion (Ftika et al., 2013). Developed RT-PCR assays indicate high sensitivity and are easy to implement (Dedkov et al.,

2019). It is the most widely used assay for diagnosing suspected VHFs (Marty et al., 2006, Masayuki et al., 2012) and proved exceptionally valuable (Marty et al., 2006). A limitation is that the viraemic period for LASV and other mammarenaviruses can be missed; therefore, cerebral spinal fluid (CSF), serum, blood, throat washings, urine or pleural fluid samples need to be collected early after the onset of illness to be detected (Buckley et al., 1970, Günther et al., 2004).

Developed LASV RT-PCRs and nucleic acid detection assays are limited attributable to a potential for primer/probe failures or false negatives (Dedkov et al., 2019, Happi et al., 2019, Mazzola et al., 2019). The high genetic diversity among the LASV strains spread across large geographical areas can result in primers/probes missing certain LASV strains which results in false negatives or failures (Dedkov et al., 2019, Happi et al., 2019, Mazzola et al., 2019). The S RNA segment of LASV is historically favoured in diagnosing Lassa fever (Vieth et al., 2007). In contrast, the L segment is seldomly used for diagnostic purposes even though it also represents a valid RT-PCR target (Vieth et al., 2007). The L gene among the LASV strains contains conserved regions, leading to conserved amino acid motifs (Vieth et al., 2007). The RT-PCR is normally combined with a serological method for a definitive diagnosis and infection stage (Raabe et al., 2017, Boisen et al., 2018).

Another nucleic acid-based technique available for rapid Lassa fever diagnosis is a one-step reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay (Fukuma et al., 2011). The assay involves cDNA synthesis and DNA amplification at a constant temperature, using four to six specific primers along with a DNA polymerase containing strand displacement activity and reverse transcriptase (Pemba et al., 2019). Fukuma et al. (2011) developed an RT-LAMP assay for LASV The RT-LAMP, employing two primer sets designed for the LASV strains circulating in Sierra Leone and Nigeria (Fukuma et al., 2011). The specificity was lower than a real-time RT-PCR (Fukuma et al., 2011). This assay does not require sophisticated equipment, such as the RT-PCR; therefore, it could be of value in the clinical diagnosis of LASV in endemic countries and may be effective if deployed in the field (Escadafal et al., 2014, Pemba et al., 2019).

While the RT-PCR is considered the ‘gold standard’ for the early and rapid Lassa fever detection, it is considered an impractical point-of-care diagnostic device in endemic regions with limited laboratory infrastructure (Günther et al., 2004, Barnes et al., 2020). Access to

reagents and a cold chain is not always available in endemic regions (Barnes et al., 2020). Innovative technologies, such as the recently developed CRISPR-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter unLOCKing) platform, provide a rapidly adaptable and deployable diagnostic device (Barnes et al., 2020). The developed SHERLOCK assay uses Cas13a RNA-targeting protein to detect viral nucleic acids (Barnes et al., 2020).

CRISPR RNA-guided (crRNA) Cas13a detection is combined with isothermal recombinase polymerase amplification (RPA), allowing for specific binding of Cas13a with the target sequence and signal amplification through Cas13a cleavage activity (Gootenberg et al., 2017, Barnes et al., 2020). When merged with heating unextracted diagnostic samples to obliterate nucleases (HUDSON), it eliminates the need for nucleic acid extraction (Barnes et al., 2020). HUDSON, through a combined heat and chemical denaturation, inactivates pathogens and nucleic acid release (Barnes et al., 2020).

This causes a safe assay with high sensitivity and specificity that can be a point-of-care diagnostic device (Barnes et al., 2020). Developed LASV SHERLOCK assays detected viral nucleic acids as low as 10 copies/µL and indicated no cross-reactivity to the Ebola virus (EBOV) or Marburg virus (Barnes et al., 2020). The developed assays also implied distinguishing between LASV lineages (Barnes et al., 2020). Nucleic acid diagnostic devices are crucial for LASV detection but are limited as they can only be used during the viraemic period.

1.6.2 Virus isolation

The isolation of the virus by cell culturing involves inoculating permissive cell lines, such as the monkey derived kidney cell line, Vero E76 with CSF, serum, blood, throat washings, urine or pleural fluid of the patient suspected of having Lassa fever (Ogbu et al., 2007, Raabe et al., 2017). A positive result is interpreted by the appearance of cell cytopathic effects after 96 hours (Charrel et al., 2003, Raabe et al., 2017). Another detection method, such as RT-PCR and subsequent sequencing, must confirm the virus (Charrel et al., 2003, Raabe et al., 2017).

Virus isolation is mainly used for research and developing reagents (viral antigens and antibodies) for additional diagnostic tests (Charrel et al., 2003, Raabe et al., 2017)). Cell culturing is a time-consuming process and can be a sensitive diagnostic technique, but depends on sample integrity and is impractical as a first-line diagnostic assay (Ogbu et al., 2007). Along

with this, VHFs, such as Lassa, require high-containment facilities (BSL-4 laboratory) (Ogbu et al., 2007).

1.6.3 Viral antigen detection

Antigen detection is used to detect viral antigens in a patient's sample or specimen, employing various techniques (ELISA, IFA, Western blot, multiplex bead assays and rapid diagnostic tests (RDT)) (Emperador et al., 2019). It is a robust method for detecting an active infection (Emperador et al., 2019). Most antigen detection methods are based on the LASV NP because it constitutes a major structural protein produced in substantial amounts (Anderson et al., 2020). A form of antigen detection requires using an antigen-capture ELISA, where wells are coated with monoclonal antibodies, targeting a specific protein (e.g., GP), and will bind to the most conserved amino acid sequence of the target viral protein when present (Saijo et al., 2007, Masayuki et al., 2012).

Antigen detection can also be performed by direct IFA, relying on fluorescently labelled antigen-specific antibodies, reacting with cells from a clinical specimen (histology specimens) or from an inoculated cell culture to detect viral antigens (Niklasson et al., 1984, Koivunen et al., 2006). The direct IFA can detect particular viral antigens through a specific antibody-antigen reaction where monoclonal or polyclonal antibodies bind to specific viral antigens present in a patient's serum (Koivunen et al., 2006, Atmar, 2014). The slides are viewed for direct visualisation of the fluorescence of the infected cells. An advantage of the direct IFA, when compared to the indirect IFA, is that it may provide enhanced specificity because of lower background fluorescence (Atmar, 2014).

The RDT for LASV uses the same antigenic/antibody reagents as an ELISA, except in a lateral flow stripped-down format (Boisen et al., 2018, Mazzola et al., 2019). A LASV RDT was developed using murine monoclonal antibodies specific for the NP, indicating reliable sensitivity and specificity using a fingerstick whole blood sample (Boisen et al., 2018, Mazzola et al., 2019). While RDTs can provide quick results for suspected cases of LASV in nosocomial settings and in the field, diagnosis would still need to be confirmed by either a molecular or serological method (Boisen et al., 2020). RDTs are, therefore, primarily used as point-of-care test (Boisen et al., 2020).

1.6.4 Serological techniques

Serological assays are used to detect specific antiviral antibodies (IgM/IgG) in a patient's blood (Broadhurst et al., 2016). It can determine if a viral infection is new or if the patient was previously infected (Broadhurst et al., 2016). Serological assays are important for LASV diagnosis because viraemia is short-lived, and PCR or antigen detection assays may miss detection outside of this period (Happi et al., 2019, Mazzola et al., 2019). Antibodies produced against LASV antigens (the LASV NP) are better conserved or cross-reactive and can aid in diagnosis (Happi et al., 2019, Papageorgiou et al., 2020). During Lassa fever infections, IgM and IgG antibodies are produced, with IgM antibodies usually developing during the first few weeks. The IgG antibodies can generally be detected a few days after IgM antibodies appear (Wulff et al., 1979, Prescott et al., 2017).

Antibodies are still produced and can diagnose infections towards the end of the viraemic period, though the antibody response is insignificant to clearing LASV infections. Limitations direct that antibodies can remain undetectable early in infections, and patients who die from VHFs often fail to seroconvert (Masayuki et al., 2012). To overcome this limitation, serological assays are used, combined with nucleic acid or antigen detection methods, considering the patient's clinical history. Several serological assays can diagnose Lassa fever, including ELISA, Western blot (WB), direct or indirect IFA and virus neutralisation assays (Masayuki et al., 2012).

The neutralisation assay is a sensitive test–the 'standard' for serodiagnostic assays, measuring the antibody response to an infection or vaccination to a wide range of human viruses (Tomori et al., 1987, Alché et al., 1988). The presence of neutralising antibodies is a beneficial indicator of protective immunity in the host (Masayuki et al., 2012). The best and most direct method to detect neutralising antibodies against VHFs is by using an infectious virus in a plaque neutralisation test. A limitation of this method is that it is time-consuming (requires 10-14 days) and involves a high-containment facility (BSL-4 laboratory) because of the high pathogenicity of the VHFs (Tomori et al., 1987, Masayuki et al., 2012). With Lassa, neutralising antibody titres may be low in Lassa fever patients (Tomori et al., 1987, Masayuki et al., 2012).

ELISAs are used to diagnose viral infections, such as LASV (Ogbu et al., 2007, Raabe et al., 2017). They are easy to use, sensitive, and specific (Ogbu et al., 2007, Raabe et al., 2017). They

can be conducted using inactivated viruses or purified antigens (Ogbu et al., 2007, Raabe et al., 2017). An IgM-specific ELISA is used for detecting new or recent infections (Masayuki et al., 2012). In certain instances, relevance in detecting acute VHF cases may depend on the duration of the illness (Masayuki et al., 2012). An IgG-specific ELISA can be useful for diagnosis in non-endemic regions (Racsa et al., 2016, Gabriel et al., 2018). In endemic areas, where IgG seroprevalence is high, a positive IgG assay result has little value in diagnosing acute infection (Racsa et al., 2016, Gabriel et al., 2018). It can have only diagnostic value if a four-fold increase in IgG antibodies in subsequent samples or seroconversion can be demonstrated (Racsa et al., 2016, Gabriel et al., 2018).

Four commercially available recombinant assays include pan-LASV ELISA kits (ReLASV® Pan-Lassa NP IgG/IgM ELISA Kit, ReLASV® Pan-Lassa Antigen ELISA Kit, ReLASV® Pan-Lassa Prefusion GP IgG/IgM ELISA Kit, ReLASV® Pan-Lassa Combo NP/Prefusion GP IgG/IgM ELISA Kit) have been developed using LASV antigens from the three most prevalent lineages, namely II, III, and IV. Two pan-LASV (reLASV) rapid antibody detection tests (ReLASV® IgG ELISA Kits–Single Lineage) produced by Zalgen labs (United States of America); however, these assays have not been validated for diagnostic purposes and are only intended for research as their performance characteristics have not yet been determined (Solutions, 2019).

Immunofluorescence assays offer an alternate approach to the serological detection of antibodies. The indirect IFA uses fixed virus-infected mammalian cells or insect/ mammalian cells expressing a specific antigen to detect antibodies in the serum by microscopy (Masayuki et al., 2012). However, growing a live virus and using virus-infected cells for LASV and other VHFs requires a BSL-4 laboratory until inactivated (Raabe et al., 2017). Previously, antibodies against LASV were detected using IFAs, but ELISAs became more widely employed for LASV diagnosis (Raabe et al., 2017).

IFAs are still used as rapid screening devices, with confirmatory testing conducted by ELISA. The IFA is not widely used to diagnose LASV, though it holds a few advantages over other used assays (Ogbu et al., 2007, Masayuki et al., 2012). The main advantage of the IFA is that results can be produced rapidly, crucial when dealing with suspected VHF cases, such as LASV, to prevent the further spread of the disease, involving contact tracing and patient

isolation (Wulff et al., 1975, Broadhurst et al., 2016). The indirect IFA can detect antibodies within seven to 10 days of infection, allowing for early diagnosis (Wulff et al., 1975).

Compared to an ELISA, the IFA is more sensitive, straightforward, produces results quicker, requires less optimisation and can detect IgM antibodies much earlier (10 days after initial infection) (Wulff et al., 1975, Ter Meulen et al., 1998, Bausch et al., 2000, Emmerich et al., 2006). IFA limitations can be challenging in interpreting the results owing to inexperience and cross-reacting antibodies from infections with related viruses (Wulff et al., 1975, Bausch et al., 2000). An advantage of traditional whole virus-based IFAs is that all LASV proteins expressed in the infected cell are potentially antigenic (Günther et al., 2004, Atmar, 2014). Antibodies targeting more than one protein can, therefore, be detected, leading to increased sensitivity (Günther et al., 2004, Atmar, 2014).

An advantage of recombinant antigen-based IFAs is that slides can be developed, targeting a specific viral antigen (Günther et al., 2004, Atmar, 2014). These IFAs can be performed outside high-containment facilities, following sample inactivation (Günther et al., 2004, Atmar, 2014). Assays relying on recombinant proteins are often more accurate (Günther et al., 2004, Raabe et al., 2017, Happi et al., 2019, Mazzola et al., 2019). They may be easier to standardise attributable to higher reproducibility and similar immunoreactivity, whereas ‘natural’ proteins from virus-infected cells display more variability owing to the number of viral particles and infection rate (Günther et al., 2004, Raabe et al., 2017, Happi et al., 2019, Mazzola et al., 2019). Including the LASV NP and LASV GP could ensure that all antibodies are detected, since most antibodies target these antigens (Saijo et al., 2007). Each viral protein produces a characteristic fluorescence pattern, increasing the assay specificity (Masayuki et al., 2012).

While a wide range of serological diagnostic assays for LASV exists, they generally require the culturing of live virus inside a BSL-4 laboratory. This would be the main challenge in the early detection and implementation of the treatment or containment actions unless slides were made and stored correctly (Branco Luis et al., 2011). The initial diagnosis of Lassa fever is essential because the infected patient can be isolated, and the spread of the disease can be prevented (Wulff et al., 1975). Developing a serological assay, such as an IFA, using recombinant antigens is, therefore, a safer, more reliable alternative to the live virus and will help circumvent several issues with diagnosing VHFs, such as LASV (Masayuki et al., 2012, Boisen et al., 2018).

1.7 Producing recombinant antigens

Producing recombinant antigens for serological assays is an effective way to diagnose VHFs, such as LASV, external from a high-containment facility once the infectious samples are heat-inactivated (Saijo et al., 2006, Bausch et al., 2014). Producing recombinant antigens can be time-consuming, though they eliminate the need to culture and inactivate live viruses; therefore, diagnosis can be conducted without a BSL-3 or 4 laboratory and can be cost-effective (Barber et al., 1990, Saijo et al., 2006). Most recombinant antigen-based assays for LASV use the LASV NP, GP or Z protein (Ter Meulen et al., 1998, Saijo et al., 2007). Recombinantly produced LASV NPs were successfully used to detect IgM and IgG antibodies towards arenaviruses, specifically the Lassa virus (Barber et al., 1990, Ter Meulen et al., 1998, Masayuki et al., 2012).

These recombinant antigens can be used in IFAs, ELISAs, and other serological assays (Hummel et al., 1992, Masayuki et al., 2012). They can be produced using a variety of hosts, such as recombinant viruses, bacteria, and mammalian cells (Hummel et al., 1992, Masayuki et al., 2012). Each system (viral, bacterial, and mammalian) has its advantages and disadvantages–discussed below, with examples. These recombinant antigen-based diagnostic assays, such as ELISA or IFA, would not only be useful in diagnosing Lassa fever but can also be used for the seroepidemiological studies of LASV to investigate antibodies in reservoir species (Masayuki et al., 2012).

1.7.1 Viral expression systems for recombinant proteins

In viral expression systems, viral vectors carry the template DNA into another cell type (mammalian, insect, bacterial or plant) to express the DNA or protein(s) (Jäger et al., 2015). These vectors can be employed for vaccine development, gene therapy or protein expression (Jäger et al., 2015, Wang et al., 2018). Viral vectors are effective vehicles for introducing foreign DNA into a host cell, but the size of the gene inserted into a host cell is often limited by the size of the vector and the requisite to maintain elements of the viral genome essential for delivery to the host cell (Burton et al., 2003).

The recombinant baculovirus system is a commonly used viral vector system. Baculoviruses are known as insect pathogens with an essential function in controlling insect populations (van Oers, 2011, Martínez-Solís et al., 2019). They also became important for expressing foreign

proteins in both mammalian and insect cells attributable to their large genomes, enabling them to accommodate large or multiple gene inserts and the ability to express large amounts of recombinant protein, resulting in them being favoured by biotechnology companies (van Oers, 2011, Martínez-Solís et al., 2019). They also can express large amounts of foreign protein early or late during an infection which is attributable to promoters, such as the polh and p10 (Hummel et al., 1992, van Oers, 2011, Martínez-Solís et al., 2019).

Insect cell lines were also modified to grow in suspension, facilitating scaling-up reactions for large-scale protein production (Martínez-Solís et al., 2019). Baculovirus vectors in mammalian or insect cells also allowed for various post-translational modifications, such as glycosylation, and protein folding, to occur (van Oers, 2011, Martínez-Solís et al., 2019). Proteins could, therefore, be properly expressed (van Oers, 2011, Martínez-Solís et al., 2019). With LASV, the LASV NP gene was expressed in the baculovirus system (Saijo et al., 2007). Subsequently, to develop antigen and antibody detection, ELISAs presented suitable sensitivity and specificity for LASV (Saijo et al., 2007). A recombinant baculovirus engineered to express the LASV GP was also produced (Hummel et al., 1992). The recombinant glycosylated protein reacted with antibodies specific for the LASV GP in both an IFA and ELISA (Hummel et al., 1992).

Animal viral vectors were also used to express unrelated genes. LASV NP and LASV GP antigens were expressed in mammalian cells with the widely used and versatile recombinant vaccinia virus expression system (Morrison et al., 1989, Barber et al., 1990, Pfleiderer et al., 1995). The vaccinia virus vectors are unique in that transcription of foreign proteins occurs in the cytoplasm of mammalian cells as opposed to the nucleus (Moss et al., 1998). The size of the genome also allows for the insertion of large genes into the vector (Moss et al., 1998). Since mammalian cells are used, post-translational modification of recombinantly expressed proteins have no complications (Moss et al., 1998).

Vaccinia virus-infected cells containing the LASV GP gene, synthesising the GPC–the mature GP1 and GP2, were identical to the protein produced by LASV (Auperin et al., 1988). Vaccinated guinea pigs, using the recombinant LASV GP expressing vaccinia virus, were protected against LASV (Auperin et al., 1988, Barber et al., 1990). Primates, however, were not protected when vaccinated, resulting in developing the severe or fatal disease–establishing this vector-based vaccine does not, in its current form, induce an effective immune response against LASV infection in primates (Auperin et al., 1988, Barber et al., 1990).

1.7.2 Bacterial expression systems for recombinant proteins

Bacterial expression systems use bacteria, such as *Escherichia coli* (*E. coli*), to express foreign proteins to develop new diagnostic assays or to study the structure and function of proteins (Primrose et al., 2006, Snyder L, 2013). The advantage of these systems is they are inexpensive, easy and quick to culture, while easy to introduce foreign DNA into bacterial cells by transformation (Primrose et al., 2006). Bacterial cells can maintain the foreign DNA and pass it to the progeny cells through a selection marker for antibiotic resistance (Primrose et al., 2006, Snyder L, 2013). A disadvantage of bacterial expression systems is they lack post-transcriptional and post-translational processing mechanisms, which can affect protein folding and conformation in bacterial cells (Primrose et al., 2006).

The LASV NP was also expressed in *E. coli* as a truncated, histidine-tagged recombinant protein used in an ELISA (Barber et al., 1987, Branco et al., 2008); however, a potential limitation with using truncated LASV NP is the loss of serological reactivity (Barber et al., 1987, Ter Meulen et al., 1998). Linear epitopes were detected to LASV NP with sensitivities and specificities comparable to other lateral flow-based assays (Barber et al., 1987, Ter Meulen et al., 1998). The recombinant LASV NP effectively detected LASV-specific IgM and IgG antibodies (Barber et al., 1987, Ter Meulen et al., 1998).

1.7.3 Mammalian expression systems

Recombinant LASV antigens were expressed in mammalian cells, such as HeLa, Vero, baby hamster kidney cells (BHK-21) and human embryonic kidney cells (HEK293) (Saijo et al., 2001, Masayuki et al., 2012) by transfecting the cells with mammalian expression vectors containing the required LASV gene or additional viral genes (Masayuki et al., 2012, Jäger et al., 2015). Stable cell lines may be developed, depending on the downstream applications (Jäger et al., 2015). Research indicates that using mammalian cells to produce recombinant LASV proteins, such as the NP, GP and Z protein, causes high expression levels and post-translational processing (Branco et al., 2010). It was also observed that recombinant LASV NP expressed in mammalian cells had a high specificity and sensitivity for detecting IgM and IgG antibodies in an ELISA and IFA (Saijo et al., 2007, Gabriel et al., 2018).

The aforementioned information for recombinant antigens expressed in bacterial, viral, and mammalian systems displays assays based on them have high specificity and sensitivity and

can, therefore, be highly effective when performing diagnosis. To help overcome the problems with batch-to-batch variations, often experienced with traditional virus-infected IFA tests, recombinant protein expression of the viral proteins can be better regulated and standardised by producing stable cell lines through antibiotic selection (Barber et al., 1990). Using these recombinant antigen-based systems for developing ELISAs or IFAs could improve the diagnoses of Lassa fever and have additional utility for the seroepidemiological studies of LASV or to investigate reservoir species (Masayuki et al., 2012).

1.8 Validation of diagnostic assays

Before a newly developed diagnostic assay can be used, it needs to be validated to ensure its suitability to achieve a particular objective (Jacobson, 1998a, Crowther, 2009). A diagnostic assay is validated if it can consistently identify samples as positive or negative for a specific analyte (antibody, antigen, or nucleic acid) while accurately determining the infection status of the individual with a certain degree of statistical significance (Jacobson, 1998b, Jacobson, 1998a, Crowther, 2009).

Several variables must be considered and standardised to validate a diagnostic assay, such as instrument calibration or error, technician error, choice of reagents, control accuracy, experiment protocol (incubation temperatures and times, buffer composition), cross-reactivity with closely related diseases, disease prevalence, diagnostic specificity, and sensitivity (Jacobson, 1998a, Jacobson, 1998b). An essential part of diagnostic assay validation is that the experimental procedures (aforementioned) need to be optimised and standardised to produce consistent results for the controls used (Jacobson, 1998a, Jacobson, 1998b, Banoo et al., 2008, Crowther, 2009).

Developing and validating a diagnostic assay can only continue once preliminary evidence of repeatability among runs of the diagnostic assay (Jacobson, 1998a, Jacobson, 1998b). Along with repeatability, a principal factor in assay validation is determining the analytical specificity and sensitivity (Jacobson, 1998b, Jacobson, 1998a, Banoo et al., 2008). The analytical sensitivity is defined as the smallest detectable amount of infectious disease (Jacobson, 1998a, Jacobson, 1998b). This is determined using an end-point dilution of a serum sample where antibodies, antigens, or nucleic acids, can no longer be detected (Jacobson, 1998a, Jacobson, 1998b). The analytical specificity of a diagnostic assay is defined as the ability of the assay to

not cross-react with samples from closely related diseases or those with similar symptoms (Banoo et al., 2008, Crowther, 2009). The analytical specificity is determined with samples, positive for closely related diseases with a similar clinical picture to determine cross-reactivity (Jacobson, 1998a, Banoo et al., 2008).

Once the analytical performances of the diagnostic assay were determined, the next step in the validation process would to determine the diagnostic specificity and sensitivity (Jacobson, 1998a). Diagnostic sensitivity is defined as the ability of an assay to identify individuals with the target disease (True Positives) (Jacobson, 1998a, Crowther, 2009). Conversely, diagnostic specificity is defined as the ability of the assay to identify individuals without the target disease (True negatives) (Jacobson, 1998a, Crowther, 2009). These parameters are determined by testing samples known to be positive or negative for a specific infection or disease (Jacobson, 1998a, Jacobson, 1998b). Known positive samples–negative during screening are classified as false negatives, and known negatives testing positive are classified as false positives (Jacobson, 1998b, Jacobson, 1998a).

For proper validation of a diagnostic assay, the number of samples analysed and the sample history are crucial (Jacobson, 1998a). The positive predictive value (PPV) is defined as the probability of samples determined to be positive that is truly positive (Banoo et al., 2008). The negative predictive value (NPV) is defined as the probability of samples determined to be negative that are truly negative (Banoo et al., 2008). It is recommended to properly validate a diagnostic assay at least 300 known positive samples and at least 1000 known negative samples be tested to determine these parameters (Jacobson, 1998a, Banoo et al., 2008, Crowther, 2009). Should this be impossible because of limited sample availability, as with most VHFs, the number of tested samples can be calculated to the statistically defined limits (Jacobson, 1998a, Banoo et al., 2008, Crowther, 2009).

When these performance characteristics are determined, the next step is to compare the newly developed assay to a used assay (serological assay for developed serological assays and nucleic acid assays for developed nucleic acid assays) or a combination of assays using the same panel (Jacobson, 1998a, Banoo et al., 2008, Crowther, 2009). Essential for recombinant antigen-based assays, is to ensure ‘parallelism’, indicating that samples detected by natural and recombinant antigens are identified in the same way.

Once all these parameters are defined a newly developed assay can be classified as fully validated and used to diagnose unknown samples. These parameters must be calculated and determined once the recombinant LASV NP and LASV GP assays are developed. The limited availability of positive LASV samples is a restrictive factor for validating newly developed LASV diagnostic assays. The total number of positive and negative samples needed to validate a new diagnostic test can be calculated based on estimated specificity, sensitivity, desired confidence interval (CI), and margin of error (Jacobson, 1998a, Banoo et al., 2008).

1.9 Project overview

There is a lack of affordable and rapid serological diagnostic tests for Lassa fever, for use with nucleic acid-based detection assays for a quick, definitive diagnosis. There are commercially available serological assays; however, these are primarily used for research since international laboratories develop in-house assays using published or unpublished protocols. Several of the serological assays (ELISA and IFA) rely on culturing and inactivation of a live virus, thus a high-containment facility is required for assay development and execution of these diagnostic assays. If the diagnosis of Lassa fever can be conducted without a BSL-4 laboratory, it would expedite diagnosis and reduce the costs. Focusing on recombinant technology for diagnostic assay development is an advantage. It is a safer alternative to using the live virus. Developing an IFA based on recombinant LASV antigen(s) for detecting Lassa-specific antibodies in a patient's serum would meet the demand for a safe, reliable, and quick diagnostic device that does not necessitate a BSL-4 laboratory.

This project was completed at the Special Viral Pathogens Laboratory (SVPL), National Institute for Communicable Diseases (NICD). The NICD is the national public health institution for South Africa, providing microbiology, epidemiology, public health, and surveillance research and training to help the Government’s response to disease threats. The SVPL is part of the NICD and contains the only BSL-4 laboratory in Africa, indicating it can respond to dangerous emerging and re-emerging zoonotic pathogens. The SVPL aims to support control, surveillance, detection, and outbreak response systems. This project falls in line with this objective because developing additional options for LASV detection using recombinant antigens would aid in diagnosis and surveillance.

Chapter 2 presents developing LASV NP and LASV GP stable cell lines. This presentation includes designing and cloning LASV NP and LASV GP expression cassettes, confirming the expression of the proteins by IFA and WB. In Chapter 3, using these cell lines and constructs in developing LASV NP and LASV GP IFA assays is presented. Evaluating these assays using a panel of known LASV negative samples and a known LASV positive sample is also displayed. The newly developed assays were compared to a virus-infected cell-based IFA.

