APPLICATION OF RECOMBINANT NUCLEOCAPSID PROTEIN-IMMUNOFLUORESCENCE ASSAY (IFA) IN HOSPITAL-BASED SEROLOGICAL SURVEILLANCE OF RIFT VALLEY FEVER IN HUMANS

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A Research dissertation submitted to the University of Zambia in partial fulfilment of the requirements for the award of the degree of Master of Science in One Health Laboratory Diagnostic Sciences

University of Zambia

Lusaka

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DECLARATION

I, Peter Chibale Mwansa do hereby declare that the contents of this dissertation being submitted herein represent genuine research I carried out and that no part of this dissertation has been previously submitted to this or another university or institution for the award of a degree or any other qualification.

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CERTIFICATE OF APPROVAL

This dissertation submitted by Peter Chibale Mwansa is approved as fulfilling part of the requirements for the award of the degree of Master of Science in One Health Laboratory Diagnostic Sciences at the University of Zambia.

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ABSTRACT

Rift Valley fever (RVF) is a mosquito-borne viral zoonosis caused by the Rift Valley fever virus (RVFV), a negative-sense RNA virus belonging to the genus Phlebovirus in the family Phenuiviridae. Transmission to humans and animals occurs through mosquito bites (*Culex* and *Aedes* spp.), direct contact with infected animal blood or organs, ingestion of contaminated animal by-products, or inhalation of aerosols from infected animals. In this study, analysis of human serum was conducted at the Virology Laboratory at the University of Zambia in Lusaka. Human serum samples were screened using a locally prepared recombinant Nucleocapsid-based Immunofluorescence Assay (IFA), where Vero E6 cells were transfected with a plasmid encoding the RVFV N gene and cultured on glass slides to express the recombinant protein. After incubation, the cells were fixed, preserved, and stored for later use. The commercial ID Screen® Rift Valley Fever Competition Multi-species enzyme-linked immunosorbent assay (ELISA) was used as confirmatory test; the tests were analysed according to the manufacturer's guidelines. From previous studies a prevalence of 9.43% of RVF antibodies was recorded in Lusaka abattoir workers dealing with cattle, while in Mazabuka district 18.63% tested were sero-positive of RVFV antibodies. However, Zambia has not recorded an outbreak of RVF for over 30 years. The current sero-prevalence and risk factors associated with RVFV in human populations is not known. This study utilized convenience sampling, obtaining archived human serum samples from selected hospitals. Sample selection was based on availability, patient records, and storage conditions at -30°C. A total of 593 archived human serum samples were analysed, revealing an overall seroprevalence of 1.7% (10/593) on both IFA and ELISA. Lukulu District Hospital had the highest prevalence 0.84% (5/593); Choma General Hospital and Kanyama Hospital both had 0.34% (2/593), while Kalabo had the lowest 0.17% (1/593). Occupation was significantly associated with RVF seropositivity ($p = 0.000$), with farmers accounting for 1.35% (8/593) of cases. The highest seroprevalence was among farmers, individuals from Western Province, and patients attending Lukulu District Hospital. The aim of the study focused on the application of the Recombinant Nucleocapsid Protein (rNP) - IFA for hospital-based serological surveillance of RVFV in humans. IFA was successfully applied for the detection of RVF-specific antibodies in human serum samples, demonstrating high sensitivity and specificity in identifying seropositive cases. These findings highlight the potential of IFA as a valuable method for hospital-based surveillance for further development of rapid, point-of-care diagnostic tests based on the recombinant RVFV Nucleocapsid protein (NP) could improve early detection in rural or resource-limited areas.

DEDICATION

I dedicate this dissertation to my beloved wife, Salome Kasamwa Mwansa, my brothers and sisters, and my dear father, Stanislaus Mwansa. With heartfelt love and cherished memories, I also honor the loving memory of my late mother, Georginah Mushibwe Mwansa.