1.10 Aim

The aim of the project was to develop and evaluate a recombinant antigen-based indirect immunofluorescence assay (IFA) for the diagnosis of Lassa fever.

1.11 Objectives

The study objectives were:

- To design and construct expression cassettes for the individual expression of recombinant LASV NP and LASV GP in a mammalian cell culture system.
- To express the recombinant LASV NP and LASV GP proteins and confirm expression by IFA and Western blot analysis.
- To produce individual recombinant LASV NP- and LASV GP-stably expressing cell lines.
- To develop a recombinant antigen-based indirect IFA to detect IgG antibodies in LASV.
- To evaluate the indirect IFA using clinical samples.

CHAPTER 2: DEVELOPING THE LASSA VIRUS, NUCLEOPROTEIN, AND GLYCOPROTEIN STABLE CELL LINES

2.1 Introduction

Lassa virus (LASV) is a viral haemorrhagic fever (VHF) causing virus belonging to the *Arenaviridae* family and the *mammarenavirus* genus (Bausch et al., 2014, Radoshitzky et al., 2019a). LASV is one of seven viruses in this family causing VHFs, and is classified as a biosafety level 4 (BSL-4) pathogen as a result (Frame et al., 1970, Charrel et al., 2003, Ogbu et al., 2007, Bausch et al., 2014). LASV is the mammarenavirus with the most significant public health impact and is endemic to West African countries, such as Sierra Leone, Guinea, Liberia, Mali, and Nigeria, indicating a high genetic diversity among the LASV strains (Frame et al., 1970, Ogbu et al., 2007). LASV was first isolated from a patient with an unknown viral disease in 1969. The virus is maintained through persistent infections of the rodent reservoir host, *Mastomys natalensis (M. natalensis)* (Frame et al., 1970, Monath et al., 1974).

Humans are often infected with LASV after inhaling released particles from rodent saliva, faeces, and urine, or contact with blood and tissue from infected rodents (Masayuki et al., 2012). The virus easily spreads from one human to another through direct contact with infected secretions (blood, saliva, urine, semen, or vomit) and mucous membranes. Nosocomial infections commonly occur during patient care when caregivers or health workers come into contact with the infected blood or secretions from patients (Fichet-Calvet, 2014).

Members of the *mammarenavirus* genus are spherical or pleomorphic, surrounded by a lipid membrane (Buckley et al., 1970, Speir et al., 1970, Charrel et al., 2003). The genomes comprise two single-stranded, ambisense RNA molecules, such as the S and L segment, with each segment coding for two gene products (Barber et al., 1990, Radoshitzky et al., 2019a). The L segment is the larger of the two segments, at 7.2 kb in size and codes for the L and Z protein. The L protein conducts virus replication and transcription (Leung et al., 1979, Fuller-Pace et al., 1989, Garcin et al., 1992).

The Z protein drives arenavirus budding and has a function in inhibiting RNA synthesis (Perez et al., 2003, Strecker et al., 2003). The S segment is 3.4 kb and codes for the two structural proteins, indicating the NP and GP (Bowen et al., 2000, Günther et al., 2004). The GP forms the virion spikes and is accountable for receptor binding and cell entry (Speir et al., 1970, Bowen et al., 2000, Fedeli et al., 2018). The NP is the most abundant protein produced by the virus, constituting a major structural protein by forming the nucleocapsid (Pinschewer et al., 2003, Günther et al., 2004).

Lassa virus has an incubation period ranging from seven to 21 days (Frame et al., 1970, Walker et al., 1975). The disease is linked with a wide range of clinical symptoms, including flu-like symptoms, haemorrhaging, and neurological symptoms (Ogbu et al., 2007, Shaffer et al., 2014). Most LASV infections (80% of cases) remain subclinical, and never progress further than mild symptoms, whereas in some cases (20% of cases), the patient could develop more severe symptoms, possibly leading to death (Sogoba et al., 2012). Diagnostic methods to diagnose LASV, include isolating the virus by cell culture, detecting the viral nucleic acids by RT-PCR, and detecting Lassa-specific antibodies by serological methods, such as ELISA or IFA (Sogoba et al., 2012, Bausch et al., 2014). Outside the endemic countries, limited laboratories globally can perform LASV diagnosis or work with the live virus because a high-containment facility (BSL-4) is required (Raabe et al., 2017).

In this respect, recombinant antigens are important for serological diagnostic assay development because they eliminate the need to culture and inactivate live viruses, negating the need for a BSL-3 or 4 laboratory while also being more cost-effective (Barber et al., 1990, Saijo et al., 2006). These recombinant antigens can be used in an IFA and ELISA, developed using the techniques discussed (Section 1.6.4) (Hummel et al., 1992, Masayuki et al., 2012). The LASV NP and LASV GP are the two major structural proteins–most antibodies produced by the host target these proteins (Günther et al., 2004, Russier et al., 2012). As beneficial immunogenic targets, LASV NP and LASV GP are excellent candidates for recombinant protein expression and diagnostic assay development based on them.

This chapter describes designing individual expression cassettes for the LASV NP and LASV GP genes and their use in developing stable, protein-expressing cell lines for each protein in mammalian cells. Expressing the LASV NP and LASV GP was conducted using the mammalian expression system (Wurm, 2004, Jäger et al., 2015). The mammalian expression

system was chosen because earlier work indicated that mammalian cells express the LASV proteins at elevated levels, allowing for post-translational processing and folding (Wurm, 2004, Jäger et al., 2015). The post-translational processing (post-translational cleavage and glycosylation) would be valuable for expressing the LASV GP because the immunologically relevant epitopes will be formed. The stable cell lines will help optimise and standardise future IFA slides by removing batch-to-batch variation among IFA slides.

2.2 Materials and methods

2.2.1 Construct design and cloning

2.2.1.1 Design of constructs and primers

The nucleotide sequence encoding a codon-optimised (mammalian expression) sequence for LASV S segment (Josiah) (GenBank NC004296) was synthesised and cloned into the pCI-neo expression vector by Dr Moolla at the NICD (pN-S Seg) (GenScript, USA) (Figure 2.1). The Josiah strain was preferred as it has a case fatality rate of 69% in those who develop clinical symptoms (Robinson et al., 2016). Figure 2.1 displays the individual pCI-neo mammalian expression vector (Promega, USA) and the pN-S Seg vector. The individual coding regions for the LASV NP and LASV GP were then PCR amplified and subcloned into the pCI-neo expression vector for individual LASV NP and LASV GP expression cassettes.

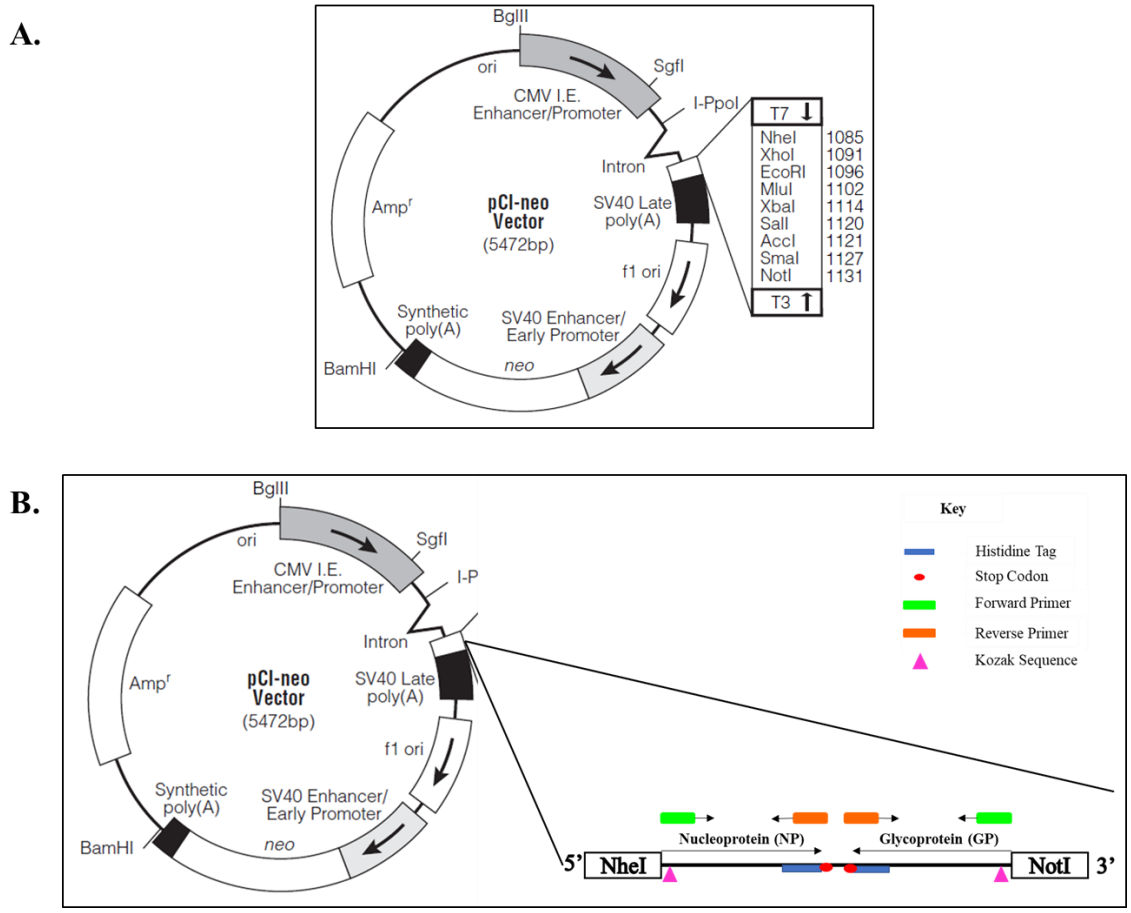

Figure 2.1: A) pCI-neo mammalian expression vector map (Promega) B) pCI-neo mammalian expression vector map showing the location of the LASV S segment insertion in the vector.

B shows the location of the histidine tag, Kozak sequence and stop codons for the LASV NP and LASV GP. The binding locations of the forward and reverse primers are indicated. The restriction enzyme (RE) digestion sites are also shown. Vector maps were obtained from Promega (Promega Corporation, USA); the image was edited to include the LASV S segment.

This expression vector was selected, containing the G-418 resistance marker, allowing stable cell selection after transfection. The expression vector also contained the ampicillin (Amp) resistance marker with the G-418 resistance marker, allowing for bacterial cell selection after transformation. Codon optimisation of the LASV S segment sequence exploits the degenerate and redundant nature of the genetic code by ensuring the chosen codons are optimised for expression in specific cells, which should facilitate improved recombinant protein expression levels.

A Kozak sequence (5'-GCCGCCACC-3') and C-terminus histidine tag (His-tag) (5'-CATCATCATCATCATCAC-3') for both the LASV NP and LASV GP proteins were

included in the synthesis of the S segment. Primers for the individual amplification of the LASV NP and LASV GP genes from the LASV S segment vector were designed. These primers contained restriction enzyme (RE) cut sites for NheI and NotI, allowing the insertion of the LASV NP and LASV GP genes into the pCI-neo expression vector (Figure 2.1). The forward primer (FP) recognised and bound to the Kozak sequence and part of the start codon while containing the RE recognition sequence for NheI (5'-GCTAGC-3') (Figure 2.1). The reverse primer (RP) recognised and bound to the His-tag and stop codon (TGA) while containing the RE recognition site for NotI (5'-GCGGCCGC-3') (Figure 2.1). The sequences for the FP and RP were:

FP: 5'–ATT GCTAGC GCCGCCACCAT–3'

RP: 5'–ACA GCGGCCGC TCAGTGATGATG–3'

The His-tag was included for both the LASV NP and LASV GP gene, detecting the LASV NP and LASV GP using anti-histidine tag antibodies during Western blot analysis and facilitating– if the need arose to purify the proteins, purification by nickel affinity.

2.2.1.2 Transformation and plasmid isolation

Working stocks of the pN-S Seg and pCI-neo mammalian expression vector were prepared. Briefly, this included the transformation of the plasmids into competent JM109 *E. coli* cells (Promega, USA) by heat shock, according to the manufacturer's instructions (Appendix B1). A total of 100 µL for each transformation culture was plated onto prepared Luria-Bertani (LB) plates with 100 µg/mL of ampicillin (Zymo Research, USA). Duplicate plates were created and incubated for 16 hours overnight (o/n) at 37°C. A negative control was made, comprising competent JM109 *E. coli* cells (Promega, USA) without the plasmid DNA. A single colony for each plasmid (pN-S Seg and pCI-neo) was selected and inoculated into 5 mL of LB broth (Sigma-Aldrich, USA) with 100 µg/mL of ampicillin (Zymo Research, USA). The LB/Amp broths were then incubated for 16 hours o/n at 37°C with shaking at 150 rpm in the Infors HT Ecotron incubator (Infors HT, USA). Glycerol stocks were made for each plasmid and stored at -70°C (Appendix B3).

Plasmids were then isolated from an o/n culture, using the Zippy™ Plasmid Miniprep Kit (Zymo Research, USA), according to the manufacturer's instructions. For this, a single colony

for each plasmid (pN-S Seg and pCI-neo) was selected and added to LB/Amp (100 µg/mL) broth as described above. Briefly, 600 µL of o/n culture was pelleted by max centrifugation (21100×g). The pellet was resuspended in 600 µL of o/n culture and cells lysed with 100 µL of 7× Lysis Buffer. Lysis was stopped by adding 350 µL of cold Neutralisation Buffer (NB) (contains 100 µg/mL of RNase A). Following centrifugation at 16000×g, the cell lysate was added to the Zymo-spin™ INN column. The Zymo-spin™ INN column was rinsed with 200 µL of Endo-Wash Buffer and 400 µL of Zyppy™ Wash Buffer centrifugated at 16000×g between wash steps. The plasmid DNA was eluted in 30 µL of Elution Buffer (10 mM tris-HCl, pH 8.5; 0.1 mM ethylenediaminetetraacetic acid (EDTA)).

A total of 5 µL of the purified plasmids was added to 1 µL of 6× loading dye (10 mM tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA) and run on a 1% agarose gel electrophoresis; it was observed, using ultraviolet (UV) illumination (Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc., USA)). The gel was prepared by adding 0.5 g of agarose (Lonza, Switzerland) to 50 mL of 1× tris-acetate EDTA (TAE) electrophoresis buffer (Thermo Fisher Scientific, USA) and 1 µg/mL of ethidium bromide (VWR Life Science, USA). The plasmid concentration was determined using the Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA) and Qubit™ dsDNA HS Assay kit (Life Technologies, Thermo Fisher Scientific, USA), according to the manufacturer's instructions (Appendix B2).

2.2.2 Polymerase chain reaction (PCR) amplification and cloning of Lassa virus nucleoprotein and Lassa virus GP genes

2.2.2.1 Polymerase chain reaction (PCR) and optimisation

The LASV NP gene was amplified and cloned for the researcher's honours project (2018). The LASV NP gene was PCR amplified from the pN-S Seg vector using the Dream Taq Polymerase (Thermo Fisher Scientific, USA) and the primers, described in Section 2.2.1.1. The reaction mix for the LASV NP PCR is displayed in Table 2.1. The LASV GP gene was amplified from the pN-S Seg vector, using the Expand High Fidelity PCR System (Roche, Germany) and the primers (Section 2.2.1.1). The reaction mix for the LASV GP PCR is indicated in

Table 2.2, using a final volume of 50 µL. The 0.2 mL PCR tubes (Thermo Fisher Scientific, USA) were vortexed and spun down before placed in the SimpliAmp™ Thermo Cycler (Applied Biosystems, Thermo Fisher Scientific, USA).

A background control (pCI-neo mammalian expression vector) and no template control (nuclease-free water (Thermo Fisher Scientific, USA)) were included. Amplification was performed in the SimpliAmp™ Thermo Cycler (Applied BioSystems, Thermo Fisher Scientific, USA) under the following conditions: 94°C for one minute, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, and followed by a final extension of 72°C for seven minutes and held at 4°C.

Table 2.1: Formulation of the PCR mixture to amplify the LASV NP gene.

Component	Volume (µL)
10× Dream Taq Buffer (Thermo Fisher Scientific, USA)	5
5U/µL Dream Taq Polymerase (Thermo Fisher Scientific, USA)	0.25 (1.25U)
25 mM dNTP mix (Thermo Fisher Scientific, USA)	0.4 (0.2 mM)
10 µM FP (Anatech, South Africa)	1
10 µM RP (Anatech, South Africa)	1
DNA template	1 (1 ng/µL)
Nuclease-Free water (Thermo Fisher Scientific, USA)	41.35
Total Volume	**50**

Table 2.2: Formulation of the PCR mixture to amplify the LASV GP gene.

Component	Volume (µL)
10× Expand High Fidelity Buffer with 15 mM $MgCl_2$ (Roche, Germany)	5
3.5U/µL Expand High Fidelity Enzyme mix (Roche, Germany)	0.75 (2.6U)
10 mM dNTP mix (Thermo Fisher Scientific, USA)	1 (0.2 mM)
10 µM FP (Anatech, South Africa)	1
10 µM RP (Anatech, South Africa)	1
DNA template	1 (1 ng/µL)
Nuclease-Free water (Thermo Fisher Scientific, USA)	39.25
Total Volume	**50**

The amplified products were added to 10 µL of 6× loading dye and run on a 1.5% agarose gel electrophoresis and viewed using UV illumination (Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc., USA)) with the GeneRuler™ 100 bp Plus DNA marker (Thermo Fisher Scientific, USA) or GeneRuler™ 1 kb DNA marker (Thermo Fisher Scientific, USA) for size verification. Bands, the correct size for the LASV NP (1743 bp) and LASV GP (1493 bp) genes, were excised from the gel and purified as described below.

The initial PCR conditions were further enhanced to best amplify the separate genes by optimising the template amount (using a dilution series PCR) and the annealing temperature by performing a temperature gradient PCR. The dilution series PCR was conducted using the primers (Section 2.2.1.1) with the pN-S Seg vector as the template in a reaction volume of 50 µL. Components were added (Table 2.1) with adjustments to the DNA template (pN-S Seg) and the final water amount for various dilutions.

Undiluted pN-S Seg (129 ng/µL) was used. Three, 10 fold serial dilutions of the DNA template (pN-S Seg) (1 ng/µL) were made (1 ng/µL to 0.1 ng/µL to 0.01 ng/µL to 0.001 ng/µL), and 1µL used as a template in each reaction. A background control (pCI-neo mammalian expression

vector) and no template control (nuclease-free water (Thermo Fisher Scientific, USA)) were included. The 0.2 mL PCR tubes (Thermo Fisher Scientific, USA) were added to the SimpliAmp™ Thermo Cycler (Applied BioSystems, Thermo Fisher Scientific, USA), and the PCR was run using the conditions as described (Section 2.2.2.1).

Once the template amount was optimised, a temperature gradient PCR was conducted to determine the optimal annealing temperature. For this, multiple reactions were assembled (Table 2.1), and a PCR was performed using the conditions from Section 2.2.2.1 but with different annealing temperatures at 55°C, 60°C and 65°C, respectively. PCR products from all PCR reactions were analysed by adding 10 µL of 6× loading dye and running the products on a 1.5% agarose gel electrophoresis. Bands were visualised using UV illumination (Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc., USA)) with the GeneRuler™ 100 bp Plus DNA marker (Thermo Fisher Scientific, USA). The gel was prepared using the same method described in Section 2.2.2.1.

2.2.2.2 DNA purification

The PCR amplified LASV NP gene product was purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), according to the manufacturer's instructions (Appendix B4). The PCR amplified LASV GP gene product was purified using the Wizard® SV Gel & PCR Clean-Up System (Promega, USA), according to the manufacturer's instructions (Appendix B5). Briefly, the pre-weighed gel slices (for the LASV NP and LASV GP genes) were dissolved in three volumes of agarose dissolving buffer (ADB) (for Zymoclean™ kit) or an equal volume of membrane binding solution (MBS) (Promega, USA).

The tubes were incubated at 55°C until the gel slices were dissolved. For the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), DNA was loaded onto the membrane in the Zymo-spin columns. Two washes were performed with 200 µL of DNA Wash Buffer with centrifugation at 14000×g between wash steps. DNA was subsequently eluted in 10 µL Elution Buffer. The concentration was determined using the Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA) as described in Appendix B2.

For the Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA), DNA was loaded onto the silica membrane on the SV minicolumn, and washes performed with 700 µL and 500 µL of Membrane Wash Buffer with centrifugation at 16000×g between wash steps. DNA was

subsequently eluted in 15 µL of nuclease-free water (Thermo Fisher Scientific, USA). The concentration was determined by adding 1 µL of the purified product to the Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

2.2.2.3 Restriction enzyme digestion

Products were digested with the Not I and Nhe I enzymes to facilitate insertion of the amplified PCR products into individual expression vectors. For this, the concentration of the purified LASV NP and LASV GP gene products was determined using the Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA), as described in Appendix B2. Following this, 0.2 µg of each of the LASV NP and LASV GP PCR products were used for cloning into the pCI-neo mammalian expression vector (Promega, USA). RE digestion with the Fast Digest NotI and NheI enzymes (Thermo Fisher Scientific, USA) was performed in a final volume of 30 µL. The following components were added to each tube; nuclease-free water (Thermo Fisher Scientific, USA) up to 30 µL, 2 µL of 10× Fast Digest Buffer (Thermo Fisher Scientific, USA), 0.2 µg of insert DNA, 1 µL of Fast Digest NotI enzyme (Thermo Fisher Scientific, USA), 1 µL of Fast Digest NheI enzyme (Thermo Fisher Scientific, USA).

The digestion reaction was then incubated at 37°C for five minutes. For digestion of the pCI-neo plasmid, in a final volume of 20 µL, nuclease-free water (Thermo Fisher Scientific, USA) up to 20 µL, 2 µL of 10× Fast Digest Buffer (Thermo Fisher Scientific, USA), 1 µg of the vector backbone (pCI-neo mammalian expression vector), 1 µL of Fast Digest NotI enzyme (Thermo Fisher Scientific, USA)–1 µL of Fast Digest NheI enzyme (Thermo Fisher Scientific, USA) was added. The digestion reaction was then incubated at 37°C for 30 minutes. The REs were inactivated by incubating the tubes at 80 °C for five minutes in a heating block. Once the enzymes were inactivated, the digestion reactions were run on a 1.5% agarose gel. Digested products were purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) (Appendix B4) or Wizard® SV Gel & PCR Clean-Up System (Promega, USA) (Appendix B5). Concentrations of the purified insert and backbone were determined using 1 µL on the Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Digested products were then used in ligation reactions.

2.2.2.4 Ligation of the Lassa virus NP and Lassa virus GP genes and the plasmid

For each ligation reaction, a final volume of 10 µL was prepared to contain the following components: 191 ng of the insert DNA (LASV NP)/ 163 ng of insert DNA (LASV GP), 100 ng of the linearised vector (pCI-neo mammalian expression vector), 5 µL of 2× Rapid Ligation Buffer (Promega, USA), 1 unit of T4 DNA Ligase (Promega, USA), and nuclease-free water (Thermo Fisher Scientific, USA) up to 10 µL. The ratio of insert DNA to linearised vector backbone DNA was optimised to favour LASV NP and LASV GP gene insertion over the re-ligation of the plasmid backbone. The 6:1 molar ratio was determined to be the optimal ratio for LASV NP and LASV GP gene insertion into the expression vector.

The reaction tube was then incubated at room temperature for 15 minutes. After that, 5 µL of the ligation reactions were used in the transformation reaction. Competent JM109 *E. coli* cells (Promega, USA) were transformed with the ligation reactions using heat shock, according to the manufacturer's instructions, as detailed in Appendix B1, plated and incubated for 16 hours o/n at 37^{o}C. The negative control comprised competent JM109 *E. coli* cells (Promega, USA) without the plasmid DNA. The pN-S-seg vector was a positive control to determine transformation efficiency.

2.2.3 Verification of Lassa virus nucleoprotein and Lassa virus glycoprotein clones

2.2.3.1 Colony polymerase chain reaction

Single colonies were selected from each plate to identify plasmids containing the LASV NP and LASV GP inserts. The selected colonies were subjected to PCR screening for the LASV NP/ GP inserts, using the primer set described in Section 2.2.1.1. For this, 50 µL of nuclease-free water (Thermo Fisher Scientific, USA) was added to 1.5 mL tubes (Eppendorf, Germany). Afterwards, part of the selected colony was picked using a sterile pipette tip and inoculated into the 50 µL nuclease-free water (Thermo Fisher Scientific, USA). The tubes were then incubated at 95°C for 10 minutes before centrifuged at 12000×g for 5 minutes. Five µL of the supernatant was used as the template in a PCR. Reaction reagents and compositions for the LASV NP and LASV GP PCRs are detailed (Section 2.2.2.1).

A no template control (nuclease-free water (Thermo Fisher Scientific, USA)) and positive control (the purified LASV NP and LASV GP genes) were included. Amplification was then

performed in the SimpliAmp™ Thermo Cycler (Applied BioSystems, Thermo Fisher Scientific, USA) under the following conditions; 94°C for 1 minute, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, and followed by a final extension of 72°C for 7 minutes and held at 4°C. The amplified products were then added to 10 µL of 6× loading dye, run on a 1.5% agarose gel, and observed with UV illumination (Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc., USA)) with the GeneRuler™ 100 bp Plus DNA marker (Thermo Fisher Scientific, USA) for size verification. Colonies indicating bands of 1743 bp (for the LASV NP) or 1494 bp (for the LASV GP) were further analysed through plasmid isolation, RE digestion, and sequencing analysis.

2.2.3.2 Plasmid isolation, restriction enzyme digestion & reading frame confirmation

Colonies positive for the LASV NP or LASV GP gene inserts by PCR screening were then inoculated into LB/Amp (100 µg/mL) broth, incubated overnight at 37 °C with shaking at 150 rpm. The plasmids were isolated using the Zippy™ Plasmid Miniprep Kit (Zymo Research, USA), according to the manufacturer's instructions (Section 2.2.1.2). The concentrations of the isolated plasmids were determined using the Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA), as described in Appendix B2 and by adding 1 µL to the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

Restriction enzyme digestions were conducted as described in Section 2.2.2.3, verifying that the inserts were in the correct orientation. This was achieved by using two restriction enzymes each of which had a single cut site each in either the plasmid backbone or LASV gene insert Based on the number and size of the bands, the orientation of the inserts were determined. The RE BamHI (New England BioLabs Inc, USA) was used with the Fast digest NheI (Thermo Scientific, USA) to verify the LASV NP and LASV GP were inserted correctly into the vector. Single digestions of the plasmids were included to control for enzyme activity. The same aforementioned digestion reactions, apart from one RE, were added to each digestion reaction. The pCI-neo mammalian expression vector digestions were also included. Undigested plasmid controls were also included. The digestion reactions were incubated at 37°C for 1 hour to ensure complete digestion, and separated on a 1.5% agarose gel.

2.2.3.3 Sanger DNA sequencing and reading frame confirmation

The LASV NP containing plasmids (pN-LASV NP) and LASV GP containing plasmids (pN-LASV GP) were sequenced to confirm that no base changes occurred during the PCR amplification. This was conducted using the BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific, USA). To formulate the sequencing reaction mixture, a final volume of 10 µL contained 2.1 µL of 5× sequencing buffer (Thermo Fisher Scientific, USA), 0.5 mM Big Dye Terminator v3.1 (Thermo Fisher Scientific, USA), 3.2 pmol of 10 µM FP used in the PCRs (Sections 2.2.1.1 and 2.2.2.1). nuclease-free water (Thermo Fisher Scientific, USA) was added to constitute 9 µL. Following that, 40 ng of the purified product (LASV NP) and plasmids (pN-LASV NP and pN-LASV GP) were added to the separate sequencing reaction tubes.