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LIST OF ACRONYMS AND ABBREVIATIONS

ACEIDHA	Africa Centre of Excellence for Infectious Diseases of Humans and Animals
AIC	Akaike's Information Criterion
aOR	adjusted Odds Ratios
BIC	Bayesian Information Criterion
CCHF	Crimean-Congo haemorrhagic fever
cDNA	Complementary DNA
CI	Confidence Intervals
cOR	crude Odds Ratios
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FN	False Negative
FP	False Positive
Gc	Carboxyterminal Glycoprotein
Gn	Amino-terminal Glycoprotein
HVFs	Haemorrhagic viral fevers
IFA	Immunofluorescence assay
LGp	Large glycoprotein
NCBI	National Centre for Biotechnology Information
NP	Nucleocapsid Protein
NSm	Non-structural protein medium segment

NSs	Non-structural protein small segment
OD	Optical Density
ODNC	Optical density of the Negative control
ODPC	Optical density of the positive control
OPD	Outpatient Department
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
RNA	Ribonucleic acid
rNP	Recombinant Nucleocapsid Protein
RT	Reverse Transcriptase
RVF	Rift Valley Fever
RVFV	Rift Valley Fever Virus
SOC	Super Optimal Broth
TN	True Negative
TP	True Positive

CHAPTER ONE

INTRODUCTION

1.1 Background

Rift Valley fever (RVF) is a mosquito-borne viral zoonosis caused by Rift Valley fever virus (RVFV). It is a negative-sense Ribonucleic acid (RNA) virus causing disease in animals and humans (Mbewana *et al.,* 2018). This is an enveloped virus belonging to the genus *Phlebovirus* of the family *Phenuiviridae* (Borrego *et al.,* 2022). Rift Valley fever virion particles are spherical in shape and approximately 100nm in size. The outer surface of the virion comprises capsomers of the structural glycoproteins Gn (Amino-terminal Glycoprotein) and Gc (Carboxyterminal Glycoprotein), which are embedded in a lipid bilayer (Pepin *et al.,* 2010). The virion contains an RNA genome that is divided into 3 segments, each named after their respective size. The large (L) segment encodes the viral RNA-dependent RNA polymerase while the medium (M) segment encodes a glycoprotein precursor that is co-translationally cleaved into Gn and Gc and 2 accessory proteins (Wichgers *et al.,* 2014).

The first of these proteins is a 14-kDa non-structural protein named NSm, which was shown to have an anti-apoptotic effect (Bird *et al.,* 2009; Pepin *et al.,* 2010). The function of the second protein, a 78-kDa protein, is presently unclear but is thought to suppress virus-induced apoptosis in the host cell. The small (S) segment encodes a Nucleocapsid (N) protein and a non-structural protein, named NSs, which counteracts host innate immune responses and is, therefore, considered the major virulence factor of the virus (Bird *et al.,* 2007). The virus is transmitted to both humans and animals directly from blood, body fluid or organs and tissue of infected animals or from infected mosquito (*Culex* and *Aedes* species) bites (Linthicum *et al.,* 1999; Nyakarahuka *et al.,* 2018). Rift Valley Fever causes death and abortion in livestock, resulting in significant economic losses.

In humans, symptoms range from mild flu-like to severe form such as retinal damage, meningo-encephalitis to haemorrhagic fever. Case fatality can be as high as 14% as was seen in an outbreak in Saudi Arabia in 2003 (Madani *et al.,* 2003). RVFV was first reported during an epidemic among sheep in Rift Valley of Kenya in 1931 (Daubney *et al.,* 1931). Although most RVF epidemics have occurred in eastern and southern Africa, RVFV distribution has been reported in most of the

countries in Africa, Egypt and Yemen (Himeidan *et al.*, 2014). The need for countries to monitor and manage RVFV outbreaks within their borders has become ever more urgent due to global warming, which has expanded the regions habitable by the mosquito vector. Rift Valley Fever is a zoonotic disease influenced by various environmental, occupational, and behavioural risk factors. Environmental conditions such as heavy rainfall and flooding create ideal breeding grounds for mosquito vectors, notably Aedes and Culex species, facilitating RVFV transmission among livestock and humans (Spickler, 2015). Occupational exposure significantly elevates infection risk, with individuals like herders, farmers, slaughterhouse workers, and veterinarians being more susceptible due to frequent contact with infected animals or their tissues. Additionally, certain behaviours, including the consumption of raw or unpasteurized milk and handling of aborted animal foetuses, have been associated with increased RVF incidence. Understanding these multifaceted risk factors is crucial for developing targeted interventions to mitigate RVF outbreaks (O'Neill *et al.*, 2024).