Another reaction mix was formulated, replacing the FP with the RP used for the PCRs (Sections 2.2.1.1 and 2.2.2.1). The six PCR reactions were then placed into the SimpliAmp™ Thermo Cycler (Applied Biosystems, Thermo Fisher Scientific, USA) under the following conditions: 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for five seconds, 60°C for four minutes, and the reaction tubes held at 4°C.

After the sequencing reaction, the products were purified to remove any unincorporated labelled dideoxynucleosides triphosphate (ddNTPs), salts, and primers, using a purification and sequencing reaction mixture. The purification mixture contained 3 µL of 3 M sodium acetate (NaOAc) (Merck, Germany), pH 4.6, 62.5 µL of 100% ethanol (Merck, Germany), and 14.5 µL nuclease-free water (Thermo Fisher Scientific, USA). After that, 10 µL of nuclease-free water (Thermo Fisher Scientific, USA) was added to the 10 µL sequencing reaction (described above) to formulate a final volume of 20 µL. The 80 µL purification mix was added to the 20 µL sequencing reaction mix and vortexed. The 100 µL purification mix was then centrifuged at max speed (21100×g) for 30 minutes. The supernatant was then carefully removed, and 250 µL of freshly prepared ice-cold 70% ethanol was added before being centrifuged at 4°C for five minutes at 21100×g. The supernatant was then removed, and 250 µL of 70% ethanol was added before centrifuged at 4°C for five minutes at 21100×g. Finally, the supernatant was removed, and the remaining ethanol could evaporate in the dark.

The sequence analysis was performed on an ABI 3500xI genetic analyser at the DNA sequencing facility in the Faculty of Natural and Agricultural Sciences, University of Pretoria. The AB1 files were analysed using BioEdit Alignment Editor Version 7 (Hall, 2004). The ends of the nucleotide sequences were trimmed, and base calling was conducted where needed. The forward and reverse sequences were aligned, using the Clustal W multiple alignment feature on BioEdit. A consensus sequence was generated and compared to other nucleotide sequences on the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) algorithm, established on the NCBI website. An alignment was conducted with the codon-optimised LASV S segment reference sequence.

The plasmids (1 µg/µL) were sent to Inqaba Biotechnology (South Africa) to be sequenced using the T7 EEV primer (5'–AAGGCTAGAGTACTTAATACGA–3') (Promega, USA) and the T3 primer (5'–ATTAACCCTCACTAAAGGGA–3') (Promega, USA) specific to the pCI-neo vector to obtain a full-length sequence for the pN-LASV NP and pN-LASV GP inserts. The T7 EEV and T3 primer were specific to sequences located on the plasmid backbone, just before the multiple cloning site (MCS). They could be used with the original primer set to generate full-length sequences for our inserts. Once the sequences for each plasmid were obtained, they were aligned to the consensus sequences obtained for each gene, using the BioEdit programme. A new consensus was generated for the LASV NP and LASV GP genes.

These sequences now contained a complete sequence and were aligned to the original codon-optimised sequences, using the Clustal W multiple alignment features on BioEdit. The consensus sequences were analysed for any point (base changes), or frameshift (deletions or insertions) mutations based on the alignment to the reference sequence. The reference sequence and consensus sequences were then translated to the amino acid sequence, using BioEdit Alignment Editor Version 7. These sequences were analysed to determine if the reading frame was correct and if any mutations that occurred resulted in a change in amino acids or the insertion of a premature stop codon. Once the sequences and reading frame were verified, glycerol stocks for the pN-LASV NP and pN-LASV GP vectors were prepared from the remaining LB/Amp (100 µg/mL) broths, containing the recombinant plasmids, as described in Appendix B3.

2.2.3.4 Plasmid preparation for transfection.

For transfection, large-scale good quality plasmid was prepared, using the Endo-Free® Plasmid Maxi Kit (Qiagen, Germany). For this, 1500 µL of the glycerol stocks were inoculated into individual 200 mL of LB/Amp (100 µg/mL) broth for each plasmid and incubated for 16 hours o/n at 37°C with shaking at 200 rpm in the Infors HT Ecotron incubator (Infors HT, USA). These overnight cultures were centrifuged at 6000×g for 15 minutes at 4°C to harvest the bacterial cells. The bacterial pellets were resuspended in 10 mL of Buffer P1 (50mM tris-Cl, pH 8; 10 mM EDTA; 100 µg/mL RNase A) and transferred to a sterile 50 mL tube. After that, 10 mL of Buffer P2 (200 mM NaOH, 1% sodium dodecyl sulphate (SDS)) was added and mixed thoroughly by inverting the tube six times before incubation at room temperature for five minutes.

The QIAfilter cartridge was prepared by screwing the cap onto the outlet nozzle of the QIAfilter Maxi cartridge during the incubation period. After that, 10 mL of chilled Buffer P3 (3.0 M potassium acetate, pH 5) was added to the lysate and thoroughly mixed before the lysate was poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 minutes. After the incubation, the lysate was filtered into a sterile 50 mL tube; 2.5 mL of Buffer ER was added to the filtered lysate, mixed thoroughly, and incubated on ice for 30 minutes. Following the incubation period, the filtered lysate was added to an equilibrated Qiagen-tip and allowed to empty by gravity. The Qiagen-tip was rinsed twice by adding 30 mL of Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7; 15% isopropanol). After the two wash steps, the DNA was eluted into a sterile tube by adding 15 mL of Buffer QN (1.6 M NaCl; 50 mM MOPS, pH 7; 15% isopropanol).

The DNA was precipitated by adding 10.5 mL of room temperature isopropanol (Sigma-Aldrich, USA), mixing, centrifuging at 4000×g for 60 minutes, and carefully discarding the supernatant. Five mL of 70% endotoxin-free ethanol (Thermo Fisher Scientific, USA) was used to wash the pellet. The tube was centrifuged at 4000×g for 15 minutes, and the supernatant was carefully removed. The DNA pellet was allowed to air-dry before resuspension in 300 µL of endotoxin-free water (Qiagen, Germany). The plasmid DNA concentration and quality were determined by adding 1 µL of the plasmid DNA to the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). The plasmid concentration was adjusted to 1 µg/µl with

sterile endotoxin-free water (Qiagen, Germany). Aliquots were prepared and stored at -20ºC for later use.

2.2.4 Cell lines and culture conditions

A frozen stock of Human Embryonic Kidney 293 cell line (HEK293) (American Type Culture Collection (ATCC), USA, C1573 passage 6) was thawed by incubation at 37°C. The thawed cells were transferred to a sterile 15 mL tube; 10 mL of Dulbecco's Modified Eagle's medium (Sigma-Aldrich, USA) was added to the HEK293 cells. The tube was centrifuged at 200×g for five minutes; the supernatant was carefully removed. The cells were gently resuspended in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) with L-glutamine (L-Glut) (Lonza, Switzerland) and 10% foetal calf serum (FCS) (HyClone, USA) (Maintenance medium).

The HEK293 cells in the maintenance medium were transferred to a T25 cell culture flask (NEST, BioMed, USA), and the cells were checked microscopically for cell clumps. The cell culture flask was incubated at 37°C in a humidified CO_2 incubator (Forma Series II Water Jacketed CO_2 Incubator, Thermo Fisher Scientific, USA). Cell growth was monitored daily; when the cells reached 80-90% confluency, they were sub-cultured into a sterile flask.

The growth medium was removed, and the cells were washed twice with 3 mL of Dulbecco's phosphate-buffered saline (DPBS) (Lonza, Switzerland) to subculture the cells; 1 mL of trypsin EDTA (Sigma-Aldrich, USA) was added to the flask, incubated for 1 minute in the CO_2 incubator to detach the cells from the flask wall. The cells were resuspended in a fresh maintenance growth medium. A proportion was moved to a clean flask at a 1:10 ratio. Additional growth medium was added; the cells were checked microscopically to ensure no cell clumps. The flask was placed in the 37ºC humified CO_2 incubator, and the sub-culturing was repeated when the cells reached 80-90% confluency.

2.2.5 Transfection of mammalian cells with recombinant expression vectors

2.2.5.1 Cell count

Cultured HEK293 cells were trypsinised as described in Section 2.2.4 and diluted in 8 mL of a fresh maintenance medium to obtain a uniform suspension with no clumps; 0.2 mL of cell suspension was then diluted in an equal volume of trypan blue (0.1% w/v trypan blue solution

in DPBS) (Lonza, Switzerland) solution and mixed well (non-viable cells stained blue). The diluted cells were added to both sides of the Neubauer haemocytometer (Precicolor–Henneberg-Sander GmbH (HBG), Germany) chamber. The cells were allowed to settle for a few minutes before counting.

The viable cells were counted in each of the four corner squares (containing 16 smaller squares each), while the cells touching the outer margins were excluded, using an inverted light microscope. Viable cells appeared white while non-viable cells stained blue and were not counted. The haemocytometer is designed, so the number of cells in the '16 corner squares' equals the cell number in a total volume of 10^{-4} cm^3. Since 1 cm^3 equals 1 mL, the subsequent cell concentration per mL (and the total number of cells) was determined using the following formula:

Cells per ml = the average count per "16 corner squares" x the dilution factor x 10^4

A suitable number of cells seeded six-well plates, 10 cm^2 individual plates, T25, or T75 flasks, for transfection reactions, based on the cell count.

2.2.5.2 Transfection of mammalian cells

The HEK293 mammalian cells were seeded in a six-well cell culture plate (CellStar, USA), twenty-four hours before transfection, at a density of 0.25×10^6 cells/mL (0.25 mL/well of cell suspension); 2 mL of fresh maintenance media were added to each well. The cells were transfected with Lipofectamine® 2000 (Invitrogen, Life Technologies, USA), according to the manufacturer's instructions. The ratio of the LASV NP and LASV GP plasmid DNA to Lipofectamine® 2000 was optimised for the best transfection efficiency (Table 2.3). The plasmid DNA and Lipofectamine® 2000 were diluted in 150 µL of Opti-MEM® Medium (Gibco, Life Technologies, USA). Then 150 µL of the diluted plasmid DNA was added to 150 µL of the diluted Lipofectamine® 2000 reagent, gently mixed and incubated for 30 minutes at room temperature. Afterwards, 300 µL of the DNA-lipid complex was added to each well of the six-well plate (CellStar, USA) and incubated at 37°C for 24 to 96 hrs in a humidified CO_2 incubator before analysing the transfected cells.

The incubation period was optimised for expressing the LASV NP and LASV GP. This was conducted by collecting cells after 24, 48, 72, and 96 hours, and testing the expression by IFA

(Section 2.2.6.1) and Western blot (Section 2.2.6.4). As controls, an Ebola (EBOV) NP plasmid (EBOV NP gene in a pCI-neo mammalian expression vector) and an empty pCI-neo mammalian expression vector were prepared and transfected into the cells. The EBOV NP plasmid was used as a control as it was known to express the EBOV NP at an elevated level. A plasmid, expressing green fluorescent protein (GFP), was also included to track HEK293 cell transfection. These were conducted at a fixed ratio of 3 µg DNA:6 µl of Lipofectamine® 2000 per well. Table 2.3 shows the amounts of plasmid DNA (pN-LASV NP/ pN-LASV GP), and Lipofectamine® 2000 added to each well, containing the HEK293 mammalian cells to optimise the transfection ratio.

Table 2.3: Optimisation of the ratio of plasmid DNA to Lipofectamine ® 2000 reagent.

Trial ratio	**Plasmid DNA (µg) LASV NP/ LASV GP**	**Lipofectamine® 2000 (µl)**
1:1	3	3
1:1.5	4	6
1:2	3	6
1:2.25	4	9
1:2.5	4	10
1:3	3	9

Troubleshooting of the transfection method was conducted at various stages to improve the LASV NP and LASV GP expression. The one method included freeze-thawed Lipofectamine 2000. The other method involved collecting the HEK293 cells and transfecting them before placing them in a six-well plate as described in these studies (Zhang et al., 2007, Sork et al., 2016).

2.2.6 Verifying Lassa virus NP and Lassa virus GP expression in mammalian cells

2.2.6.1 Immunofluorescence assay (IFA)

The transfected cells were collected at 24-hour intervals from 24 to 96 hours post-transfection and screened for the expression of the LASV NP and LASV GP. The growth medium was removed from each of the wells. The cells were rinsed with 1 mL of DPBS (Lonza, Switzerland) and trypsinised (Section 2.2.4), then collected in a sterile tube. Cells were collected by low-speed centrifugation (300×g), the supernatant discarded, and the cells resuspended in 300 µL DPBS; 10 µL of each cell solution was added to the individual wells on sterile, degreased eight-well IFA slides (MP Biomedicals LLC, USA). Two slides were prepared for each transfected plasmid. The slides were initially incubated at 37°C to air-dry before being fixed in 100% cold acetone (Sigma-Aldrich, USA) at -20°C for at least one hour.

The excess acetone was allowed to air-dry from the slides at room temperature. Each slide was probed individually with 10 µL/well of control antibody serum (Lassa positive and Lassa negative) diluted 1:10 in DPBS (Lonza, Switzerland). For the positive control, Lassa IgG positive serum (from the SVPL, NICDs collection of diagnostics known IgG Lassa positive human sera), collected from a LASV survivor during the convalescent-phase of the disease during the early 1970s, was used. For the negative control, a known negative serum obtained from the South African National Blood Services, screened for rabies and VHFs endemic to South Africa, with routine serological assays at the NICD, was used. The slides were placed in a humidified container (with wet tissue paper) and incubated at 37°C for 30 minutes.

The slides were rinsed with DPBS (Lonza, Switzerland) for five minutes at room temperature and then dipped briefly in distilled water (dH_2O) before air-dried. Following that, 10 µL of the antibody conjugate (Goat anti-human IgG fluorescein isothiocyanate (FITC) (1 mg/mL), Sigma-Aldrich, USA), at a dilution of 1:10 in DPBS (Lonza, Switzerland), was added to the wells. Evans blue dye (100%) (Thermo Fisher Scientific, USA) was added to the conjugate at a 1:500 dilution as a counterstain. The slides were positioned in a humidified container and incubated at 37°C for 30 minutes. The slides were then washed with DPBS (Lonza, Switzerland) for five minutes at room temperature and dipped in dH_2O and air-dried, as before. The slides were glycerol (Thermo Fisher Scientific, USA) mounted and viewed under a

fluorescent microscope (Floid™ imaging system, Thermo Fisher Scientific, USA) at 20× objective magnification. Five fields for each well were observed.

2.2.6.2 Protein isolation for SDS-PAGE and WB analysis

Forty-eight hours post-transfection (Section 2.2.5.2) cells (from the six-well plate) were collected by centrifugation at 200×g, washed with DPBS (Lonza, Switzerland), and lysed with 1% triton X-100 (Thermo Fisher Scientific, USA) in DPBS (Lonza, Switzerland). For this, 150 µL of 1% triton X-100 was added to the cells (from each well); they were incubated on ice for 20 minutes before centrifuged at max speed (20000×g) for 15 minutes; the supernatant was transferred to a sterile tube. The cell lysate and supernatant were analysed using SDS-PAGE and Western blot to determine LASV NP and LASV GP expression.

Fifty µL of the LASV NP, LASV GP, and control (EBOV NP and pCI-neo mammalian expression vector) lysates were transferred to a sterile tube and mixed with 50 µL of loading buffer (0.125 M tris-HCl (Sigma-Aldrich, USA); 4% SDS (BDH Laboratory Supplies, England); 20% glycerol (Sigma-Aldrich, USA); 0.2 M DTT (Thermo Fisher Scientific, USA)), pH 6.8). All samples with loading buffer were heated at 75 °C for five minutes; the prepared lysates were loaded onto the SDS-PAGE gels. The remaining lysates were stored at -20°C. Two mL of the cells supernatant, from each of the wells, was collected in a sterile tube and 50 µL was mixed with the loading buffer as described above. The remaining supernatants were stored at -20°C

2.2.6.3 SDS-PAGE

The glass plates (10.1×7.3 cm, 10.1×8.2 cm) (Bio-Rad Laboratories Inc., USA) with integrated spacers (Bio-Rad Laboratories Inc., USA) were thoroughly cleaned before casting the gel, by wiping them with water and 70% ethanol (Thermo Fisher Scientific, USA). The plates were then set up in the gel casting stand (Bio-Rad Mini-PROTEAN 3 Electrophoresis System (Bio-Rad Laboratories Inc., USA)). To formulate a 9% running gel, 5.9 mL of acrylamide (Sigma-Aldrich, USA), 5 mL of 4× running gel buffer (1.5M tris-HCl (Sigma-Aldrich, USA), pH 8.8), 200 µL of 10% SDS (BDH Laboratory Supplies, England), 8.7 mL of distilled water (dH_2O) was added to a tube. The 4% stacking gel was prepared by adding 1.76 mL of acrylamide (Sigma-Aldrich, USA), 3.32 mL of 4× stacking gel buffer (0.5M tris-HCl (Sigma-Aldrich,

USA), pH 6.8), 132 µL of 10% SDS (BDH Laboratory Supplies, England), and 8.12 mL of distilled water to a sterile tube.

Before the stacking and running gels were poured, 200 µL of 10% ammonium persulfate (APS) (Bio-Rad Laboratories Inc., USA) and 20 µL of tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories Inc., USA) was added to the running gel. For the stacking gel, 150 µL of 10% APS (Bio-Rad Laboratories Inc., USA) and 15 µL of TEMED (Bio-Rad Laboratories Inc., USA) was added to the stacking gel. The running gel was poured ¾ of the way to the top of the glass sandwich; isopropanol (Sigma-Aldrich, USA) was added on top of the gel, allowed to set for 30 minutes.

Once the running gel was set, the isopropanol (Sigma-Aldrich, USA) was removed; the gel top was rinsed with distilled water. The stacking gel was poured on top of the running gel, and a 10-well comb was inserted. The gel was allowed to set for 30 minutes. Once both gels had set, they were placed into the clamping frame and electrode assembly, put into the tank, and the inner chamber filled with 1× Tank Buffer (0.025 M tris (Sigma-Aldrich, USA), 0.192 M glycine (Sigma-Aldrich, USA), 0.1% SDS, pH 8.3) to above the lower glass plate. The outer chamber was filled with a 1× Tank Buffer.

Prepared cell lysates and supernatants (30 µL) were run alongside 5 µL of Bio-Rad Precision Plus Protein standard molecular weight marker (Bio-Rad Laboratories Inc., USA). The gel was initially run at 100V until the samples were through the stacking gel, then the voltage was increased to 180V. The controls used are described in Section 2.2.6.2.

2.2.6.4 Western blot

The Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories Inc., USA) transferred the gel to a PVDF membrane for use in the Western blot. The gel was first equilibrated in transfer buffer (40 mL of 5× Tank Buffer (0.025 M tris (Sigma-Aldrich, USA), 0.192 M glycine (Sigma-Aldrich, USA), 0.1% SDS, pH 8.3), with 20 mL of 100% methanol (Sigma-Aldrich, USA) and ddH_2O up to 200 mL for 10 minutes. To set up the transfer cell, one thick fibre pad (Bio-Rad Laboratories Inc., USA) was dipped into transfer buffer and added to the transfer cell. The transfer buffer was then poured on top of the fibre pad (Bio-Rad Laboratories Inc., USA), ensuring that the pad was covered entirely. The PVDF membrane (0.2 µm PVDF membrane, Bio-Rad Laboratories Inc., USA) was cut to fit the size of the gel; the membrane

was activated, placing it in 100% methanol (Sigma-Aldrich, USA) and rinsed with dH_2O. The PVDF membrane was then soaked in transfer buffer.

The PVDF membrane (0.2 µm PVDF membrane, Bio-Rad Laboratories Inc., USA) was positioned on the fibre pad on the transfer cell and the air bubbles removed. The equilibrated gel was placed on the membrane, and air bubbles were carefully removed. The other pre-soaked fibre pad was placed on top of the gel, and air bubbles were removed by carefully rolling a pencil over the membrane. The transfer lasted 20 minutes at 20V at maximum power and current (120 Watts and 600 mA) settings. The fibre pads were removed from the transfer cell, and the membrane was carefully removed and transferred to the blocking buffer.

The membrane was blocked overnight at 4 °C with 10 mL of blocking buffer (5% milk (Spar Fat-free milk powder) in TTBS (1% tween in tris buffer saline (25 mM tris (Sigma-Aldrich, USA), 150 mM NaCl, 2 mM KCl (Merk, Germany), pH 7.4)). The blocking buffer was removed, and the membrane was washed with 1% tween in TBS (TTBS). All antibodies were diluted in TTBS with 2% milk. For detection by Western blot, membranes were probed with antibodies specific for the LASV NP or LASV GP proteins or an anti-histidine antibody directed against the His-tag of the expressed recombinant proteins.

For the protein-specific antibodies, the primary antibody (10 mL) (a commercial rabbit anti-Lassa N antibody (5 mg/mL) (Cusabio, USA), and a commercial rabbit anti-Lassa GP complex (1 mg/mL) (Absolute Antibody, United Kingdom) was added to the membrane at a dilution, yielding final antibody concentration of 1µg/mL and incubated for two hours at room temperature with shaking. The membrane was washed three times with TTBS (10 minutes each time) at room temperature with shaking.

The secondary antibody (10 mL) goat anti-rabbit antibodies (from KPL TrueBlue™ HRP conjugated) (1 mg/mL) was added to the membrane at a dilution that yielded a final concentration of 0.2 µg/mL and incubated for one hour at room temperature with shaking and the wash steps repeated. For detection using the histidine tag, the membrane was incubated with a His-probe-HRP (Thermo Fisher Scientific, USA) (1 µg/mL final concentration) for one hour at room temperature with shaking. The membrane was washed three times with TTBS (10 minutes each time) at room temperature with shaking.

The SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, USA) working solution was formulated by adding 1 mL of stable peroxide solution (Thermo Fisher Scientific, USA) to a clean 2 mL tube and 1 mL of luminol/enhancer solution (Thermo Fisher Scientific, USA) was added to the same tube. The working solution (Thermo Fisher Scientific, USA) was poured onto the PVDF membrane covering the entire surface, followed by a five-minute incubation at room temperature. The membrane was then viewed for chemiluminescence (Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc., USA)) to visualise the presence of the LASV NP and LASV GP. The controls used are described in Section 2.2.6.2.

2.2.7 Generating stable cell lines

HEK293 mammalian cells were cultured and maintained as described in Section 2.2.4 to generate individual stable cell lines for the LASV NP and LASV GP. The HEK293 mammalian cells were counted using the Neubauer haemocytometer (Precicolor HBG, Germany) (Section 2.2.5.1). The HEK293 mammalian cells were seeded and transfected with the expression vectors, containing the individual LASV NP and LASV GP genes, using Lipofectamine® 2000 (Invitrogen, Life Technologies, Netherlands) (Section 2.2.5.2) into 10 cm^2 culture plates (CellStar, USA). The transfected HEK293 mammalian cells were analysed for LASV NP and LASV GP expression at 48 hours post-transfection. The maintenance medium was changed to a medium containing 500 µg/mL of the antibiotic, G-418 (Gibco, Life Technologies, USA).

This selective growth medium was changed every two to three days for three to five weeks until drug-resistant clones appeared and grew. A negative (un-transfected HEK293 mammalian cells) control was included to monitor the selection process. Two methods were employed to select clones to obtain pure individual LASV NP and LASV GP clones. In the first method cloning rings (Sigma-Aldrich, USA) were used to select individual clones from the plates when drug-resistant clones appeared. Individual cloning rings were placed onto visible colonies, using sterilised tweezers, to surround the entire colony.

The cells in the centre were then trypsinised, transferred into a fresh selective growth medium described above, placed into individual wells of a 96-well plate (CellStar, USA) and incubated at 37°C in a CO_2 incubator and maintained (Section 2.2.4). The selected stable clones were scaled up from the 96-well plate to a six-well plate (CellStar, USA). The individual HEK293

mammalian cell clones were individually screened for LASV NP and LASV GP expression once they reached confluence in the six-well plate by IFA (Section 2.2.6.1) and Western bot (Sections 2.2.6.2, 2.2.6.3 and 2.2.6.4).

Those expressing the desired proteins at elevated levels (70% of cells fluorescing) were scaled up to a T25 cell culture flask (Nest, Biomed, USA) and maintained (Section 2.2.4). The second method to select pure LASV NP and LASV GP stable cell lines was by serial dilution of the selected stable cell lines that were a mixture of expressing and non-expressing cells. The transfected mammalian cells were seeded onto a 96-well cell culture plate (CellStar, USA); a serial dilution was performed across the plate to dilute the cells. This was conducted by adding 100 µL of maintenance medium containing 500 µg/mL of the antibiotic, G-418 (50 mg/mL) (Gibco, Life Technologies, USA), to each well.

Following this, 100 µL of the cell suspension was added to each well in the first column of the plate. Next, 100 µL of the cell suspension from the wells in the first column was added to the adjacent well. This was repeated across the 96-well cell culture plate (CellStar, USA), resulting in a two-fold dilution of cells across the plate. A chance existed that the latter dilutions would have individual cells in a well by serially diluting the cells this way, which could be clonally expanded, resulting in a single uniform cell population.

The HEK293 mammalian cells were incubated in a humidified CO_2 incubator (Forma Series II Water Jacketed CO_2 Incubator, Thermo Fisher Scientific, USA) at 37°C. Once the cells in the last few columns were near full confluence, the cells were trypsinised and scaled up to a 48-well plate (CellStar, USA). This step was repeated until the selected clones were in a six-well plate (CellStar, USA). They were then screened by IFA (Section 2.2.6.1) and Western blot (Sections 2.2.6.2, 2.2.6.3, and 2.2.6.4) to identify those expressing the LASV NP and LASV GP proteins, respectively. Those expressing the desired proteins at elevated levels were scaled up to a T75 cell culture flask (Nest, Biomed, USA) and maintained (Section 2.2.4). The stable LASV NP and LASV GP clones were frozen down and stored in liquid nitrogen until needed (Appendix B6).

2.3 Results

2.3.1 Construct design and cloning

2.3.1.1 PCR amplification of the Lassa virus NP and Lassa virus GP genes

The LASV NP and LASV GP genes were individually amplified using the designed primers from the LASV S segment plasmid (Figure 2.2). The expected product sizes for the LASV NP and LASV GP were 1743 bp and 1493 bp, respectively (Figure 2.2). The band shown above 3000 bp was taken as the full-length S segment (which would also be amplified by the primer set), while the bands seen between 1500 and 2000 bp were the LASV NP (1743 bp) and LASV GP (1493bp) gene PCR products, respectively.

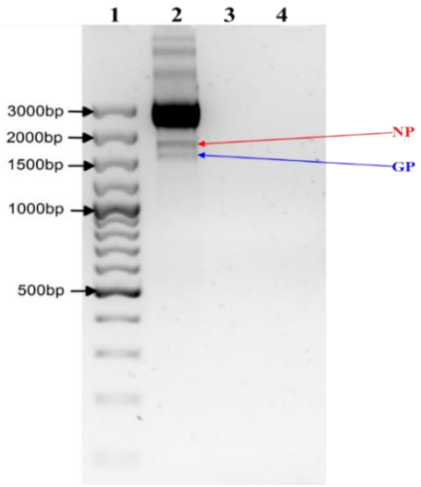

Figure 2.2: Initial PCR amplification of the LASV NP and LASV GP gene from the pCI-neo expression vector with Lassa virus S segment.