In Zambia, RVFV was first reported in humans in Chisamba District, Central Province, in 1984 where it caused some deaths (Davies *et al.*, 1992). Further evidence of the disease was provided in subsequent studies conducted in the early 1990s that indicated that cattle-dominated regions with higher amounts of rainfall had high seropositivity in the herds tested (Samui *et al.*, 1997). However, for the past three decades, no reports of outbreaks have been noted in humans or animals defying the epidemic resurgence pattern of 10-15 years as previously proposed (Pepin *et al.*, 2010). However, a Zambian study using a locally-developed assay screened traditional cattle sera from five districts for anti-RVFV antibodies through authentic antigen-based and rNP-based IFAs. Antibody prevalence rates in the five districts were 1.3% to 13.5% in the authentic antigen-based indirect IFA and 6.0% to 21.4% in the rNP-based IFA (Saasa *et al.*, 2018). The results indicated that despite no reports of active cases of RVF in these provinces of Zambia, the virus is circulating among cattle herds.

Rift Valley fever often presents with nonspecific manifestations such as fever, headache, joint pain, and general malaise, leading to misdiagnoses like malaria, dengue fever, yellow fever, leptospirosis, brucellosis, typhoid fever, chikungunya virus, influenza-like illnesses, hepatitis, Crimean-Congo haemorrhagic fever (CCHF), and other nonspecific febrile illnesses (Zhao *et al.*, 2020). Conducting serological surveillance for RVF in humans is essential, because knowledge gathered is useful in guiding healthcare providers in hospitals toward understanding the sero-prevalence and

risk factors associated with hospital-based patients for RVFV infection and aids in prioritizing resources for vaccination, implementation of measures to prevent, manage, vector control or control of the disease, and public awareness initiatives (Davies, 2010; Chengula *et al*, 2013).

1.2 Statement of the Problem

Rift Valley fever is a re-emerging viral disease that is of public health and veterinary importance in Africa (Dautu *et al.*, 2012). Infection of humans is associated with symptoms (e.g., fever, headache, joint pains) that can easily be mistaken for other diseases (Zhao *et al.*, 2020). In severe cases, humans experience haemorrhagic fever, neurological disorders, or blindness and death can occur (Chevalier *et al.*, 2010). The outbreak that occurred in 2000 resulted in 124 deaths in Saudi Arabia and 166 deaths in Yemen. In Sudan, the 2007 outbreak resulted in 222 human deaths (Himeidan *et al.*, 2014).

A study conducted in northern Tanzania between 2017 and 2019 retrospectively analyzed livestock abortion samples using serology and reverse transcription quantitative polymerase chain reaction (RT-qPCR). The findings revealed a previously undetected outbreak of Rift Valley fever (RVF) among peri-urban dairy cattle, with evidence of RVFV nucleic acids in milk samples. This outbreak, occurring below national surveillance detection thresholds, underscores the significant threat RVF poses not only to livestock and human health but also to the socio-economic stability of the region (Glanville *et al.*, 2022). In Malawi RVF suspected outbreak occurred in March, 2006, following a period of heavier than usual rainfall and widespread flooding. The suspected outbreak spread to many districts in the Southern region in July 2007 (Kothowa *et al*., 2021). A study conducted in Malawi detected both the genome and antibodies of Rift Valley fever virus, indicating active circulation of the virus in the region (Kainga, 2023). In a study conducted in Democratic Republic of the Congo (DRC) on sera collected from apparently healthy cattle between 2014 and 2015 found an overall true seroprevalence of 12.37% (95% CI 10.86-14.05) (Tshilenge *et al.*, 2018). Zambia has not recorded any outbreak of RVF in the recent past, and as such little is known about the current seroprevalence and risk factors associated with RVFV infection in human populations in the country (Kasongamulilo *et al.*, 2025). There is also scarce information regarding prevalence of RVF in hospital-based patients. A lack of knowledge about these factors and the absence of testing for RVF in Zambia may result in inadequate prevention, control, and management of the disease if it occurs in the country. The limited epidemiological data on human

RVF in Zambia poses significant challenges for effective disease prevention and control. The availability of highly sensitive, cost-effective diagnostic methods offers a valuable opportunity, to identify extent of at-risk human populations and establish a foundation for preventive interventions. High levels of morbidity and mortality in humans and livestock have previously been caused by several RFV outbreaks, leading to significant economic loss in the affected countries (Sindato *et al.,* 2011).