Lane 1: GeneRuler™ 100 bp Plus DNA marker. Lane 2: Isolated pCI-neo expression vector with LASV S segment. Lane 3: Negative/Background control (isolated pCI-neo expression vector without LASV S segment). Lane 4: No template control (nuclease-free water). The LASV NP gene is shown in Figure 2.2 by a red arrow at 1743 bp, while a blue arrow at approximately1493 bp is the LASV GP gene.

The initial PCR attempts yielded low levels of LASV NP and LASV GP PCR products. Amplifying the full S segment was favoured under the provided PCR conditions. To improve the efficiency of the PCR reaction and favour amplifying the individual genes, a dilution series was performed with varying amounts of input template DNA and a temperature gradient PCR to optimise the template concentration and annealing temperatures, respectively (Figure 2.3).

The dilution series PCR (Figure 2.3A) was conducted to determine the optimum concentration of the template DNA to amplify the LASV NP and LASV GP genes with a range of DNA template concentrations (1 ng to 1 pg was tested). The study established (Figure 2.3A) that the optimal template DNA concentration was 1 ng for amplifying both the LASV NP and LASV GP genes. Using 1 ng of template yielded sufficient product and adequate size separation for the LASV NP and LASV GP PCR products to be easily excised and purified from the gel. Next, the annealing temperature was optimised to efficiently amplify the LASV NP and LASV GP genes.

The temperature gradient PCR results are shown in Figure 2.3B; three annealing temperatures (55°C, 60°C, 65°C) were tried. The optimum annealing temperature for the primers was determined at 55°C (Figure 2.3B). Based on these results, all subsequent PCR reactions were performed at an annealing temperature of 55°C.

Having optimised the PCR conditions, a PCR reaction was performed, and the LASV NP and LASV GP PCR products were excised from the gel and purified (Figure 2.4). Agarose gel electrophoresis confirmed successful purification and a single PCR product for each target (Figure 2.4). These purified LASV NP and LASV GP PCR products were used as templates in subsequent PCR reactions to increase the yield of the purified product required for cloning the individual genes into the pCI-neo mammalian expression vector. The LASV NP gene was referred for sequencing to confirm the sequence was correct as the DNA polymerase used to amplify the gene was not a high-fidelity enzyme. The sequencing results are discussed in subsequent sections.

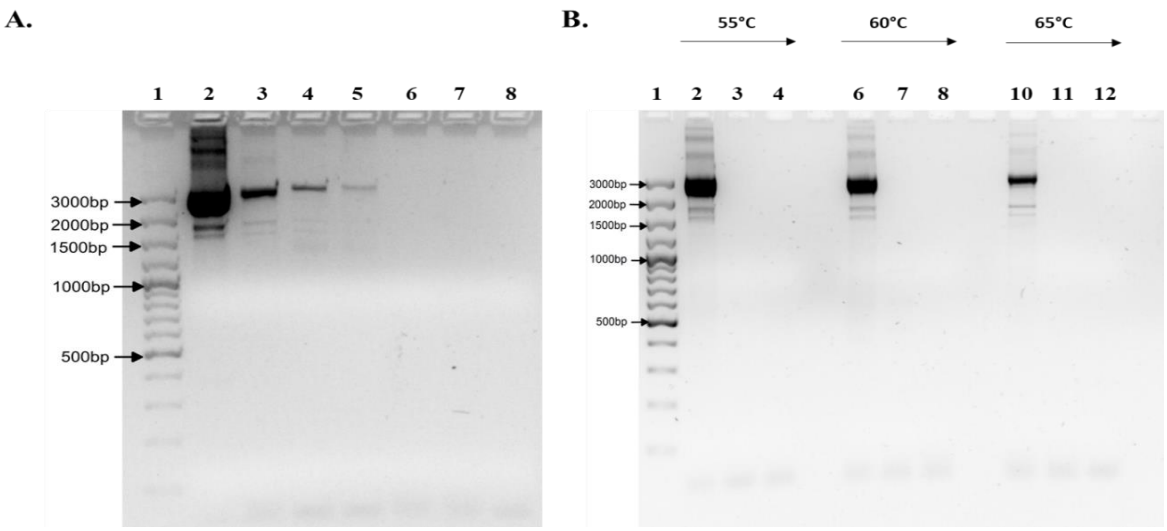

Figure 2.3: Optimising template amount and annealing temperature for PCR amplification of the LASV NP and LASV GP genes.

A) PCR amplification products for the dilution series PCR for the LASV NP and LASV GP genes are shown. Lane 1: GeneRuler™ 100 bp Plus DNA marker. Lane 2: Template at 129 ng. Lane 3: Template at 1 ng. Lane 4: Template at 0.1 ng Lane 5: Template at 0.01 ng. Lane 6: Template at 0.001 ng Lane 7: Negative control (pCI-neo mammalian expression vector without LASV S segment insert). Lane 8: No template control (nuclease-free water). B) PCR amplification products following the temperature gradient PCR for the LASV NP and LASV GP genes are shown Lane 1: GeneRuler™ 100 bp Plus DNA marker. Lane 2, 6, 10: pCI-neo mammalian expression vector with LASV S segment. Lane 3, 7, 11: Background control (pCI-neo mammalian expression vector without LASV S segment). Lanes 4, 8, 12: No template control (nuclease-free water). Lanes 2 to 4 show an annealing temperature of 55°C. Lanes 6 to 8 show an annealing temperature of 60°C. Lanes 10 to 12 show an annealing temperature of 65°C.

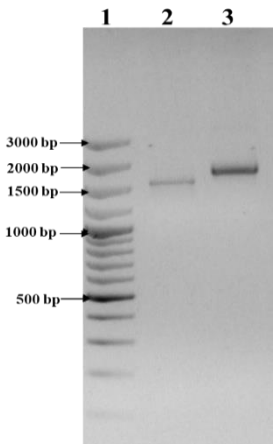

Figure 2.4: Confirmatory gel of the purified LASV NP and LASV GP gene amplification products.

Lane 1: GeneRuler™ 100 bp Plus DNA marker. Lane 2: Purified LASV GP gene, Lane 3: Purified LASV NP gene.

2.3.1.2 Cloning of Lassa virus NP and Lassa virus GP genes

The purified LASV NP gene product concentration was determined to be 110 ng/µL and the LASV GP was 119.7 ng/µL. Once the concentration of the PCR products was determined, 1µg of each product was successfully digested with Not I and Nhe I; 0.2 µg of the digested products was used in the ligation reaction as described. Following ligation, the separate LASV NP and LASV GP ligation reactions were transformed into competent JM109 *E. coli* cells and incubated overnight. LB/Amp agar plates had bacterial growth in single colonies, whereas the negative control plate indicated no growth.

Colonies were selected from the LB/Amp agar plates and screened using colony PCR to identify colonies containing the pN-LASV NP and pN-LASV GP vectors. The PCR reactions were conducted using the same primer set used for cloning and under the same PCR conditions. Colonies containing the LASV NP insert were expected to have a band in 1743 bp, and those with the LASV GP insert at 1493 bp. Five colonies were selected from the LASV NP LB/Amp transformation plate and screened, with three being positive for the LASV NP gene. Fifteen colonies were selected from the LASV GP LB/Amp transformation plate and screened, with three being positive for the LASV GP gene. Figure 2.5A and Figure 2.5B present the positive colonies for the pN-LASV NP and pN-LASV GP vectors. The pN-LASV NP and pN-LASV GP clones were further verified by RE digestion with BamHI and NheI.

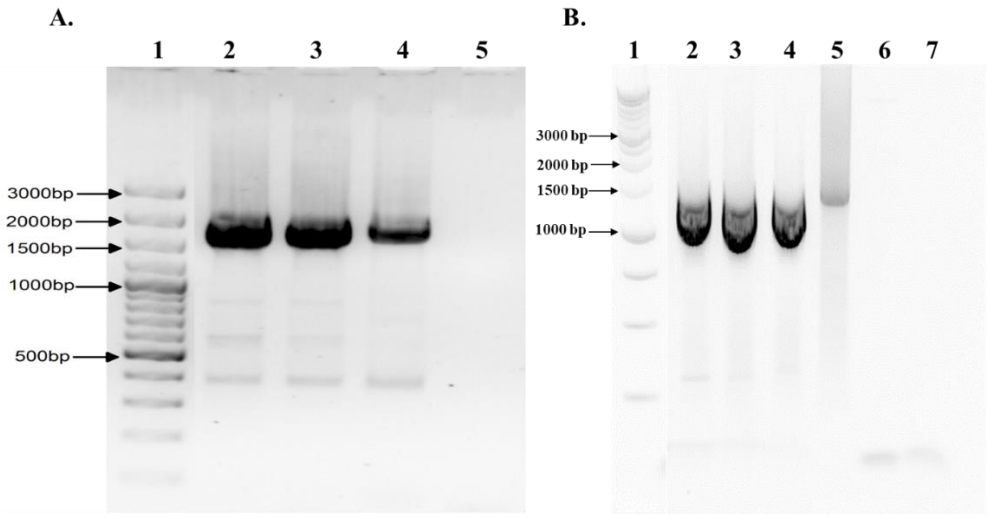

Figure 2.5: Representative gels showing amplification products from the individual colony PCRs to identify colonies with the LASV NP and LASV GP gene inserts.

A) LASV NP colony PCR. Lane 1: GeneRuler™ 100 bp Plus DNA marker. Lane 2: Selected bacterial colony 1. Lane 3: Selected bacterial colony 2. Lane 4: Purified LASV NP gene (control). Lane 5: No template control (nuclease-free water). B) LASV GP colony PCR. Lane 1: GeneRuler™ 1 kb DNA marker. Lane 2: Selected bacterial colony 1. Lane 3: Selected bacterial colony 2. Lane 4: Selected bacterial colony 3. Lane 5: Purified LASV GP gene (control). Lane 6: Negative control (pCI-neo mammalian expression vector). Lane 7: No template control (nuclease-free water).

2.3.1.3 Verifying Lassa virus NP and Lassa virus GP clones by restriction enzyme (RE) digestion

RE digestion was conducted on the PCR positive clones to verify the LASV NP and LASV GP clones, followed by sequencing the insert and vector backbone. The RE digestion was achieved using the NheI and BamHI, ensuring that the LASV NP and LASV GP genes were correctly orientated on insertion into the vector backbone and that the recombinant plasmids were 'pure'. BamHI and NheI were used as single cut sites existed for each in the plasmid backbone and MCS of the plasmid, respectively. The study could, therefore, determine if the plasmid had the insert in the correct orientation, based on the fragment sizes.

For the LASV NP clones, the RE digestion was expected to produce two bands at 3172 bp and 4043 bp (Figure 2.6). For the LASV GP clones, the RE digestion was expected to have two bands at 3172 bp and 3794 bp (Figure 2.7). Control single digestions with the NheI and BamHI were included to establish the activity of the RE. They were expected to yield linearised

plasmids of 7215 and 6966 bp for the LASV NP and LASV GP clones, respectively. Figure 2.6 displays the LASV NP clones with bands of the expected size for the double and single digestions. Figure 2.7 demonstrates that two out of the three LASV GP clones (Clone 1 & 2) had bands of the predicted size for the double and single digestions. One of the three clones (Clone 3 – Lanes 4, 8, 12, 16) indicated extra bands and was discarded. The LASV NP and LASV GP clones, indicating the correct size bands, were sequenced to further verify and validate the plasmids before examining LASV NP and LASV GP expression.

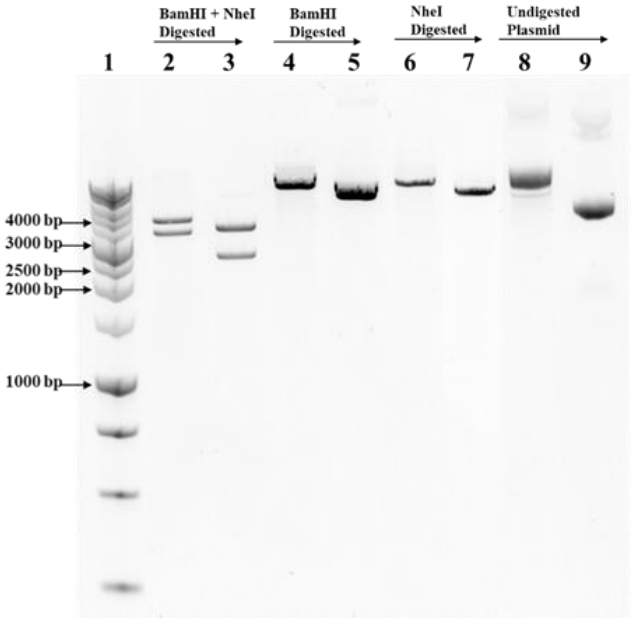

Figure 2.6: Representative gel showing restriction enzyme digestion with BamHI and NheI for the LASV NP containing plasmids.

Lane 1: GeneRuler™ 1 kb DNA Ladder. Lane 2, 4, 6: digestion products for one LASV NP containing plasmids following double (lane 2) and single digests (lane 4 and 6). Similarly, Lanes 3, 5, 7 show the digestion products for empty: pCI-neo mammalian expression vector control. Lanes 8 and 9 show the undigested plasmids for LASV NP containing clone and pCI-neo, respectively.

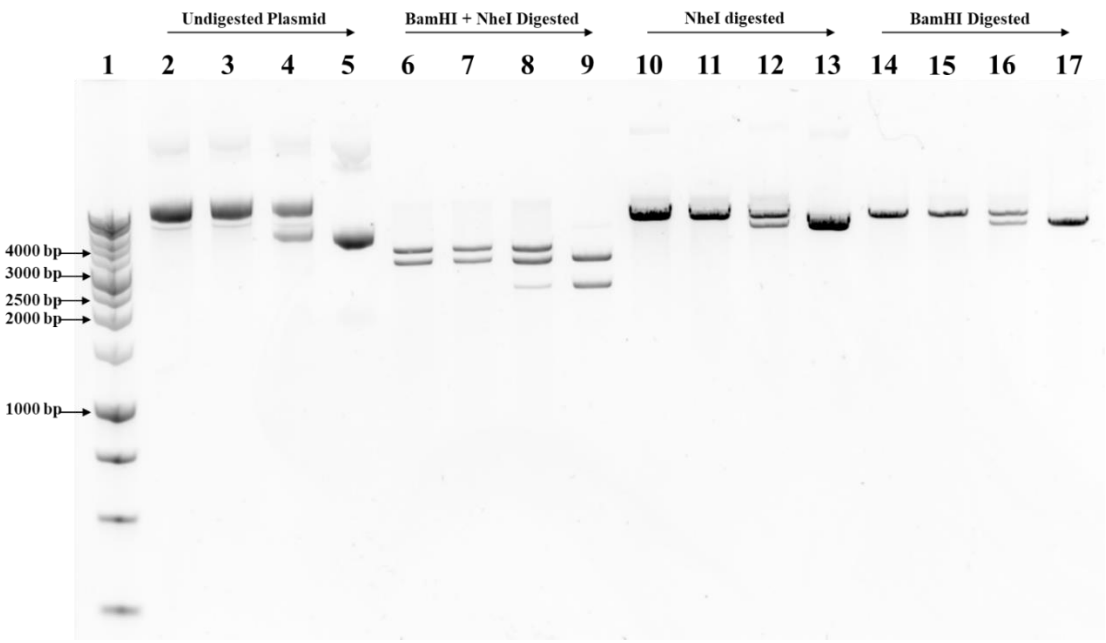

Figure 2.7: Representative gel showing restriction enzyme digestion with BamHI and NheI for the LASV GP containing plasmids.

Lane 1: GeneRuler™ 1 kb DNA Ladder. Lane 2, 6, 10, 14: LASV GP Clone 1. Lane 3, 7, 11, 15: LASV GP Clone 2 Lane 4, 8, 12, 16: LASV GP Clone 3. Lane 5, 9, 13, 17: pCI-neo mammalian expression vector.

2.3.1.4 Verifying Lassa virus NP and Lassa virus GP plasmids by sequencing

Since the LASV NP gene was amplified from the LASV S segment using a DNA polymerase that was not a high-fidelity enzyme, an extra sequencing step after the initial PCR reactions and before cloning was added. This ensured that the amplified LASV NP gene was correct with no mutations. These results indicated that no mutations were present; cloning was performed, using this gene insert (data not shown as it was conducted during my honours project).

The clones were sequenced, following the RE digestion of the individual LASV NP and LASV GP clones, to verify the LASV NP and LASV GP inserts with the plasmid backbones were correct. The originally designed primers and the T7 EEV and T3 primers, specific to the pCI-neo vector, were used. For the pN-LASV NP clones, three plasmids were sequenced (in-house) with the cloning primers and aligned to a codon-optimised LASV S segment (Figure 2.8). Analysis of these sequences confirmed that all three of the selected plasmids had base changes, and two had deletions of nucleotide bases. The plasmids containing the deletions were discarded as the reading frame was affected. The single remaining amino acid sequence was

checked to ascertain if the base changes affected the protein sequence. It was established that these were silent mutations that would not affect the amino acid sequence.

The study established, upon sequencing an early pN-LASV GP clone backbone, that the plasmid was not 'pure' and contained another plasmid in the mixture. This could affect downstream processes; therefore, these clones were discarded, and the cloning steps were redone to ensure that the LASV GP insert, and plasmid backbone were accurate. For the new pN-LASV GP clones, one plasmid was selected and sequenced, using the same set of primers from the PCRs to ensure no base changes. The selected plasmid (pN-LASV GP Clone 2) had no base changes or deletions, and the reading frame was accurate compared to the codon-optimised LASV S segment (Figure 2.9). The amino acid sequence was also correct.

As a final check, the selected pN-LASV NP (clone 1) and pN-LASV GP (clone 2) vectors were further sequenced, using primers for the pCI-neo vector to verify 'ligation joint' end sequences, confirming His-tag and Kozak sequence's presence. The added sequencing step ensured that the recombinant plasmid was pure, with no contamination from another plasmid. The sequencing results for the pN-LASV NP and pN-LASV GP clones indicated that the plasmid backbone was correct and that the His-tag and Kozak sequence were present (Figure 2.10). These plasmids were subsequently called pN-LASV NP and pN-LASV GP. Full length sequence alignments to the codon optimised pN-S seg for both the pN-LASV NP and pN-LASV GP have been included in Appendix C.

Glycerol stocks were prepared for each plasmid. Plasmid preparation was conducted for each, using a maxi-prep endotoxin removal kit. The concentration of the endotoxin free pN-LASV NP vector was determined as 2590.9 ng/µL with an OD 260/280 ratio of 1.93. The concentration of the endotoxin-free pN-LASV GP vector was determined as 4145.3 ng/µL with an OD 260/280 ratio of 1.87. Both plasmids were diluted to 1 µg/µL and stored at -20^{o}C for use in LASV NP and LASV GP expression experiments.

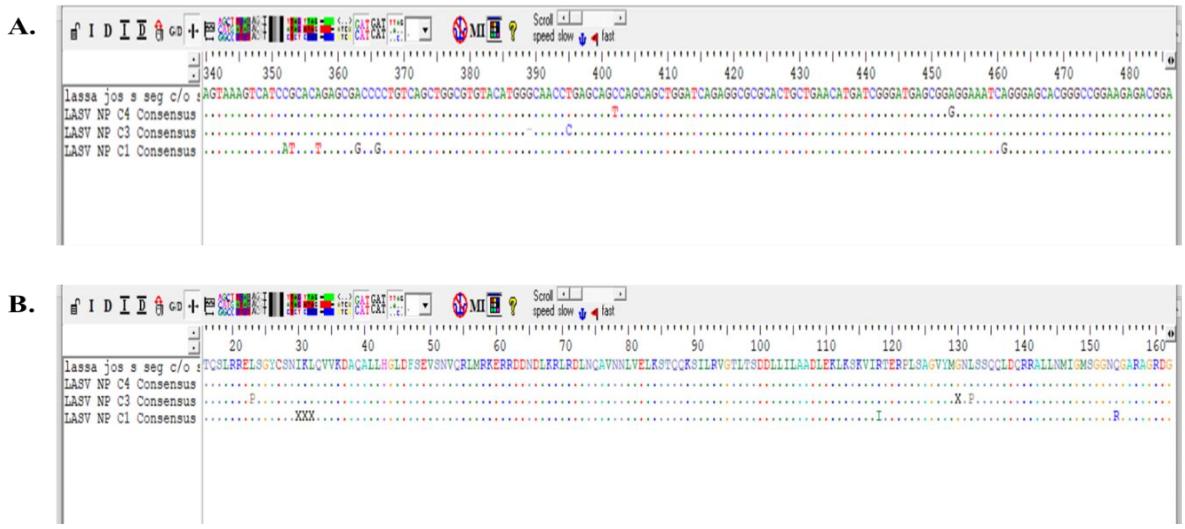

Figure 2.8: LASV NP consensus sequences and amino acid sequences for the pN-LASV NP clones' alignment to the codon-optimised LASV S segment sequence.

A) LASV NP consensus sequences for clones' alignment to the codon-optimised LASV S segment. B) LASV NP consensus amino acid sequences, for the clones, alignment to the codon-optimised LASV S segment amino acid sequence. Only the base or amino acid changes are shown in the figure. Nucleotides are noted only when they differ from the reference sequence; when identical, they are indicated by dots.

Figure 2.9: LASV GP consensus sequences and amino acid sequences for the pN-LASV GP clones' alignment to the codon-optimised LASV S segment sequence.

A) LASV GP consensus sequences for the clone alignment to the codon-optimised LASV S segment. B) LASV GP consensus amino acid sequences, for the clones, alignment to the codon-optimised LASV S segment amino acid sequence. Only the base or amino acid changes are shown in the figure. Nucleotides are noted only when they differ from the reference sequence; when identical, they are indicated by dots.

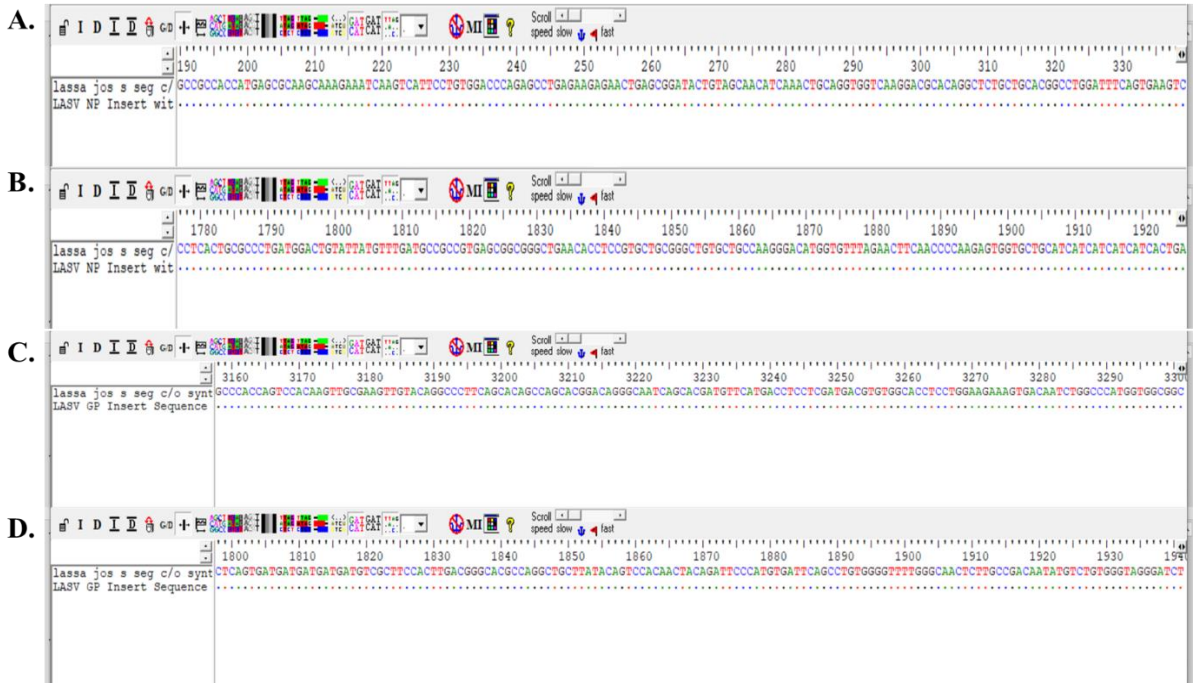

Figure 2.10: Alignment of the 3' and 5'termini of the LASV NP and LASV GP nucleic acid sequences.

A) 5' pN-LASV NP plasmid consensus sequence alignment to codon-optimised LASV S segment confirming the Kozak sequence. B) 3' pN-LASV NP plasmid consensus sequence alignment to codon-optimised Lassa virus S segment confirming the presence of the Histidine tag sequence. C) 5' pN-LASV GP plasmid consensus sequence alignment to codon-optimised LASV S segment confirming the presence of the Kozak sequence. D) 3' pN-LASV GP plasmid consensus sequence alignment to codon-optimised LASV S segment confirming the histidine tag sequence. Nucleotides are noted only when they differ from the reference sequence; when identical, they are indicated by dots.

2.3.2 Optimising and verifying Lassa virus NP and Lassa virus GP expression in mammalian cells

2.3.2.1 Immunofluorescence antibody assay

To verify expression, the optimal transfection conditions had to be determined while maximising the expression of the proteins from the vectors pN-LASV NP and pN-LASV GP; transient transfections followed by verification of expression by IFA were performed. Transfected cells were collected daily after 24 hours until 96 hours to determine the optimal incubation period for expression of the LASV NP and LASV GP proteins, following transfection. It was established that the optimal transfection ratio for the pN-LASV NP vector

was 3 µg DNA and 6 µL Lipofectamine. For the pN-LASV GP vector, the optimal ratio was 4 µg DNA and 9 µL Lipofectamine. The optimal incubation time following transfection of HEK293 cells to maximise expression of the LASV NP and LASV GP proteins was determined to be 72 hours post-transfection (Figure 2.11). A control, EBOV NP plasmid, was included to provide relative expression levels, expressing at high levels.

The research observed that by comparison, the EBOV NP plasmid (Figure 2.11A) showed a greater number of positive fluorescing cells than pN-LASV NP (Figure 2.11C) and pN-LASV GP (Figure 2.11E) plasmids. All cells were counterstained with Evans blue dye, resulting in a red background, contrasting the FITC secondary antibody detection of expressed proteins for improved visualisation. It was observed for the expressed LASV NP cells that the fluorescence pattern was observed throughout the cytoplasm of the HEK293 cells, appearing more granular (Figure 2.11C). For the expressed LASV GP, the fluorescence pattern was more diffuse, with a localisation towards the outer cell membrane and was more pronounced (Figure 2.11E). The LASV NP and LASV GP were being expressed in the HEK293 mammalian cells, based on the IFA results.

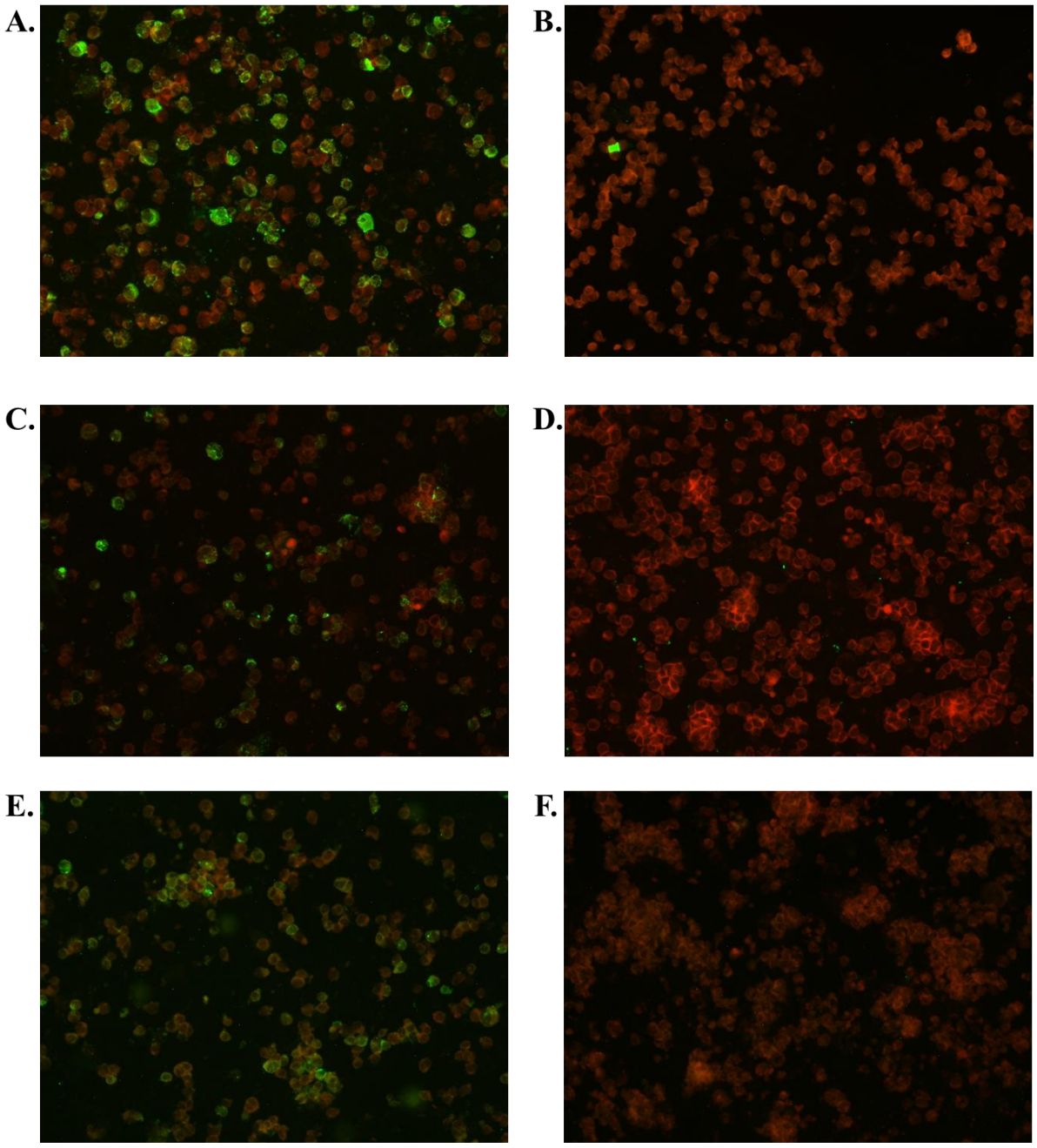

Figure 2.11: Staining patterns of the transfected EBOV, LASV NP, and LASV GP expressing cells.

A) Positive control–plasmid with the Ebola NP with Ebola positive serum. B) Negative control–pCI-neo mammalian expression vector with Lassa positive serum C) pN-LASV NP with Lassa positive serum D) pN-LASV NP with Lassa negative serum E) pN-LASV GP with Lassa positive serum F) pN-LASV GP with Lassa negative serum The pictures were taken using the Floid™ Cell Imaging Station (Life Technologies). The images shown are representative IFA results for the respective proteins obtained by staining with the primary antibodies at a dilution of 1:10 and using secondary goat anti-human FITC antibody (Sigma Aldrich) also diluted 1:10; following transient transfections.

2.3.2.2 Western blot

Western blots were conducted using specific anti-LASV antibodies and an anti-histidine antibody to further verify the LASV NP and LASV GP expression, and to detect the His-tag on the proteins (His-probe-HRP) (Figure 2.12 and Figure 2.13).

LASV NP is a 63 kDa protein. Western blot analysis of the LASV NP transfected HEK293 mammalian cells indicated a unique band between 50 and 75 kDa, corresponding to the correct size for the NP (63 kDa). The LASV NP was detected on a Western blot, using a specific rabbit anti-LASV antibody (Figure 2.12A) and a His-probe, detecting the His-tag in the construct (Figure 2.12B).

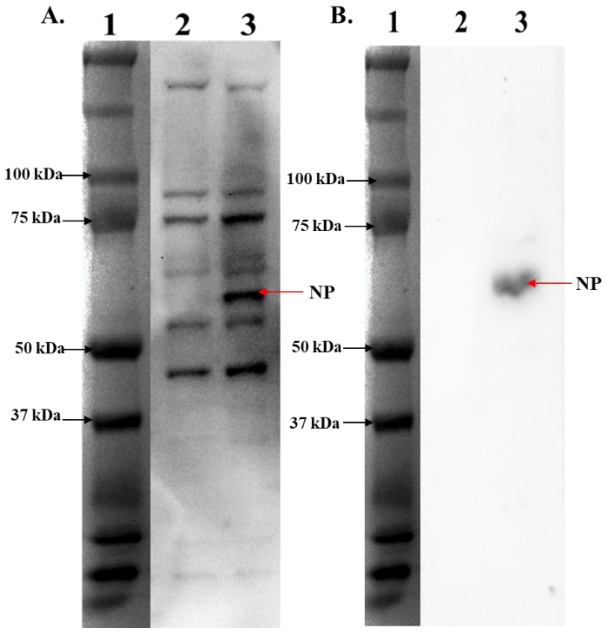

Figure 2.12: Western blot images showing expression of the LASV NP in mammalian cells.

A) Western blot with rabbit anti-Lassa antibodies. Lane 1: Bio-Rad precision plus protein standard. Lane 2: Negative control (pCI-neo transfected HEK293 cell line) cell lysate. Lane 3: LASV NP transfection cell lysate pool. B) Western blot with Histidine probe (His-probe-HRP). Lane 1: Bio-Rad precision plus protein standard. Lane 2: Negative control (pCI-neo transfected HEK293 cell line) cell lysate. Lane 3: LASV NP transfection cell lysate.

LASV GP produces a 76 kDa precursor protein processed into two subunits of 42 or 37 kDa each. The study could not detect the LASV GP by Western blot analysis (Figure 2.13A Figure 2.13B). Ample background binding was observed, despite efforts to troubleshoot this by

changing the conditions. Attempts were made to alter the antibody dilutions for both the anti-LASV antibodies and His-probe, increase the wash steps and the length of each wash. The blocking buffer used was changed to tween 20 tris-buffered saline (TTBS) with bovine serum albumin (BSA) at various percentages (0.5–5%) to remove the background binding. The blocking buffer and antibody incubation periods were also altered by prolonging these periods, changing to shaking from not shaking or reverse, and changing from incubating at room temperature to 4°C incubations; however, the observed background remained the same. Additionally, the WB was attempted with the known LASV positive human sera which produced high background and no unique bands for the LASV GP (data not shown). The sequencing was repeated to verify the construct integrity again. The results were identical to the original sequencing results with no mutations or deletions.

The study attempted to improve the expression levels of the LASV GP by undertaking various transfection methods. One of the methods included freeze-thawed lipofectamine 2000, and the other method involved collecting the HEK293 cells and transfecting the collected cells before placing them in a six-well plate (Zhang et al., 2007, Sork et al., 2016). While these methods improved the expression levels observed with the IFA, it could not be verified with the Western blot with remaining high amounts of background. As the study signified the expression of the LASV GP on the IFA using the anti-LASV-specific antibody and an anti-His antibody (data not shown), a LASV GP stable cell line was developed. Reasoning that a stable cell line would have more expressing cells, increasing the LASV GP for detection by Western blot, as failure to detect the LASV GP on a Western blot was thought to be due to the amount of protein being expressed.

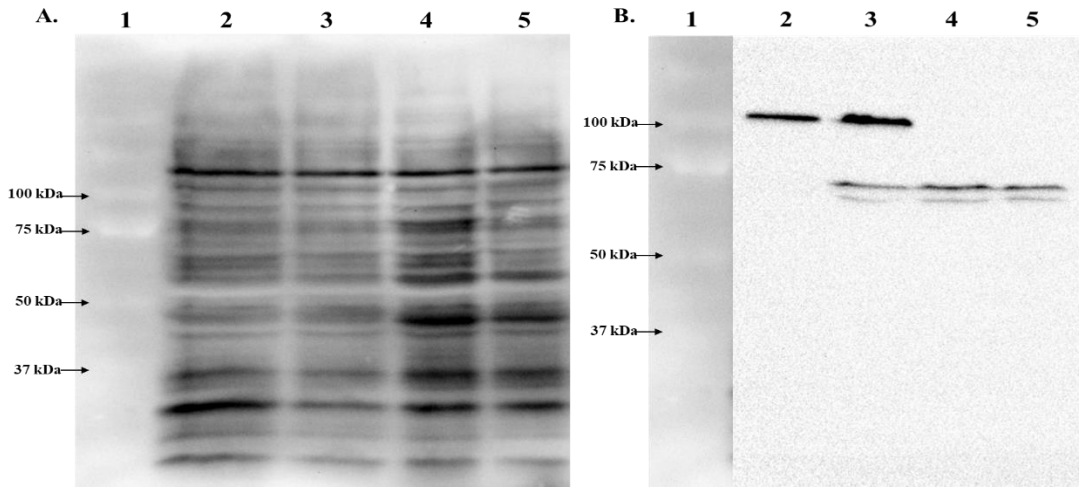

Figure 2.13: Representative, Western blot images obtained upon screening for the expression of the LASV GP in mammalian cells.

A) Western blot results when probing with a rabbit anti-Lassa GP complex antibody. Lane 1: Bio-Rad precision plus protein standard. Lane 2 & 4: Negative control (pCI-neo mammalian expression vector) cell lysate. Lane 3 & 5: LASV GP transfection cell lysate. B) Western blot result when probing with a His-probe. Lane 1: Bio-Rad precision plus protein standard. Lane 2: Ebola NP (positive control). Lane 3: Ebola NP transfection cell lysate (positive control). Lane 4: Negative control (pCI-neo mammalian expression vector) cell lysate. Lane 5: LASV GP transfection cell lysate.

2.3.3 Developing Lassa virus NP and Lassa virus GP, expressing stable cell lines

2.3.3.1 Developing Lassa virus NP, expressing stable cell line

The pN-LASV NP vector was used to develop a stable LASV NP expressing cell line after verifying protein expression. The stable cell line development was conducted to standardise the IFA, preventing batch-to-batch variation, occurring with prepared virus-infected or transient transfection IFA slides. Following the selection period (three to five weeks), clones were selected and scaled up to a six-well plate and screened for expression of the LASV NP through an IFA (Figure 2.14). Following the initial screening of clones by IFA, clones showing the best expression (those estimated to have an expression in > 50% of the cells), were scaled up to a T75 flask before verifying expression by Western blot (Figure 2.14). Two stable LASV NP expressing clones, were selected. LASV NP expressing stable clone 1 was determined to be the most suitable as the fluorescence detected was brighter, appearing to hold more positive cells than LASV NP clone 2.

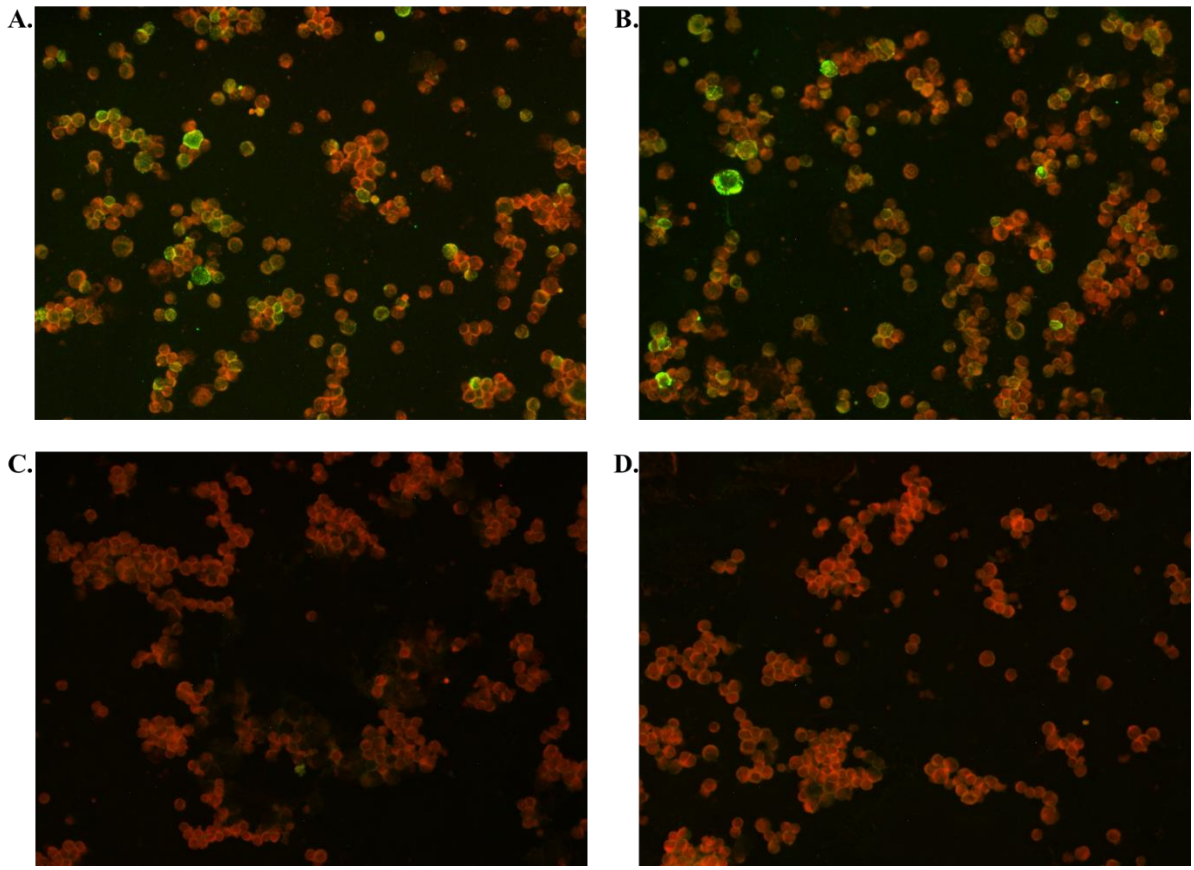

Figure 2.14: Visualising immunofluorescence antibody assay showing the expression of the two selected LASV NP expressing stable cell lines by fluorescence microscopy.

A) LASV NP expressing stable cell line clone 1 probed with Lassa positive serum, 1:10 dilution. B) LASV NP expressing stable cell line clone 2 probed with Lassa positive serum, 1:10 dilution. C) HEK293 mammalian cells probed with Lassa positive serum, 1:10 dilution. D) LASV NP expressing stable cell line clone 1 probed with a known negative serum, 1:10 dilution and using secondary goat anti-human FITC antibody (Sigma Aldrich) also diluted 1:10 for all.

Western blot analysis (Figure 2.15) indicated unique bands between 50 and 75 kDa, corresponding to the correct size for the LASV NP (63 kDa). The LASV NP expressing clones 1 and 2 were frozen and stored in liquid nitrogen until needed.

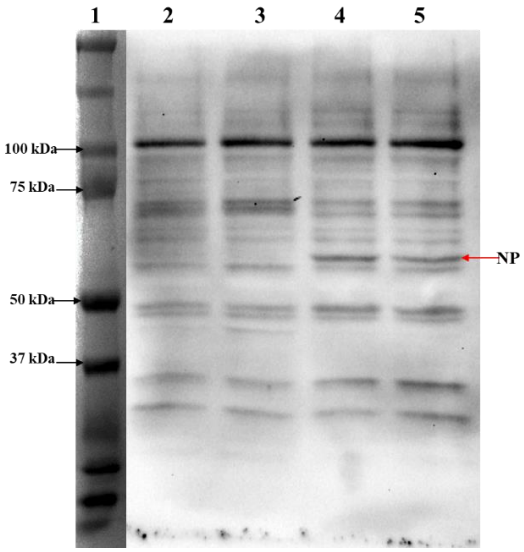

Figure 2.15: Western blot analysis of LASV NP expression in selected LASV NP expressing stable cell lines.

Lane 1: Bio-Rad precision plus protein standard. Lane 2: HEK293 mammalian cells cell lysate (control). Lane 3: HEK293 mammalian cells cell lysate (control). Lane 4: LASV NP expressing stable cell line clone 1 cell lysate. Lane 5: LASV NP expressing stable cell line clone 2 cell lysate. The red arrow indicates the unique band in both clones that represent the LASV NP. The WB was conducted using a LASV NP-specific antibody (commercial rabbit anti-Lassa N antibody (Cusabio, USA)).

2.3.3.2 Developing Lassa virus GP stable cell line

The study followed the same procedure for developing a LASV GP, expressing stable cell lines as for the LASV NP, expressing stable cell lines. After the three-to-five-week selection period, initial screening of the LASV GP expressing stable cell line revealed that the stable cell lines were expressing the LASV GP but not at remarkably elevated levels. While the initial results indicated expression of the LASV GP in the developed stable cell lines, over time, the number of expressed cells decreased until there were a few expressing cells. Figure 2.16 shows the decrease in expression of the LASV GP over time by an IFA. After unsuccessful attempts at developing and selecting ‘pure’ LASV GP expressed stable cell lines, due to time constraints in completing the project, we were not able to develop LASV GP expressing stable cell lines.

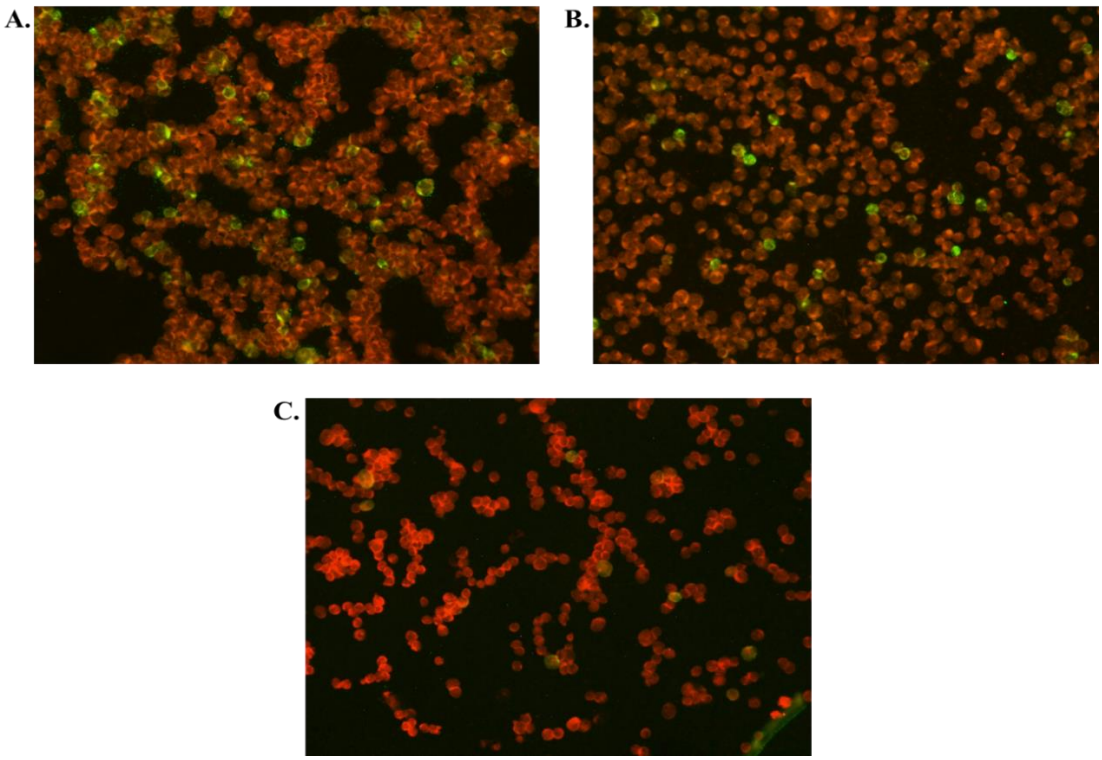

Figure 2.16: Immunofluorescence antibody assay analysis of the LASV GP expressing stable cell line, demonstrating the reduced detection of fluorescing cells over time.

A) Image of LASV GP expressing stable cell early after selection (1:10 dilution of LASV positive serum). B) Image of LASV GP expressing stable cell line two weeks later (1:10 dilution of LASV positive serum). C) Image of LASV GP expressing stable cell line after three weeks (1:10 dilution of LASV positive serum) and using secondary goat anti-human FITC antibody (Sigma Aldrich) also diluted 1:10 for all

2.4 Discussion

This chapter aimed to develop recombinant LASV protein-expressing stable cell lines to replace the need to culture live virus under high containment when producing LASV IFA slides for diagnostics. The LASV NP and LASV GP proteins were chosen as candidates for recombinant expression because both are major structural proteins of LASV, the target of the host's immune system (Günther et al., 2004, Russier et al., 2012). Other researchers have successfully expressed these proteins in recombinant expression systems (Ter Meulen et al., 1998, Masayuki et al., 2012).

Expression cassettes for the LASV NP and LASV GP proteins developed the individual stable cell lines for these proteins by subcloning from a synthesised codon-optimised LASV S segment expression cassette. The individual LASV NP and LASV GP expression cassettes

were cloned into the mammalian expression vector, pCI-neo. The sequences and reading frame were confirmed as accurate. During the LASV NP gene cloning, two silent mutations were introduced into the original codon-optimised sequence; however, since they did not affect the amino acid sequence, the study proceeded with this construct. The mutations likely arose because a high-fidelity polymerase enzyme was not used during the amplification of the LASV NP construct. A high-fidelity polymerase enzyme was used when the LASV GP gene was subcloned to ensure this did not recur.

To verify expression of the recombinant proteins from these cassettes, cells were transfected, and IFA and Western blot analysed protein expression. To obtain good protein expression, the transfection conditions had to be optimised for each construct. The expression of LASV NP and the LASV GP by IFA was detected while confirming LASV NP expression by Western blot. Initial protein expression analysis for both the pN-LASV NP and pN-LASV GP vectors was conducted following transient transfection of the constructs to ensure that the expressed proteins were functional and could be detected by LASV-specific antibodies before developing stable cell lines.

At first, detection of the LASV NP and LASV GP protein expression by IFA was low, indicating suboptimal expression; therefore, conditions for the transfection reaction and the IFA detection protocol were optimised. The IFA results for the LASV NP indicated a fluorescence pattern throughout the cytoplasm of the HEK293 cells (Schlie et al., 2010, Loureiro et al., 2019). Staining was more granular, following what was expected for the LASV NP (Schlie et al., 2010, Loureiro et al., 2019). Recombinant NP was shown to localise as ‘dot-like’ structures evenly distributed in the cytoplasm (Schlie et al., 2010). The observations for the LASV NP IFA were consistent with those made by Schlie et al (Schlie et al., 2010).

Initial IFA results for the LASV GP indicated expression lower than the LASV NP, owing to lower transfection efficiency (data not shown). This challenge was conquered while indicating expression of the LASV GP at levels comparable to the LASV NP with the optimised transfection reaction and IFA conditions as depicted in Figure 2.11. When observing the expression pattern for the LASV GP, a more diffuse pattern with localisation more towards the outer cell membrane, which was expected for LASV GP, was presented (Schlie et al., 2010, Loureiro et al., 2019, Ibukun, 2020). This follows other studies of LASV GP localisation,

confirming the LASV GP in membranous structures in the cytoplasm, representing ER, and in vesicle-like structures and at the plasma membrane (Schlie et al., 2010).

For the LASV NP, besides the IFA results, the study confirmed that the protein was expressed and the correct size (63 kDa) by Western blot analysis. A unique band was observed in the LASV NP cell lysate when probing with a LASV-specific antibody, detecting the His-tag on the protein (His-probe). The Western blot using the rabbit anti-LASV antibody, specified several non-specific bands with a unique band (63 kDa) in the LASV NP cell lysate sample. To improve the number of non-specific bands, the Western blot conditions (antibody ratios, incubation times, blocking buffer and time, wash buffer and length of wash steps) were optimised; however, the non-specific reactions with the antibody remained.

This was owing to crude preparation of the cell lysate resulting in other proteins besides the target protein still being present while increasing the potential for non-specific binding. Another possibility is that because the primary antibody was a polyclonal antibody, it recognised more than one epitope of the LASV NP while cross-reacting with proteins produced by the host cell in the crude cell lysate with epitopes, similar to the LASV NP.

Detecting the LASV GP by WB was unsuccessful with both the anti-LASV GPC antibody and the His-probe. The rabbit anti-LASV GPC antibody Western blot (Figure 2.13A) had several non-specific bands, complicating the identification of unique bands in the GP cell lysate, owing to cross-reactivity with other proteins in the lysate preparation. It was expected that detection using the His-probe might overcome this since it is directed towards the His-tag; however, this was not the case. Several options were considered to overcome this, such as more extended antibody incubation periods, using various antibodies (rabbit anti-LASV GPC or human anti-LASV), various antibody amounts, increasing the length and number of wash steps, and changing the blocking buffer (to TTBS with BSA at varying percentages (0.5–5%)).

The LASV GP was still not detected while optimising the Western blot conditions slightly reduced the number of non-specific bands. Failure to detect the protein by the His-probe was perplexing, as we knew the His-tag was present from our sequencing analysis and by performing an IFA with an anti-histidine antibody (data not shown). We postulate that the lack of detection by the His-probe could be due to the His-tag being hidden or removed during protein folding or processing.

The cleaved LASV GP products were primarily established intracellularly in the ER or vesicle-like structures, while the complete LASV GP structure would be trafficked towards the outer cell membrane (Schlie et al., 2010, Loureiro et al., 2019, Ibukun, 2020). If our cell lysis method is inefficient, we could, therefore, lose most of the LASV GP in the pellet after centrifugation. This was not further investigated because we thought the issue was linked to the amount of LASV GP being expressed. As we had shown expression of the LASV GP on the IFA using the anti-LASV-specific antibody and an anti-His antibody, we continued developing a LASV GP stable cell line, reasoning that the stable cell line has more expressing cells, increasing the amount of LASV GP for detection by Western blot.

While we were unsuccessful at detecting the LASV GP by Western blot, Illick et al. successfully expressed the LASV GP in mammalian cells (Illick et al., 2008). This study indicated high expression of the LASV GPC, GP1 and GP2 in HEK293T and Vero mammalian cells (Illick et al., 2008). The plasmid backbone used in this experiment was the pcDNA3.1 (+) (Invitrogen) and contained the entire 1.5-kbp cytomegalovirus (CMV) major immediate-early (CMV-MIE) promoter with the intron-A sequence (Illick et al., 2008). The CMV-MIE with intron-A sequence would have significantly enhanced the intracellular expression of LASV GP1 and GP2 (Illick et al., 2008). The cell lysis method was also different, using a lysis buffer, containing protease inhibitors to prevent protein degradation in the supernatant, possibly reducing the background (Illick et al., 2008).

The background in this experiment was further reduced by probing and blocking the membrane, performed in 1× PBS, pH 7.4, 5% non-fat dry milk, 0.05% Tween-20, and 0.1% thimerosal. In observing the study methodology, to improve detection of the LASV NP and LASV GP by Western blot, each sample could be prepared differently by either attempting to purify the target proteins from the cell lysate or by ultracentrifugation. We could have cloned the LASV NP and LASV GP genes using various expression vectors, such as the pcDNA3.1 (+) vector, or included the intron-A sequence in our original expression vector. Using the pcDNA3.1 (+) vector would not change developing stable cell lines as the vector contains the geneticin resistance gene–used to develop stable cell lines. The manufacturers, however, recommend linearising the vector to increase the chance of integration and not disrupting elements necessary for expression.

We attempted to develop stable protein-expressing cell lines for the LASV NP and LASV GP, assuming LASV GP expression succeeded based on IFA results only. Stable cell lines integrated the target gene into the host genome, and clones, expressing the desired protein at high levels can be selected. Developing the LASV NP stable expressing cell line was a time-intensive procedure. Identifying a 'pure' LASV NP expressing clone from mixed clones, not always expressed at high levels, proved difficult. Our perseverance paid off, and we produced two clones, indicating excellent expression of the LASV NP by the IFA analysis (Figure 2.14). As a measure of purity, we decided that visual estimation of over 85% of the cells being positive was sufficient for clones to be considered 'pure' since it is known that not every expressing cell will show fluorescence on an IFA. Ideally, during the development of a stable cell line, expressing cells should be sorted from non-expressing cells using direct staining and sorting with flow cytometry. We attempted to 'sort" by serial dilution of positive clones and were content that this was sufficient for the purposes of this project. The LASV NP expression was also detected for both clones on a Western blot, using a commercial rabbit anti-LASV NP antibody. These results demonstrated that both stable cell lines had successfully integrated the LASV NP gene into their genomes, and the expressed proteins could be detected.

Initially, the LASV GP stable cell line selection went well, and IFA analysis of the mixed stable cell lines looked promising with diverse clones expressing the LASV GP being selected. Over time the expression levels decreased until few cells expressed the LASV GP. It is not uncommon for stable cell lines to have low efficiencies of stable integration and persistent expression, and maybe this occurred with our LASV GP (Richards et al., 2002, Mutskov et al., 2004, Wurm, 2004, Schiedner et al., 2008). An explanation for losing expression over time is the random integration of the LASV GP gene into an inactive heterochromatin site (Zahn-Zabal et al., 2001, Wurm, 2004, Matasci et al., 2011, Liu et al., 2014). When a foreign gene is integrated into an inactive heterochromatin site of the host cell, the expression levels of the foreign gene does not remain stable over time, and the genes are silenced, resulting in a lack of protein expression (Zahn-Zabal et al., 2001, Wurm, 2004, Matasci et al., 2011).

HEK293 mammalian cells were used throughout this project since they are commonly used to express recombinant proteins while also being easy to grow and maintain. To improve the expression of the LASV GP, a different cell line, such as Vero cells, could have been used. Vero mammalian cells are derived from the kidneys of African green monkeys; a cell line well characterised, susceptible to VHF viruses, including LASV (Ammerman et al., 2008). Chinese

hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) cells were used successfully by Schlie et al., expressing the LASV NP and LASV GP, while showing the formation of virus-like particles (Schlie et al., 2010).

This chapter's aims are primarily achieved with the successful design, cloning, and expression of the LASV NP and LASV GP genes. We also successfully developed a stable LASV NP expressing cell line. Developing a stable cell line expressing the LASV GP protein requires additional work. Chapter 3 maintains these achievements, using the study's LASV NP expressing stable cell line and the LASV GP expression cassette to develop and evaluate LASV NP and LASV GP-specific IFAs.

CHAPTER 3: DEVELOPING AND EVALUATING THE LASSA VIRUS-SPECIFIC, INDIRECT IMMUNOFLUORESCENCE ANTIBODY ASSAY

3.1 Introduction

As detailed in the previous two chapters, LASV is the mammarenavirus with a substantial public health impact infecting 500 000 individuals annually, with a case fatality rate of <2% (Ogbu et al., 2007, Bausch et al., 2014, Meyer et al., 2018, Radoshitzky et al., 2019a). This virus is endemic to West Africa and maintained in nature through persistent infections of the reservoir host, *Mastomys natalensis (M. natalensis)*, one of the most widespread and common mammals in Africa (Monath et al., 1974, Colangelo et al., 2013, Gryseels et al., 2017). LASV has an incubation period of seven to 21 days, causing a wide range of clinical, appearing indistinguishable from other febrile diseases. Diagnosis based on clinical characteristics is onerous, complicating clinical diagnosis leading to inaccuracy (Ogbu et al., 2007, Kerber et al., 2015).

Diagnostic methods to detect LASV include isolating the virus by cell culture, detecting the viral nucleic acids by RT -polymerase chain reaction (RT-PCR), and detecting Lassa-specific antibodies by serological methods, such as ELISA or immunofluorescence antibody assay (IFA) (Sogoba et al., 2012, Bausch et al., 2014). The RT-PCR is the ‘gold standard’ for early and rapid diagnosis of Lassa fever (Günther et al., 2004). This technique involves the RT of the viral RNA into cDNA before the PCR and may be conducted using inactivated specimens (Drosten et al., 2003). A limitation is that the viraemia for LASV and other mammarenaviruses can be missed and, therefore, CSF, serum, blood, throat washings, urine or pleural fluid samples need to be collected soon after the onset of illness (Buckley et al., 1970, Günther et al., 2004).

ELISAs are used to diagnose viral infections, such as LASV, because they are easy to use, sensitive and specific, and can be performed using inactivated viruses or purified antigens (Ogbu et al., 2007, Raabe et al., 2017). IFAs are used as rapid screening devices, with confirmatory testing conducted by ELISA. The IFA is more sensitive, straightforward, requires

less optimisation, and can detect IgM antibodies much earlier (10 days after initial infection) (Wulff et al., 1975, Ter Meulen et al., 1998, Bausch et al., 2000, Emmerich et al., 2006).

Since it can cause VHF, LASV, and other VHFs are classified as biosafety level 4 (BSL-4) pathogens, the live virus and infectious samples can be handled only in a high-containment laboratory (Charrel et al., 2003, Bausch et al., 2014). This limits laboratories that can perform diagnostic testing for LASV, as limited high-containment facilities exist globally.

Diagnostics for LASV are further hampered attributable to a lack of an affordable and rapid diagnostic serological test for Lassa fever (Emperador et al., 2019). A need exists to develop an improved diagnostic test that can be developed and used with no high-containment facilities (Mazzola et al., 2019). This will allow quicker diagnosis, resulting in the speedy implementation of preventive measures and the quarantine of infected patients, therefore, limiting the spread of the virus (Happi et al., 2019).

The indirect IFA is a diagnostic device, using fixed virus-infected cells or mammalian cells, expressing a specific antigen to detect antibodies in the serum (Masayuki et al., 2012). The main advantage of the IFA is that results can be produced rapidly–crucial when dealing with suspected VHF cases, such as LASV, to prevent the further spread of the disease (Wulff et al., 1975, Broadhurst et al., 2016). For VHF diagnostics, recombinant technology is valuable to develop diagnostic assays, allowing for safe diagnosis outside a high-containment facility once infectious samples were heat-inactivated (Saijo et al., 2006, Bausch et al., 2014). Developing an IFA based on recombinant LASV antigen(s) to detect Lassa-specific antibodies in a patient's serum, would meet the demand for a safe, reliable, and quick diagnostic device that does not require a BSL-4 laboratory.

For the work described in this chapter, we sought to build on the work conducted in Chapter 2 with the main aim of developing an individual LASV NP and LASV GP IFA diagnostic device while evaluating the performance characteristics of these assays. The LASV NP and LASV GP were chosen because both form the major structural proteins for LASV. Most antibodies, therefore, are produced against these proteins (Günther et al., 2004, Russier et al., 2012). The developed LASV NP, expressing stable cell line, produced a batch of LASV NP IFA slides while a batch of LASV GP IFA slides was produced by transient transfection of cells with the LASV GP expressing plasmid. A stable cell line would be beneficial as we know the expression

level on each slide would be consistent and would allow standardisation of the assay, but this was only available for the LASV NP.

Results through the LASV NP and LASV GP assays were measured against those obtained with LASV IFA, using virus-infected cells. A measure of detection accuracy of the IFA assays for LASV was determined with a panel of 30 known LASV negative serum samples and positive control. This was a preliminary evaluation only due to constraints with obtaining a larger sample set of known LASV positives. A measure of the sensitivity of the IFA assays was obtained, applying a serial dilution of a known LASV IgG positive serum sample.

3.2 Materials and methods

3.2.1 Developing and optimising Lassa virus-specific nucleoprotein and Lassa virus-specific GP IFAs

3.2.1.1 Developing Lassa virus-specific NP and Lassa virus-specific GP IFAs

- Preparing the virus-infected cell-based LASV, LASV NP and LASV GP IFA slides

The LASV NP and LASV GP recombinant antigen-based IFAs (Based on the LASV Josiah strain) were compared to traditional LASV-specific IFA slides prepared from whole live virus in the BSL-4 laboratory at the SVPL, NICD. The slides comprised a mixture of mammalian cells (Vero cells (C1008, ATCC)) infected with LASV (comprising LASV strains from three lineages (Liberia (Josiah), AV, and Nigeria (Nig)) and Vero cells, not infected with LASV (Johnson et al., 1981). A single well on each virus-infected IFA slide was spotted with control Vero cells only as a negative control. The optimal ratio of1:3 non-infected to infected cellsas determined in the laboratory (data not shown) was used for spotting the whole virus-infected cells to non-infected cells (Johnson et al., 1981).

For the LASV NP IFA slides, the newly developed LASV NP expressing stable cell line was employed. The stable LASV NP expressing cell line was grown and maintained in a T75 cell culture flask (Nest, Biomed) (Section 2.2.4). For the LASV GP IFA slide preparation, HEK293 mammalian cells were transfected with the pN-LASV GP vector seventy-two hours before the slides were prepared. HEK293 mammalian cells (control HEK293 cells) were also maintained in a T75 cell culture flask (Nest, Biomed) (Section 2.2.4). The standard protocol (Section 2.2.6.1) was used initially to develop the LASV NP and LASV GP-specific IFAs.

The optimal ratio of expressing cells to non-expressing cells needed to be determined before detecting the LASV NP and LASV GP-specific IFA slides. Mixing expressing and non-expressing cells better distinguish true positive non-specific reactivities. Various ratios of LASV NP expressing stable cells or LASV GP expressing transfected cells to HEK293 mammalian cells were assessed to determine the optimal ratio. These included ratios of 1:1 1:2, 1:3, 1:4, and 1:5. 10 µL of the cells was spotted on individual wells with an eight-well microscope slide (MP Biomedical LLC) with a 6 mm diameter. A control well was spotted with HEK293 cells only, probed with LASV IgG antibody-positive serum. A second control where the LASV NP expressing stable cell line/ LASV GP expressing transfected cells (undiluted) was spotted and probed with LASV antibody-negative serum. The IFA was conducted, according to the standard procedure (Section 2.2.6.1).

A mixture was prepared and spotted onto eight-well microscope slides (MP Biomedicals LLC) for each protein, LASV NP, and LASV GP, once the optimal ratio of protein-expressing non-expressing cells was determined for each protein. A single well on the IFA slides was spotted with control HEK293 cells. Figure 3.1A shows the slide layout for the LASV NP IFA and LASV GP IFA. Figure 3.1B illustrates the slide layout for the traditional LASV IFA. Figure 3.1 also suggests where the positive, negative, and test serum was added. Once spotted, the slides were stored at -70 °C until needed.

3.2.1.2 Optimising conditions for the Lassa virus-specific IFA

The IFA protocol steps (serum dilution, temperature, and length of incubation period, washing steps) (Section 2.2.6.1) were optimised for the best results before evaluating the LASV NP and LASV GP IFAs with the panel of 30 known negative samples. This was conducted with the LASV antibody-positive and negative sera (controls).

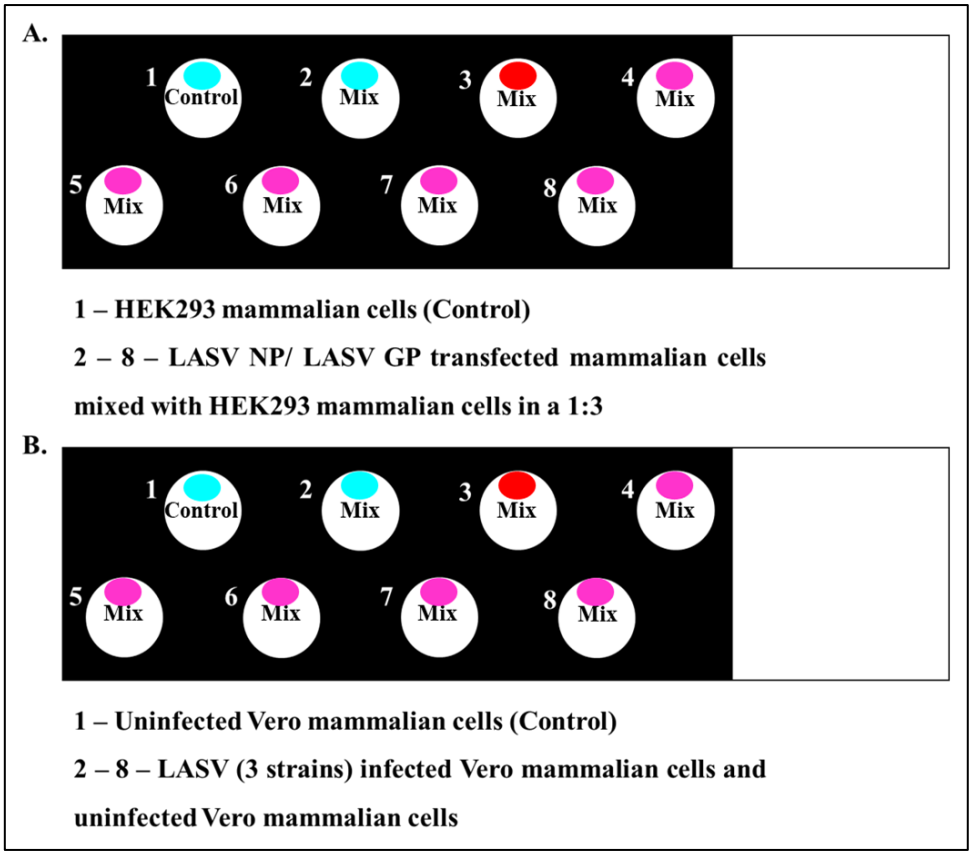

Figure 3.1: Slide layout for the LASV NP, LASV GP, and traditional LASV IFAs.

A) Slide layouts for the LASV NP IFA using the stable LASV NP cell line and LASV GP IFA using pN-LASV GP transfected cells. B) Traditional LASV infected IFA slide. The figure also shows the slide layout regarding where the control serum was added, and which wells were for the test samples. The dots on each well indicate whether a positive, negative or test serum was added. The blue dot indicates the positive control serum. The red dot indicates the negative serum, and the pink dot indicates the test serum.

3.2.2 Preliminary evaluation of developed Lassa virus-specific IFAs

3.2.2.1 Sample selection and preparation

The samples to evaluate the LASV NP and LASV GP IFAs comprised a panel of 30 clinical serum samples from the NICD collection and control LASV IgG positive and LASV IgG negative serum samples. The panel of 30 clinical samples was previously confirmed as negative for LASV, using RT-PCR and in-house IgG and IgM ELISAs or IFA assays during diagnostic work-up of the sample, at the NICD. Samples were not re-tested for this project but results were reviewed and panel established based on the available data. The panel of 30 clinical samples was submitted to the NICD to be tested for additional pathogens (Rabies (from healthy

individuals for antibody titres), malaria, Crimean-Congo haemorrhagic fever (CCHF), yellow fever, tick bite fever, meningitis, influenza B, paratyphoid fever). The ethical clearance obtained for the project does not cover disclosure of the pathogen for which each sample tested positive.

These clinical samples were positive for other VHFs/ viruses with similar clinical presentations. Each clinical sample was heat-inactivated at 60^{o}C for 30 minutes and diluted (1:10) in DPBS. The LASV positive serum sample was a convalescent-phase serum sample collected from a Lassa fever survivor in the 1970s. This sample was previously confirmed as positive for LASV antibodies, employing an in-house ELISA at the NICD. A negative serum (obtained from the South Africa National Blood Services and was screened for VHFs endemic to South Africa using the routine serological assays at the NICD) was included as a negative control.

3.2.2.2 Evaluating Lassa virus-specific IFAs

To measure how sensitive the assay was for detecting LASV antibodies, the highest detectable dilution of a known Lassa positive sample was determined for each of the traditional virus-infected LASV, LASV NP, and LASV GP IFAs. The titre of this control LASV IgG positive serum was unknown, but the sample was serially diluted to a 1:2000 ratio (1:10, 1:100, 1:200, 1:400, 1:1000, 1:2000). We then determined the highest dilution producing a positive result for each specific IFA. The procedure described in Section 2.2.6.1 was employed to determine this dilution limit. The slide layout was similar (Figure 3.1), with minor changes. Wells 1 and 3 were spotted with the known LASV positive serum sample at a 1:10 ratio. Well 2 was spotted with the LASV negative serum sample in a 1:10 ratio. Wells 4 to 8 were spotted with the serially diluted known LASV positive serum sample.

The panel of 30 clinical samples was exploited to perform the preliminary evaluation of the traditional virus-infected LASV and recombinant LASV NP and LASV GP-specific IFAs. These samples were negative for LASV; however, positive for other VHFs with similar clinical presentations. The procedure described in Section 2.2.6.1 was employed to determine the detection accuracy of the IFAs. The slide layout in Figure 3.1 presents spotting the IFA slides with the panel of known LASV negative serum samples. Wells 1 and 2 were spotted with a known LASV positive serum sample in a 1:10 dilution; Well 3 was spotted with a known

negative serum sample in a 1:10 dilution. The remaining wells were spotted with the panel of known LASV negative serum samples in a 1:10 dilution. The reproducibility of the results was also confirmed by testing in duplicate and on alternative days. The IFA slide results were also read and checked by a second individual (Dr Moolla), in a blinded fashion, to account for reader bias. Test wells were analysed and determined as positive or negative after examining the positive and negative control wells.

3.2.2.3 Statistical analysis

The recombinant LASV NP and LASV GP IFAs were analysed as a single assay as in a true laboratory diagnostic setting; they would not be used individually but always in combination. The detection accuracy for the recombinant antigen-based IFAs and the traditional virus-infected LASV IFA were individually determined using a 2×2 table (refer to Appendix D). The results for the panel of known LASV negative samples was recorded as either positive, discordant, or negative. For this project, the assay detection accuracy was defined as the ability of the assay to identify individuals without the disease (true negatives). The accuracy of the LASV-specific IFAs was calculated with a 95% CI, for the individual IFAs after each round of testing. Finally, the consensus results were calculated using the combined LASV NP and LASV GP IFAs and compared to the traditional virus-infected IFA in a true diagnostic setting.

The detection accuracy of the assay was calculated using the following formula:

$$Accuracy = \frac{Number\ of\ true\ negatives}{Number\ of\ true\ negatives + Number\ of\ discordant\ results} \times 100$$

The 95% CI was calculated using the following formula:

$$CI = (P \pm 1.96\sqrt{\frac{P(1-P)}{n}})$$

Where P = Determined detection accuracy and n = Number of known positive or negative samples.

3.3 Results

3.3.1 Developing the Lassa virus-specific NP and Lassa virus-specific GP IFAs

3.3.1.1 Developing the Lassa virus-specific NP IFA

The developed LASV NP expressing stable cell line was successfully maintained in T75 cell culture flasks. Mixtures of LASV NP expressing cells and control HEK 293 cells identified the best expression ratio to non-expressing cells for the IFA. The optimal ratio of LASV NP expressing cells to non-expressing (control) cells was 1:3. The 1:4 and 1:5 ratios had too few positive fluorescing cells, and the 1:1 and 1:2 ratios had too many positive fluorescing cells, distinguishing between specific interaction and non-specific binding trickier. The optimised ratio of 1:3 was applied to spot 15 IFA slides. Figure 3.2 shows the IFA results for the LASV NP expressing cell line, ratio optimisation.

3.3.1.2 Developing the Lassa virus-specific GP IFA

Transient transfection in a T75 cell culture flask was constructed with the cloned LASV GP expressing plasmid as a LASV GP expressing stable cell line was not developed. The transfected cells were used to spot the LASV GP IFA slides. The optimal ratio in a six-well cell culture plate (4 µg DNA and 9 µL Lipofectamine 2000) for the LASV GP expressing plasmid determined in the previous chapter was scaled up (20 µg DNA and 45 µL Lipofectamine 2000) for a T75 cell culture flask to transfect the HEK293 mammalian cells.

Concerning the ratio optimisation performed for LASV NP to control cells, the optimal ratio of LASV GP expressing to control cells was determined to spot the slides. Figure 3.3 shows these results. The optimal ratio for the LASV GP expressing cells to control cells was determined as 1:3. The 1:4 and 1:5 ratios were determined to have too few positive cells, which could have resulted in positive samples being diagnosed as negative. The 1:1 and 1:2 ratios indicated too many positive cells. Once the optimal ratio was determined, 15 IFA slides were created and stored at -70 degrees C until use.

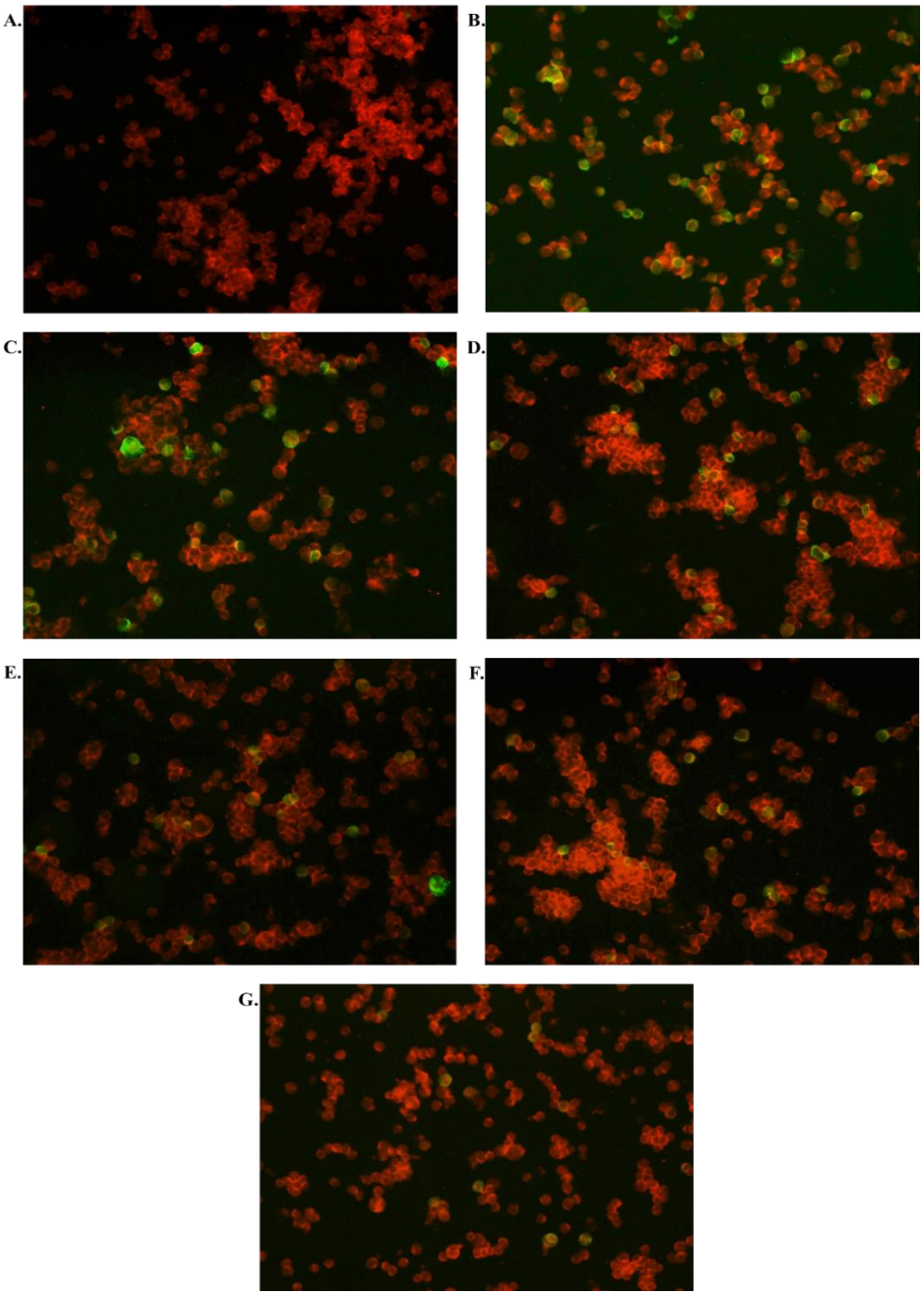

Figure 3.2: Representative images of IFA results for various ratios of the LASV NP, expressing stable cell line to control cells.

A) Control: HEK293 mammalian cells with LASV positive serum. B) Undiluted LASV NP expressing stable cell line with LASV positive serum. C) LASV NP expressing stable cell line diluted 1:1 with control cells. D) LASV NP was expressing stable cell line diluted 1:2 with control cells. E) LASV NP expressing stable cell line diluted 1:3 with control cells. F) LASV NP expressing stable cell line diluted 1:4 with control cells. G) LASV NP expressing stable cell line diluted 1:5 with control cells. For A-G, LASV positive serum diluted to 1:10 was usedand secondary goat anti-human FITC antibody (Sigma Aldrich) also diluted 1:10 was used as the detecting antibody.

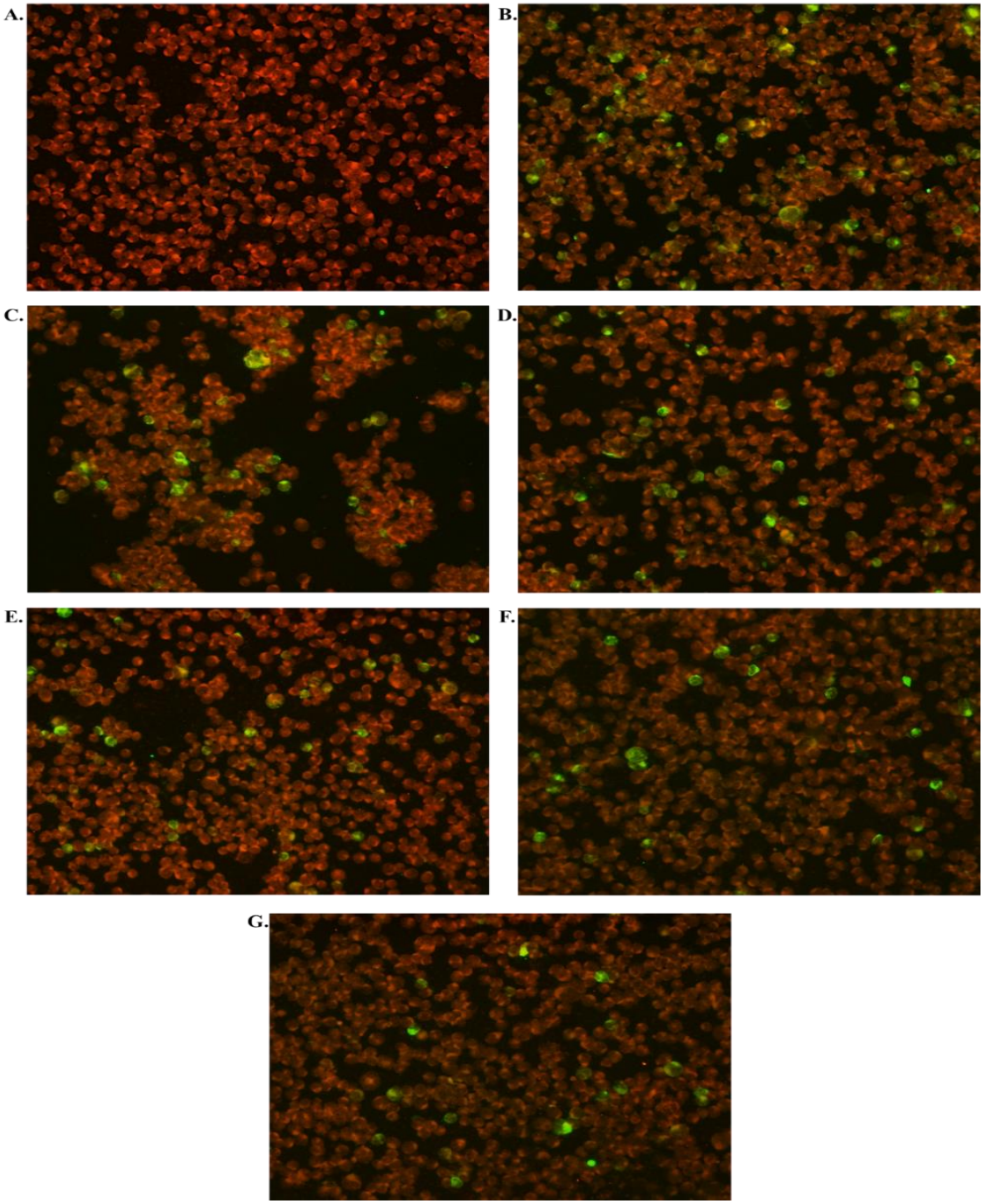

Figure 3.3: Representative images of the IFA results for various ratios of the LASV GP transiently expressing cell lines to control cells.

A) Control: HEK293 mammalian cells with LASV positive serum. B) Undiluted LASV GP transiently expressing cell line with LASV positive serum. C) LASV GP transiently expressing cell line diluted 1:1 with control cells. D) LASV GP transiently expressing cell line diluted 1:2 with control cells. E) LASV GP transiently expressing cell line diluted 1:3 with control cells. F) LASV GP transiently expressing cell line diluted 1:4 with control cells. G) LASV GP transiently expressing cell line diluted 1:5 with control cells. For A-G, LASV positive serum diluted to1:10 was used and detected using secondary goat anti-human FITC antibody (Sigma Aldrich) also diluted 1:10

3.3.2 Preliminary evaluation of recombinant antigen-based Lassa virus-specific IFAs

3.3.2.1 Determining the dilution limit of Lassa virus NP and Lassa virus GP-specific IFAs

The control LASV IgG positive serum sample was used to determine the dilution limit for a known positive sample to measure the relative sensitivity of the recombinant antigen-based IFA to the virus-infected based IFA. Figure 3.4 shows the results. Each serum dilution was assessed in duplicate on each IFA in two runs. The highest dilution at which antibodies could be detected for the recombinant LASV NP IFA was determined at a dilution of 1:400. Fluorescence observed at the 1:200 and 1:400 dilutions was weak. The 1:1000 and 1:2000 dilutions were negative, with no fluorescing cells observed, indicating that the developed LASV NP IFA could detect LASV antibodies in our positive serum only up to a 1:400 dilution.

For the recombinant LASV GP IFA, the dilution limit was at a dilution of 1:400. Similarly to the recombinant LASV NP based assay, the fluorescence at 1:200 and 1:400 was weak; no fluorescence was observed for the 1:1000 and 1:2000 dilutions. This signified that the LASV GP IFA could detect LASV antibodies in our positive serum only up to a 1:400 dilution.

By comparison, the traditional virus-infected LASV IFA detected the known LASV positive serum sample up to a 1:2000 dilution. The fluorescence observed at the highest dilution was more intense/brighter than that detected with the recombinant antigen-based assays. The controls used all indicated the expected results with the negative control not showing any fluorescence and the positive control showing fluorescence.

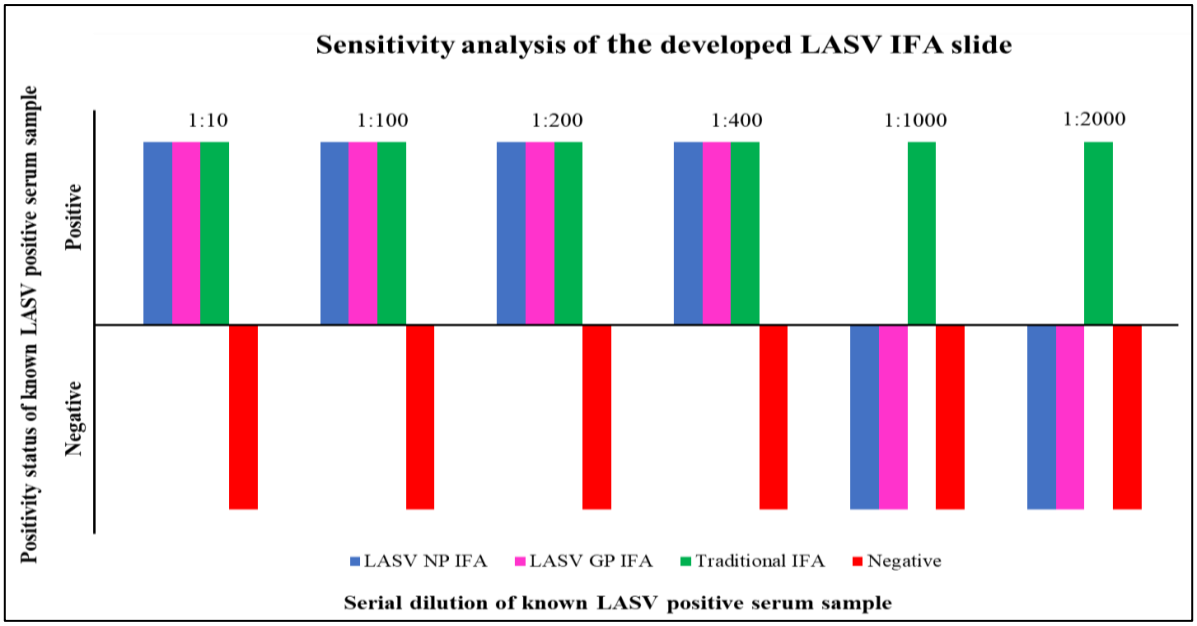

Figure 3.4: Preliminary evaluation of the recombinant antigen-based LASV NP and LASV GP IFAs and the whole virus-infected IFA using a serial dilution of a known LASV positive serum sample.

The three IFAs were tested against the serially diluted IgG known LASV positive serum sample in duplicate. The results for both rounds were identical for the three IFAs–the combination of both rounds are shown here. The negative control also indicated no positive fluorescence in the wells. Repeats were not shown here as the dilution value was consistent between the two rounds.

3.3.2.2 Preliminary evaluation of the recombinant Lassa virus IFAs

Having established that the recombinant LASV NP and LASV GP based IFAs could detect LASV antibodies, we needed to evaluate both IFAs. The prepared LASV NP and LASV GP IFA slides were used to screen a panel of 30 known LASV negative serum samples to determine a preliminary efficacy for detecting true LASV negative samples. (Refer to Appendix D for the IFA raw data). We used the traditional virus-infected LASV IFA slides prepared with LASV infected Vero cells for comparison. The recombinant LASV NP and LASV GP IFAs were analysed as a single assay. In a true laboratory diagnostic setting, they would not be used individually but always combined.

Table 3.1 and Figure 3.5 show the performance of all three IFAs after each sample was tested twice with the number of discordant results obtained for the individual assays. Discordant results were defined as those samples for which too much background/ non-specific binding

was seen and could not be determined to be positive or negative. No samples tested positive–only samples were difficult to read, therefore, recorded as discordant. This is a known phenomenon with IFA testing; therefore, we tested each sample twice, as often testing in the second round will yield a readable result. We did not test the samples on control cell only slides seeing as our developed IFA slides consisted of both positive and negative cells. This allowed us to distinguish between specific and non-specific reactivity when reading the slides. Based on this, we calculated the accuracy of the assays to identify known LASV negative serum samples correctly; these data are shown for each round and the final consensus result.

Based on this approach, the number of discordant results for combining the LASV NP and LASV GP IFA indicated three discordant results, providing an accuracy rate of 90% (95% CI: 74.4% to 96.5%) in calling of true negative samples. (Figure 3.5). By comparison, traditional virus-infected LASV IFA indicated only one discordant result, providing accuracy in calling true negative samples of 96.67% (95% CI: 83.3% to 100%) (Figure 3.5). The large 95% CIs were because the sample size was not large. As the panel of samples increases, the CI also becomes smaller. The traditional virus-infected LASV IFA had higher accuracy in calling the true negative samples than the recombinant antigen-based IFAs. The recombinant antigen-based IFA was still accurate and could correctly diagnose negative samples.

The acceptability level for newly developed IFAs ranges from a detection accuracy of 85% to 100%; therefore, our recombinant antigen-based IFA is comparable to other developed IFAs, based on preliminary evaluation with limited samples (Atmar, 2014, Lipkin et al., 2014, Raabe et al., 2017). However, larger panel of known LASV negative and LASV positive samples must be evaluated to determine the true performance characteristics (diagnostic specificity and sensitivity) of the developed LASV NP and LASV GP assays.

Table 3.1: Clinical performance of the individual LASV NP and LASV GP IFAs compared to the traditional virus-infected LASV IFA.

	LASV NP IFA	LASV GP IFA	Traditional LASV IFA
The total number of samples tested	30	30	30
First round accuracy at calling negative samples (no. of discordant results)	80% (6)	76.7% (7)	80% (6)
Second round accuracy at calling negative samples (no. of discordant results)	76.7% (7)	76.7% (7)	83% (5)
Consensus accuracy at calling negative samples (95% CI)	90% (74.4% - 96.5%)		96.67% (83.3% - 100%)

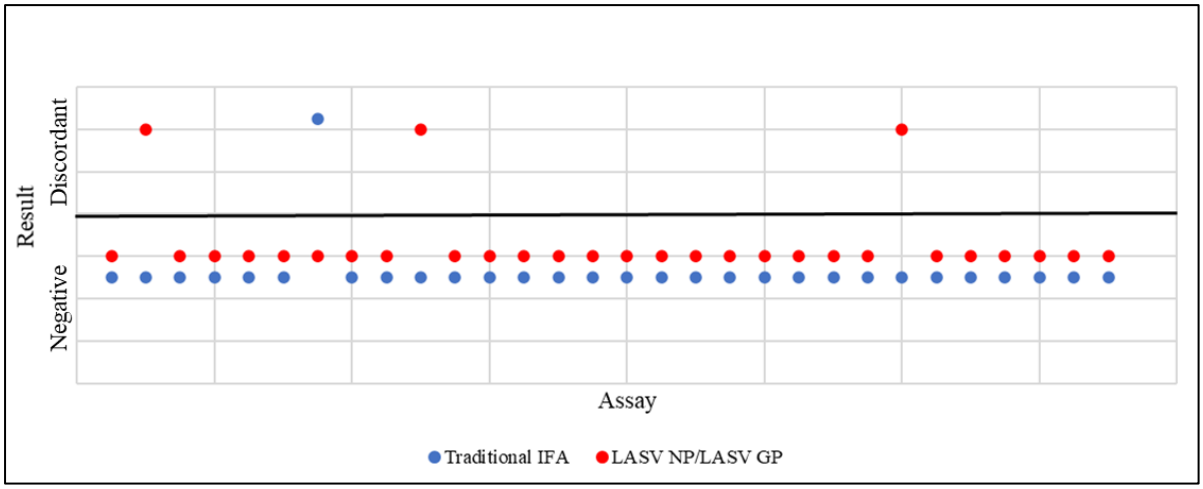

Figure 3.5: Scatterplot depicting the consensus results for the recombinant LASV NP, LASV GP, and traditional LASV IFA on the LASV negative validation panel.

The scatterplot shows the number of discordant and negative results for each assay. The dots indicate the consensus results for each sample. The solid line indicates the cut-off for a result to be classified as discordant or negative.

3.4 Discussion

Lassa virus is a VHF, posing a significant public health impact and is classified as a BSL-4 pathogen (Ogbu et al., 2007, Bausch et al., 2014). The live virus can, therefore, only be handled under high containment by limited laboratories performing LASV diagnosis and studying the

virus. This is because of the infectious nature of the samples and virus unless inactivated (Bausch et al., 2014). A lack exists for an affordable and rapid serological diagnostic test for Lassa fever that can be used with nucleic acid- or antigen-based detection assays for a quick, definitive diagnosis. With an observation to developing a recombinant antigen-based IFA for LASV and approaching this need, in Chapter 2, we successfully cloned expression cassettes for the LASV NP and LASV GP proteins, expressed these recombinant proteins in a mammalian cell line, and developed a stable expression cell line for the LASV NP protein.

This approach was followed, as recombinant antigens are safer to use than live viruses. The mammalian expression system was used to allow for higher expression levels and post-translational modifications. Next, we aimed to use these cassettes to develop individual LASV NP and LASV GP-specific IFAs and establish their utility as diagnostic assays. In this chapter, we report on the successful production of IFA slides based on recombinant LASV NP (from a stable cell line) and LASV GP (transiently expressed) proteins. These IFAs were compared to a traditional virus-infected LASV IFA, using LASV infected cells regarding their preliminary evaluations.

When the traditional virus-infected LASV IFA was compared to the combined IFA based on recombinant antigens, there was a clear difference in the highest detectable dilution of the positive control sample used. We established that the recombinant antigen-based LASV IFA (both the LASV NP and LASV GP) could detect the positive control sample up to a dilution of 1:400. By comparison, the traditional IFA detected antibodies in the same positive sample up to a dilution of 1:2000. We used this to indicate the relative sensitivity of the assays. The difference in the dilution limit for the sample between the recombinant based IFAs and the traditional LASV IFA is thought to be due to all viral antigens in the whole virus-based IFA, whereas the recombinant assays have only single antigen targets (Günther et al., 2004, Atmar, 2014).

While the dilution limit for the sample was lower for the recombinant IFAs compared to the traditional virus-infected LASV IFA, samples would be screened using a dilution between 1:10 and 1:100 in a normal diagnostic setting (Bausch et al., 2000, Gabriel et al., 2018). Our recombinant antigen-based IFAs should, therefore, detect positive samples in a diagnostic setting at these dilutions; however, the ability to detect low positive samples will be known

only once the true sensitivity of the LASV NP and LASV GP IFAs is determined using a panel of known LASV positive samples, with a range of antibody levels.

The recombinant antigen-based LASV NP and LASV GP IFAs were based on the LASV Josiah strain. The traditional whole virus-infected IFA used three LASV strains (Liberia (Josiah), AV and Nig strains). Using these three strains would mean better exposure across the LASV lineages so more antibodies can be detected owing to differences in antigens epitopes (Bowen et al., 2000, Mazzola et al., 2019, Ibukun, 2020). The antibodies in the control LASV positive serum could have better reactivity to one of the other strains even though cross-reactivity to the others occurs. Previous research by Heinrich et al, showed that LASV NP and LASV GP antigens produced from lineages II-IV are able to cross-react with antibodies from LASV survivors across multiple lineages (Heinrich et al., 2020). However, it was shown that the LASV Z protein produced more variability and had little cross-reactivity with the LASV Z protein across the different lineages (Heinrich et al., 2020). Subtle changes in the genetic sequences among the diverse LASV strains could result in better recognition of a specific antigen, increasing the dilution limit as measured of the assay (Bowen et al., 2000, Dedkov et al., 2019, Mazzola et al., 2019, Ibukun, 2020).

These changes do not alter the protein structure, indicating cross-reactivity can occur among the lineages (Mazzola et al., 2019, Ibukun, 2020). These differences may affect the sensitivity of recombinant LASV NP and LASV GP IFAs, with some lineages having better reactivity than others. The assays should detect positive samples across the lineages as the structural integrity of the protein and specific epitopes are maintained.

Once the highest detectable dilution was determined, the next step was to measure the accuracy of detecting LASV-specific antibodies for the recombinant antigen-based LASV NP and LASV GP IFAs using the panel of 30 known LASV negative serum samples (Table A.2 Appendix D). This panel was used to determine cross-reactivity to other VHFs with similar clinical presentation as LASV. The recombinant antigen-based LASV NP and LASV GP IFAs had an accuracy rate of 90% in calling negative results with three discordant results. This was lower than the traditional virus-infected IFA, which was accurate in calling negatives 96.67% of the time and had only one discordant result.

This preliminary evaluation of the assays indicated they were comparable in reliability and accuracy to the traditional virus-based IFA. While we could not directly compare to other developed serological assays, owing to the limited evaluation, we observed that our initial evaluation was encouraging concerning the accuracy of detecting LASV-specific antibodies (Gabriel et al., 2018, Takah et al., 2019). One limitation of this evaluation was that we did not perform another test, such as an ELISA, to verify that the selected samples were Lassa antibody-negative (Jacobson, 1998a). This meant that while the samples were selected based on an alternate diagnosis, we could not guarantee these individuals did not previously have LASV exposure or infection (Pfleiderer et al., 1995).

The discordant results for each IFAs were due to non-specific binding or high background, complicating determining positive or negative samples. The background was not observed in the positive control wells or the serial dilution of the known LASV positive serum sample. It is common for patient serum samples to display non-specific antibody reactivity (Atmar, 2014, Joshi et al., 2017). In a diagnostic setting, this problem would be overcome by titration of the sample or a request for a new sample. We lacked access to a high titre Lassa IgM positive sample for a similar evaluation to be conducted on the LASV GP and LASV NP IFA for IgM antibody detection.

The most significant limitation for evaluating the recombinant antigen-based LASV NP and LASV GP IFAs was the difficulty in obtaining samples to assess. This limitation meant that we could not determine the true diagnostic sensitivity and specificity of the recombinant antigen-based LASV NP and LASV GP IFAs. A larger, more representative panel of LASV positive and negative serum samples could determine the true diagnostic sensitivity and specificity of the recombinant LASV NP and LASV GP IFAs. Ideally, when determining the diagnostic specificity and sensitivity, the panel should comprise samples known to be positive for LASV with antibody titres at various levels and representative of the LASV strain diversity to determine if low positives can be detected and if there is cross-reactivity among the lineages (Jacobson, 1998a, Banoo et al., 2008). The negative samples should comprise samples known to be negative for LASV but positive for diseases with similar clinical symptoms to determine cross-reactivity (Jacobson, 1998a, Banoo et al., 2008).

The recombinant antigen-based LASV NP and LASV GP IFAs show promise as diagnostic screening tools for Lassa fever. Developing a LASV GP stable cell line is still warranted to prevent batch-to-batch variation on IFA slides, resulting in varied expression levels between transfection or infection reactions. A LASV GP stable cell line for IFA slides would signify that the LASV GP is expressed in equal amounts, facilitating standardisation. Our initial evaluation of the recombinant LASV antigen IFAs indicated that they have potential application as diagnostic tests and are comparable in their reliability and accuracy to the whole virus-based IFA. Based on this, our recombinant antigen-based LASV IFA warrants further development and evaluation as a diagnostic device for Lassa fever.

CHAPTER 4: CONCLUSION AND FUTURE PERSPECTIVES

This project aimed to develop and validate a recombinant antigen-based indirect immunofluorescence antibody assay for LASV. Individual gene expression cassettes for the LASV NP and LASV GP proteins were successfully cloned, and IFA and Western blot presented protein expression for LASV NP and IFA for LASV GP. We developed IFAs to detect antibodies against LASV NP and LASV GP, employing these plasmids. For the LASV NP IFA, we standardised the batch-to-batch slide preparation through a stable LASV NP expressing cell line, developed as part of this project. The preliminary evaluations of these IFAs were promising, indicating their potential for diagnostic application in the future (once fully validated). This would meet the need for a quick, standardised serological test for LASV that can be used outside high-containment facilities.

The cloned LASV NP and LASV GP expression cassettes can be used in technology transfer with laboratories in endemic regions. This would allow the laboratories in the endemic areas to set up serological diagnostic assays that can be used outside of a BSL-4 laboratory while providing an accurate, sensitive, and cost-effective method to detect LASV antibodies. Sharing these expression cassettes with other laboratories could lead to their use in developing vaccines or therapeutic agents for LASV.

As discussed in Chapter 2, LASV GP expression needs to be confirmed by Western blot. The steps from plasmid transfection to Western blot will need to be examined and optimised to confirm expression. Since we know the LASV GP insert sequence is correct and should, therefore, be expressed, these steps must be optimised, allowing for enhanced LASV GP expression. A viable way to improve expression is to attempt an alternative expression system or vector. This can be conducted through a viral vector to transport the LASV GP gene into mammalian or insect cells.

Another option would be to attempt the LASV GP expression with a different expression vector, such as the pcDNA3.1 (+) expression vector (Invitrogen). The transfection reaction would need to be optimised. Various transfection reagents and methods must be attempted to accomplish this–if the transfection reaction is optimised, the LASV GP expression will

increase. The pellet should be run on an SDS-PAGE and Western blot to ensure we are not losing the LASV GP in the pellet during cell lysis. Alternative cell lysis methods should be evaluated because, from our original IFA results, there was expression of the LASV GP, but it was lost during cell lysis and Western blot. Future work could also consider purifying the LASV using the histidine tag before running a Western blot.

The antigenicity of the recombinant material was important as the primary objective of this work was to establish a safer, hazard-free, serological assay to detect LASV infection. We compared the performance of the recombinant antigen-based IFA to virus-infected inactivated Lassa virus-based IFAs. In an immunofluorescence format, the performance of these antigens followed those obtained using the conventional immunofluorescence test through Lassa virus-infected cells. The conclusion, based on the preliminary results, was that the recombinant antigen-based IFA results are sufficiently close to the virus-infected IFA results, allowing efficient detection of antiviral antibodies. Future work on this assay would, therefore, mandate a full validation of its analytical sensitivity and specificity.

The recombinant LASV NP and LASV GP antigens can be used to develop other diagnostic serological assays, such as an ELISA. This developed ELISA could be used with the recombinant IFAs or RT-PCR for LASV diagnosis, presenting a confirmatory test for IFAs. An additional feature of the ELISA is that it could be a quantitative assay that could measure and monitor seroconversion in LASV patients, advantageous in any large-scale seroepidemiological study. The developed ELISA, with these two features, could be employed in clinical trials to monitor natural immunity and vaccine-induced immunity.

Purifying these antigens would allow their immunogen application to produce monoclonal antibodies against each antigen. These antibodies could be further used in other diagnostic assays, such as an IFA or Western blot, to identify the target LASV antigens. The developed monoclonal antibodies could develop novel therapeutic reagents to treat Lassa fever. Developing these antibodies could initiate similar antibodies for other VHF causing viruses.

This work adds to the ‘proof-of-principle’ data for developing similar assays for other Old World mammarenaviruses and other VHF causing viruses. Developing similar assays for other VHFs is necessary because most VHFs present similar clinical symptoms, requiring BSL-4 laboratories. This indicates a need for diagnostic assays that can be used outside of high-

containment facilities. These assays could have applications in generating much-needed surveillance studies for Lassa and other VHFs of concern. Applying this assay for screening rodent populations could lead to discovering reservoir hosts of certain Old World mammarenaviruses or new mammarenaviruses. The recombinant antigens could develop vaccines against LASV.

In conclusion, recombinant LASV NP and LASV GP IFAs were developed, employing the individual pN-LASV NP and pN-LASV GP expression cassettes. The recombinant LASV NP and LASV GP IFAs detected LASV antibodies with little risk of infection; it is comparable in their reliability, sensitivity and accuracy to the whole virus-based IFA. Our recombinant protein-based LASV IFA, therefore, warrants further development and evaluation as a diagnostic device for Lassa fever.

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APPENDICES

Appendix A: Phylogenetic tree GenBank sequence accession numbers

Table A.1: *Arenaviridae* family member accession numbers used to generate the phylogenetic tree in Figure 1.1.

	S segment accession number
antennavirus	
Hairy antennavirus	MG599867
Striated antennavirus	MG599864
hartmanivirus	
Haartman hartmanivirus	KR870017
Muikkunen hartmanivirus	MH483026
Schoolhouse hartmanivirus	MH483024
Zurich hartmanivirus	MH483031
reptarenavirus	
California reptarenavirus	JQ717262
Giessen reptarenavirus	KR870012
Golden reptarenavirus	JQ717264
Ordinary reptarenavirus	KX527575
Rotterdam reptarenavirus	KC508669
mammarenavirus	
Allapahuayo mammarenavirus	AY012687
Alxa mammarenavirus	KY432893
Argentinian mammarenavirus	AY358023
Bear Canyon mammarenavirus	AY924391
Brazilian mammarenavirus	U41071
Cali mammarenavirus	K02734
Chapare mammarenavirus	EU260463
Chevrier mammarenavirus	MF414202
Cupixi mammarenavirus	AF512832
Flexal mammarenavirus	AF512831
mammarenavirus	**S Segment Accession Number**
Gairo mammarenavirus	KJ855308

	S segment accession number
Guanarito mammarenavirus	AY129247
Ippy mammarenavirus	DQ328877
Lassa mammarenavirus strain Pinneo-NIG-1969 Lineage I	KM822128
Lassa mammarenavirus strain Nig08-04 Lineage II	GU481068
Lassa mammarenavirus strain Nig08-A19 Lineage III	GU481072
Lassa mammarenavirus Josiah Lineage IV	J04324
Lassa mammarenavirus strain Soromba-R Lineage V	KF475765
Lassa mammarenavirus strain KAK-428 Lineage VI	KT992425
Lassa mammarenavirus Alzey Isolate Lineage VII	LT601602
Latino mammarenavirus	AF512830
Loei River mammarenavirus	KC669698
Lujo mammarenavirus	FJ952384
Luna mammarenavirus	AB586644
Lunk mammarenavirus	AB693150
lymphocytic choriomeningitis mammarenavirus	AY847350
Machupo mammarenavirus	AY129248
Mariental mammarenavirus	KM272987
Merino Walk mammarenavirus	GU078660
Mobala mammarenavirus	AY342390
Mopeia mammarenavirus	AY772170
Okahandja mammarenavirus	KM272988
Oliveros mammarenavirus	U34248
Paraguayan mammarenavirus	AF485261
mammarenavirus	**S segment accession number**
Pirital mammarenavirus	AF485262
Planalto mammarenavirus	MF317490
Ryukyu mammarenavirus	KM020191

	S segment accession number
Serra do Navio mammarenavirus	AF485256
Solwezi mammarenavirus	AB972428
Souris mammarenavirus	KP050227
Tacaribe mammarenavirus	M20304
Tamiami mammarenavirus	AF485263
Wenzhou mammarenavirus	KJ909794
Whitewater Arroyo mammarenavirus	AF228063
Xapuri mammarenavirus	MG976577

Appendix B: Additional materials and methods

B1: Transformation of competent *Escherichia coli* cells

Working stocks of the plasmid expression and vector plasmids were prepared. For this, the plasmid containing the synthesised S segment and the pCI-neo expression vector was transformed into competent JM109 *E. coli* cells (Promega, USA) by heat shock according to the manufacturer's instructions (Promega, USA). The transformation was conducted by adding 3 µL of 1 ng/µL of both sets of plasmids to 50 µL of competent single-use JM109 *E. coli* cells (Promega, USA) and the tubes incubated on ice for 20 minutes. After the incubation step, the cells underwent heat shock by placing the tubes into a heating block at 42 °C for precisely 42 seconds. The cells were then placed on ice for 2 minutes. A total of 950 µL of room temperature super optimal broth with catabolite repression (SOC) medium (Thermo Fisher Scientific, USA) was added to each of the transformation reactions before the tubes were incubated at 37 °C for 90 minutes with shaking at 150 rpm. A total of 100 µL for each transformation culture was then plated onto prepared LB plates with 100 µg/mL of AMP (Zymo Research, USA). Duplicate plates were made and incubated overnight at 37 °C in the Infors HT Ecotron incubator (Infors HT, USA). A negative control was made, comprising competent JM109 *E. coli* cells (Promega, USA) without the plasmid DNA.

B2: Determination of plasmid concentrations (Qubit™)

The Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA) and Qubit™ dsDNA HS Assay kit (Life Technologies, Thermo Fisher Scientific, USA) were used to determine the concentrations of the plasmid stocks (pN-S Seg and pCI-neo without LASV S segment). A Qubit™ working solution was prepared in a 1.5 mL tube by adding 4 µL of the Qubit™ dsDNA HS Reagent to 996 µL Qubit™ dsDNA HS Buffer. Thereafter, four Qubit™ assay tubes were labelled (two for the Qubit™ Standards and two for the isolated plasmids). A volume of 190 µL of the Qubit™ working solution was added to the two assay tubes for the Qubit™ standard solutions. Thereafter, 199 µL of the Qubit™ working solution was added to the two individual assay tubes labelled for the isolated plasmids. Subsequently, 10 µL of Qubit™ Standard 1 (Life Technologies, Thermo Fisher Scientific, USA) and Qubit™ Standard 2 (Life Technologies, Thermo Fisher Scientific, USA) were added to the assay tubes labelled Standards 1 and 2 to make a final volume of 200 µL. Thereafter, 1 µL of the two samples was

added to the individual assay tubes labelled for each of the samples. The four assay tubes were then vortexed, and the two standards were then perused individually by the Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA) first to calibrate the machine. Once calibrated, the two samples were then placed into the Fluorometer individually, and the concentrations were determined in ng/µL.

B3: Plasmid glycerol stock production

Glycerol stocks for both plasmids (pN S-Seg and pCI-neo) were prepared from the LB/Amp broth cultures of the *E. coli* cells transformed with the individual plasmids. A 50% glycerol solution was made by diluting 100% sterile glycerol (Sigma-Aldrich, Merck, Germany) in sterile distilled water. The stocks were made by adding 500 µL of the LB/Amp broth to 500 µL of 50% glycerol solution to make a final solution with 25% glycerol. The glycerol stock tubes were then stored in the -80 °C freezer until needed.

B4: DNA purification (Zymoclean™ Gel DNA Recovery Kit (Zymo Research))

The correct size band1743 bp (for the LASV NP gene) was excised from the gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). The excised band was weighed in a pre-weighed 1.5 mL tube, after which three volumes of ADB were added. The tubes were then incubated at 55 °C in a heating block until the agarose was completely dissolved. The melted agarose was transferred to individual Zymo-spin columns in separate collection tubes and centrifuged for 1 minute at 14000×g. A volume of 200 µL DNA Wash Buffer was then added to the column before being centrifuged at 14000×g for 30 seconds, and the wash step repeated. After that, 10 µL Elution Buffer was added directly to the column matrix, placed in a clean 1.5 mL tube, and incubated at room temperature for five minutes. Finally, the tubes were then centrifuged at 14000×g for 1 minute to elute the DNA. After that, 1 µL of the purified DNA was run on a 1.5% agarose gel with the GeneRuler™ 100 bp Plus DNA marker (Thermo Fisher Scientific, USA) to verify the purification was successful. The concentration of the purified products was determined using the Qubit™ Fluorometer 3.0 (Life Technologies, Thermo Fisher Scientific, USA) as described in Appendix B2.

B5: DNA purification (Wizard® SV Gel & PCR Clean-Up System (Promega))

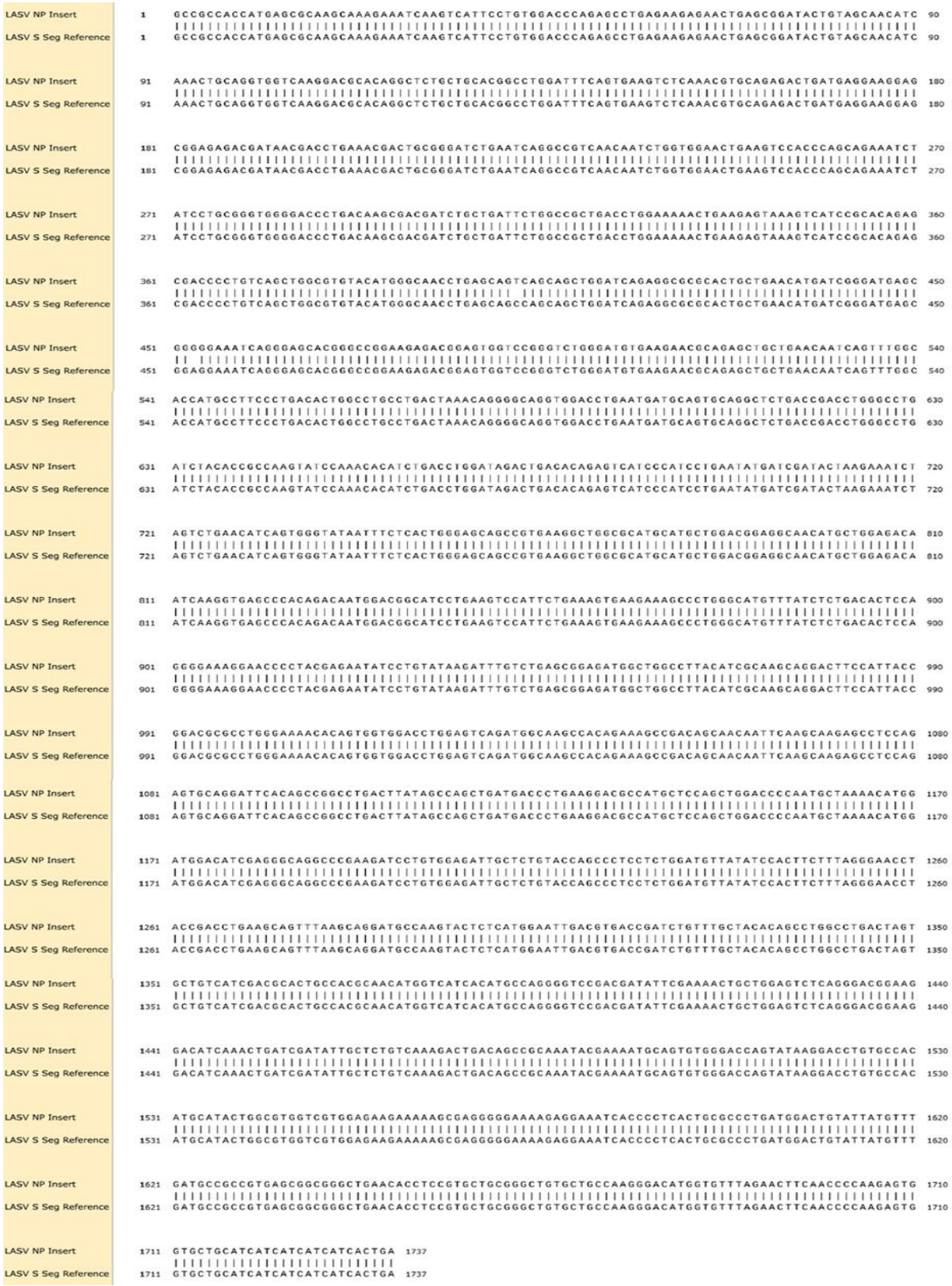
The correct size band (1493 bp for the LASV GP gene) was excised from the gel and purified using the Wizard® SV Gel & PCR Clean-Up System (Promega, Madison, USA). The excised bands were weighed in a pre-weighed 1.5 mL tube, after which an equal amount of MBS (Promega, USA) was added. The tubes were incubated at 55 °C in a heating block until the gel slice was completely dissolved. The dissolved gel mixture was added to an SV minicolumn in a collection tube and incubated for 1 minute at room temperature before centrifuging at 16000×g for 1 minute. The flowthrough was discarded, and 700 µL of Membrane Wash Buffer (Promega, USA) was added to the minicolumn before centrifuging at 16000×g for 1 minute. The flowthrough was discarded, and 500 µL of Membrane Wash Buffer (Promega, USA) was added to the minicolumn. The minicolumn was centrifuged at 16000×g for five minutes. The flowthrough was discarded and the minicolumn recentrifuged at 16000×g for 1 minute with the microcentrifuge lid open to allow excess ethanol to evaporate. The SV minicolumn was then placed into a clean 1.5 mL tube, and 15 µL of nuclease-free water was added directly to the column matrix. The column was incubated at room temperature for 1 minute before being centrifuged at 16000×g for 1 minute. The amplified products were then added to 10 µL of 6× loading dye, run on a 1.5% agarose gel electrophoresis, and viewed using UV illumination (Molecular Imager Gel Doc™ XR+ (Bio-Rad Laboratories Inc., USA)) with the GeneRuler™ 1 kb DNA marker (Thermo Fisher Scientific, USA) for size verification.

B6: Freezing mammalian cells

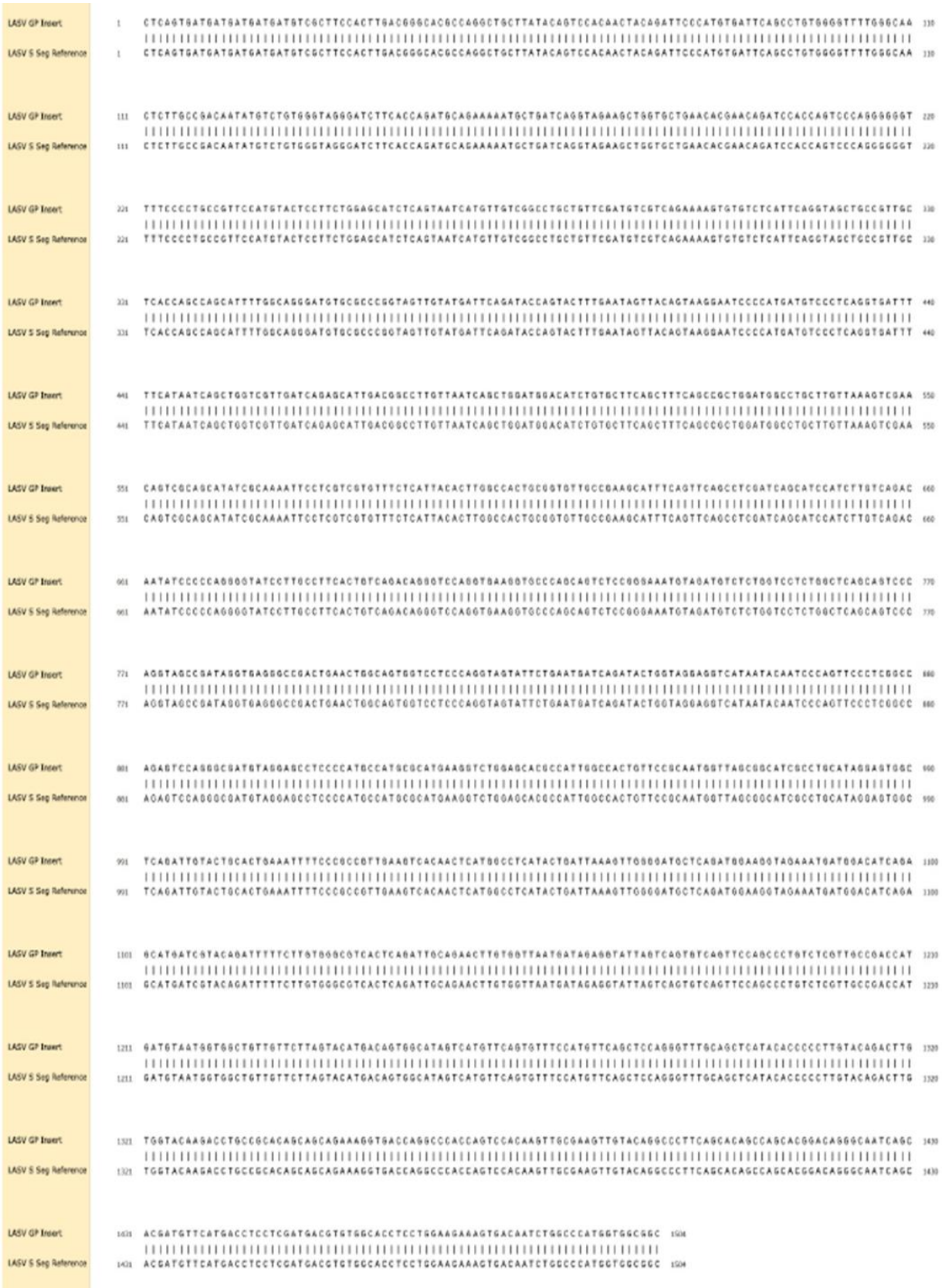
The mammalian cells were sub-cultured and maintained as described in Section 2.2.4. Cells were resuspended in 10 mL of DMEM (Sigma-Aldrich, USA) with L-glutamine (L-glut) (Lonza, Whitehead Scientific, Switzerland) and 10% FCS (Lonza, Whitehead Scientific, Switzerland) before being counted as described in Section 2.2.5.1. Once counted, the mammalian cells were centrifuged in 200×g for five minutes before the supernatant was discarded. Cells were resuspended in cryoprotective medium (DMEM with 15% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Merck) and an adjusted FCS percentage) to a cell concentration of 1×10^6 cell/mL. The resuspended cells were aliquoted into 1.5 mL cryotubes and stored in a freezing container with isopropanol (Sigma-Aldrich) at -70 °C overnight. The next day, the cryotubes were moved and stored in liquid nitrogen.

Appendix C: Full Sequence Alignment

LASV NP full sequence alignment to reference LASV S segment sequence generated using SnapGene



LASV GP full sequence alignment to reference LASV S segment sequence generated using SnapGene



Appendix D: Raw immunofluorescence assay results

Table A.2: Results for the 30 known negative samples on the Lassa virus nucleoprotein and glycoprotein, and Lassa virus-infected cells immunofluorescence assays.

Sample ID	IFA							
	Recombinant LASV NP		Recombinant LASV GP		Overall result	LASV infected cells		Overall result
	1st Test	2nd Test	1st Test	2nd Test		1st Test	2nd Test	
62/20	*	-ve	*	-ve	N	-ve	*	N
449/17	-ve	-ve	*	*	?	*	-ve	N
EBOV Pos Con 1	-ve	-ve	-ve	-ve	N	-ve	-ve	N
EBOV Pos Con 2	-ve	-ve	-ve	-ve	N	-ve	-ve	N
409/17	-ve	-ve	-ve	-ve	N	-ve	*	N
54/20	-ve	-ve	-ve	*	N	-ve	-ve	N
65/20	-ve	-ve	-ve	*	N	*	*	?
232/18	-ve	*	-ve	-ve	N	-ve	-ve	N
59/20	*	-ve	*	-ve	N	-ve	-ve	N
545/17	*	-ve	*	*	?	*	-ve	N
53/20	-ve	-ve	-ve	-ve	N	-ve	*	N
140/18	-ve	-ve	-ve	-ve	N	-ve	-ve	N
139/18	-ve	-ve	-ve	*	N	-ve	-ve	N
345/20	-ve	-ve	-ve	-ve	N	-ve	-ve	N
69/20	-ve	*	-ve	-ve	N	*	-ve	N
52/20	-ve	-ve	-ve	-ve	N	-ve	-ve	N
60/20	-ve	-ve	-ve	-ve	N	-ve	-ve	N
64/20	*	-ve	-ve	-ve	N	*	-ve	N

Sample ID	IFA							
	Recombinant LASV NP		Recombinant LASV GP		Overall result	LASV infected cells		Overall result
	1st Test	2nd Test	1st Test	2nd Test		1st Test	2nd Test	
492/18	-ve	-ve	-ve	-ve	N	-ve	-ve	N
67/20	-ve	*	-ve	*	N	-ve	-ve	N
349/20	-ve	-ve	-ve	-ve	N	-ve	-ve	N
233/18	-ve	-ve	-ve	-ve	N	-ve	-ve	N
293/17	-ve	-ve	*	-ve	N	-ve	-ve	N
68/20	-ve	*	*	*	?	-ve	-ve	N
70/20	*	-ve	-ve	-ve	N	*	-ve	N
63/20	*	*	-ve	-ve	N	-ve	*	N
66/20	-ve	-ve	-ve	-ve	N	-ve	-ve	N
58/20	-ve	*	*	-ve	N	-ve	-ve	N
410/17	-ve	-ve	-ve	-ve	N	-ve	-ve	N
51/20	-ve	*	-ve	-ve	N	-ve	-ve	N
					N = 27			N = 29
					? = 3			? = 1

Key:

* - Inconclusive Result (Too much background/ nonspecific binding)

? – Discordant Result

N – Negative Result

Table A.3: 2×2 Table showing the raw data used to calculate the detection accuracy for the combined recombinant LASV IFAs vs the traditional LASV infected IFA with the panel of known LASV negative samples

		Traditional IFA		
		Positive/ Discrepant	**Negative**	
LASV NP & LASVGP IFA	**Positive/ Discrepant**	0	3	**3**
	Negative	1	26	**27**
		1	**29**	**30**

Appendix E: Project clearance certificates

2021 ethical clearance certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

15 April 2021

Approval Certificate
Annual Renewal

Ethics Reference No.: 257/2019
Title: Development and evaluation of a recombinant antigen-based indirect immunofluorescence assay for the diagnosis of Lassa fever

Dear Mr JG Bell

The **Annual Renewal** as supported by documents received between 2021-03-25 and 2021-04-14 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-04-14 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2022-04-15.
- Please remember to use your protocol number (257/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Professor Werdie (CW) Van Staden
MBChB MMed(Psych) MD FCPsych(SA) FTCL UPLM
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 46 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Dikgerese tša Maphelo

2020 ethical clearance certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

21 April 2020

Approval Certificate
Annual Renewal

Ethics Reference No.: 257/2019
Title: Development and evaluation of a recombinant antigen-based indirect immunofluorescence assay for the diagnosis of Lassa fever

Dear Mr JG Bell

The **Annual Renewal** as supported by documents received between 2020-03-24 and 2020-04-08 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-04-08.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-04-21.
- Please remember to use your protocol number (257/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2016 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tweelopelle Building
University of Pretoria, Private Bag x323
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Email: deepeka.behari@up.ac.za
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

2019 ethical clearance certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

31 May 2019

Approval Certificate
New Application

Ethics Reference No.: 257/2019
Title: Development and evaluation of a recombinant antigen-based indirect immunofluorescence assay for the diagnosis of Lassa fever

Dear Mr JG Bell

The **New Application** as supported by documents received between 2019-05-02 and 2019-05-29 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-05-29.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-05-31.
- Please remember to use your protocol number (257/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Genetically modified organism certificates

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Genetic Resources, Private Bag X973, PRETORIA, 0001
Harvest House Room 165, 30 Hamilton Street, Arcadia, Pretoria, 0002

From: Ms N L Mkhonza
Tel: (012) 319 6165 • **Fax:** (012) 319 6298 • **E-mail:** BathobileM@daff.gov.za
Enquiries: Ms B Mahlangu • **Ref:** 39.2/NICD/NHLS – 14/008

Prof. J. T Paweska
National Institute for Communicable Diseases of the National Health Laboratory Services
Centre for Emerging and Zoonotic Diseases (CEZD)
Special Viral Pathogens Laboratory (SVPL)
1 Modderfontein Road
Sandringham
2131

Tel: +27 (0) 11 386 6376
Fax: +27 (0) 11 882 3741

DEPT AGRICULTURE, FOR. STRY & FISHERIES
DIRECTORATE GENETIC RESOURCES
2015 -11- 23
PRIVATE BAG X973 PRETORIA 0001
REGISTRAR - GMO ACT

Dear Prof J. T Paweska

RE: REGISTRATION OF FACILITY

With reference to the application to register a facility, submitted in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997). Registration number **39.2/NICD/NHLS-15/115.**

The facility is hereby registered; please find attached your certificate which serves as proof of registration. Please familiarize yourself with the Standard Operating Procedure approved for Regulation 2(2) to determine whether your current activities or any future activities would require an additional contained use permit or not.

Please consult the website of the Department at www.daff.gov.za (Branches, Agricultural Production, Health & Food Safety/ Genetic Resources/ Biosafety) for the latest application forms and the SOP document referred to in the above paragraph.

If any of the provisions of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), including any condition of any permit issued in terms of the GMO Act, is not complied with at all times, you will be subject to prosecution in terms of Section 21 of the GMO Act, 1997.

Yours sincerely

Ms N L Mkhonza
Registrar: Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

CERTIFICATE OF REGISTRATION

Registration Number: **39.2/NICD/NHLS - 15/115**

It is hereby certified that the facility situated at:

Prof. J. T Paweska
National Institute for Communicable Diseases of the National Health Laboratory Services
Centre for Emerging and Zoonotic Diseases (CEZD)
Special Viral Pathogens Laboratory (SVPL)
1 Modderfontein Road
Sandringham
2131

has been registered in the name(s) of: **Prof. J. T Paweska**

on behalf of: **NICD/NHLS**

in terms of Regulation 8 of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997) in respect of the following-

type of facility: **LABORATORY AND ANIMAL CONTAINMENT**

containment level: **TWO (2), THREE (3), FOUR (4)**

for period: **23 NOVEMBER 2015 – 23 NOVEMBER 2018**

Date issued: 23 NOVEMBER 2015

DEPT. AGRICULTURE, FORESTRY & FISHERIES
DIRECTORATE GENETIC RESOURCES
2015 -11- 23
PRIVATE BAG X973 PRETORIA 0001
REGISTRAR - GMO ACT

Registrar for Genetically Modified Organisms

Date

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Genetic Resources, Department of Agriculture, Forestry and Fisheries
Private Bag X973, Pretoria 0001

Enquiries: Bathobile Mahlangu • **Tel:** 012 319 6165 • **Fax:** 012 319 6298 • **E-mail:** BathobileM@daff.gov.za •**Ref:** 39.2/ NICD/NHLS – 19/030

Prof. J. T Paweska
National Institute for Communicable Diseases of the National Health Laboratory Services (NICD/NHLS)
Centre for Emerging and Zoonotic Diseases (CEZD)
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DEPT. AGRICULTURE, FORESTRY & FISHERIES
DIRECTORATE GENETIC RESOURCES
2019 -10- 25
PRIVATE BAG X973 PRETORIA 0001
REGISTRAR - GMO ACT

Dear Prof J. T Paweska

RE: REGISTRATION OF FACILITY

With reference to the application to register a facility, submitted in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997). Registration number **39.2/NICD/NHLS-19/115.**

The facility is hereby registered; please find attached your certificate which serves as proof of registration. Please familiarize yourself with the Standard Operating Procedure approved for Regulation 2(2) to determine whether your current activities or any future activities would require an additional contained use permit or not.

Please consult the website of the Department at www.daff.gov.za (Branches, Agricultural Production, Health & Food Safety/ Genetic Resources/ Biosafety) for the latest application forms and the SOP document referred to in the above paragraph.

If any of the provisions of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), including any condition of any permit issued in terms of the GMO Act, is not complied with at all times, you will be subject to prosecution in terms of Section 21 of the GMO Act, 1997.

Yours sincerely

Ms N L Mkhonza
Registrar: Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997

agriculture,
forestry & fisheries
Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

CERTIFICATE OF REGISTRATION

Registration Number: **39.2/NICD/NHLS - 19/115**

It is hereby certified that the facility situated at:

Prof. J. T Paweska
National Institute for Communicable Diseases of the National Health Laboratory Services (NICD/NHLS)
Centre for Emerging and Zoonotic Diseases (CEZD)
Special Viral Pathogens Laboratory (SVPL)
1 Modderfontein Road
Sandringham
2131

has been registered in the name(s) of: **Prof. J. T Paweska**

on behalf of: **NICD/NHLS**

in terms of Regulation 8 of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997) in respect of the following-

type of facility: **LABORATORY AND ANIMAL CONTAINMENT**

containment level: **TWO (2), THREE (3), FOUR (4)**

for period: **25 OCTOBER 2019 – 25 OCTOBER 2022**

Date issued: 25 OCTOBER 2019

DEPT. AGRICULTURE, FORESTRY & FISHERIES
DIRECTORATE GENETIC RESOURCES
2019 -10- 25
PRIVATE BAG X973 PRETORIA 0001
REGISTRAR - GMO ACT

Registrar for Genetically Modified Organisms

Date

NICD ethical clearance letter

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

Center for Emerging Zoonotic and Parasitic Diseases
1 Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 386 6400 Fax: +27 (0)11 882 0596
Reference: testing of samples

17 April 2019

University of Pretoria

To whom it may concern

RE: ACCESS TO HUMAN CLINICAL PANELS FOR EVALUATION OF NEWLY DEVELOPED SEROLOGICAL ASSAYS FOR LASSA FEVER

This letter serves to confirm that Jason Bell (UP student number: 14036194) is a postgraduate student partaking in collaborative research between the University of Pretoria and the NICD. This work will be conducted for the project titled: Development and evaluation of a recombinant antigen-based indirect immunofluorescence assay for the diagnosis of Lassa fever. The study involves the development of new and improved diagnostic assays for determining serological responses in patients suspected or confirmed to have Lassa fever. The student will have access to the human clinical sample bank for previously investigated cases of viral haemorrhagic fever including Lassa fever. Testing will be conducted at the laboratories of the CEZPD, NICD. The work will be done the under supervision of Dr N Moolla, a full-time employee of the NICD, and co-supervision of Prof Wanda Markotter, University of Pretoria.

Ethical clearance for the retrospective use of samples submitted to SVPL, CEZPD, NICD-NHLS (for laboratory investigation for suspected viral haemorrhagic fever) for the evaluation and validation of new and improved diagnostic assays for viral haemorrhagic fevers, was obtained, from the University of the Witwatersrand Human Ethics Committee, clearance # M180219. The sample panel will be selected based on the outcome of previous diagnostic investigations and will be provided blindly with reference only to a laboratory reference number (for example SVPL 01/2019) with no reference to any patient identifying information (including age, sex, location of case).

Yours sincerely

Naazneen Moolla (PhD)
Senior Medical Scientist
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Appendix F: Language editing certificate

Nr: 20185

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LANGUAGE EDITING CERTIFICATE

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Authors:	Jason Bell
Institution:	University of Pretoria
Date Issued:	1 February 2022

This document certifies that the manuscript listed above was edited for proper English language, grammar, punctuation, spelling, and overall style. Neither the research content nor the author's intentions were altered in any way during the editing process. Documents receiving this certification should be English ready for publication; however, the author has the ability and choice to accept or reject our suggestions and changes.

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Warm regards

Elizabeth Marx

Attended the EFA International Editors' Conference – Chicago: August 2019 https://www.the-efa.org/efas-2019-conference-announcement/