Recent serological studies suggest that RVFV is circulating in human populations in Zambia, despite the country having not recorded an outbreak of the disease for several decades (Kasongamulilo *et al.,* 2025). Humans that live in close proximity to livestock or carry out slaughtering and processing of meat in infected areas are at high risk of RVF infection (Liu *et al*., 2017). Knowledge gathered can help with implementation of measure to prevent, manage or control the disease (Davies, 2010; Chengula *et al*, 2013).

1.3 Justification

The zoonotic nature of RVFV and prior evidence of the virus in livestock highly suggest that human infections are present. Therefore, this study aimed to assess the serological status of RVFV infection and identify potential risk factors associated with virus exposure in a non-RVF-indicated human population across selected hospitals in Lusaka, Southern, and Western Provinces of Zambia. The study screened human serum samples using a rNP-based IFA, with confirmation via the ID Screen® RVF Competition Multi-species ELISA. The ELISA test confirmed the presence of specific IgG antibodies, which emerge around 8 days post-infection and may persist for several years (Paweska *et al.*, 2005).

The scientific knowledge and epidemiological data this research generated raise public health awareness, support better policy formulation, and encourage a multidisciplinary approach to controlling zoonotic and emerging diseases.

1.4 Research Questions

(i) What is the prevalence of RVFV among individuals attending Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, Kalabo and Lukulu District Hospitals in Western Province, Zambia?

(ii) Do specific demographic factors (such as age or gender) influence RVFV exposure among patients attending Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, Kalabo and Lukulu District Hospitals in Western Province, Zambia?

1.5 Objectives

1.5.1 General objective

To evaluate the application of the Recombinant Nucleocapsid Protein (rNP)-based Immunofluorescence Assay (IFA) for hospital-based serological surveillance of Rift Valley Fever Virus (RVFV) in humans across Lusaka, Southern, and Western Provinces of Zambia.

1.5.2 Specific objectives

(i) To clone the NP gene of RVFV into the pCAGGS vector for expression in Vero E6 cells facilitating detection of human serum antibodies through IFA.

(ii) To determine the seroprevalence of RVF in non-RVF indicated human population in Zambia.

(iii)To determine the risk factors associated to RVF infection among hospital based patients in Zambia

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical overview of Rift Valley Fever

Rift Valley fever is a viral zoonosis that primarily affects animals but can also infect humans. Its etiological factor is the RVF of the *Phlebovirus* genus and *Bunyaviridae* family. It is transmitted mainly by the *Aedes* and *Culex* mosquito species. Contact with the blood or organs of infected animals may infect humans (Kwaśnik *et al.,* 2021). Rift Valley Fever leads to severe disease in livestock, with up fatality rates of up to 70% in young animals and as high as 20% - 30% in adult ruminants. In addition, infected pregnant animals undergo spontaneous abortion at an alarmingly high rate, in the range of 40–100% (Gogovi *et al.*, 2019).

More than a century ago, in June 1912, an outbreak of "an unknown disease that resulted in high mortality among lambs", which briefly had a negative impact on the sheep industry in Kenya was reported (Bron *et al.*, 2021). Eighteen years later, in 1930, the likely causative agent, RVFV, was isolated (Daubney *et al.*, 1931). Nearly 100 years later, this primarily mosquito-transmitted virus, now known as Rift Valley fever phlebovirus (Pepin *et al.*, 2010), still causes morbidity and mortality in animals with spill over to people throughout the African continent, Indian Ocean, and Middle East. Outbreaks lead to severe recurring economic losses, disrupting the livelihoods of often poor communities. Due to its economic impact, pathogenicity, and unpredictable (re) emergence, RVFV is recognized as a danger for both human and animal populations (Mehand *et al.*, 2018).

Since the 1970s, periodic epidemics of RVF have been reported in many countries in Eastern and Southern Africa including Kenya, Somalia, Sudan, Tanzania, Zimbabwe, and South Africa (Nderitu *et al*., 2010). The RVF epidemic of 1997–1998 that affected Kenya, Somalia, and Tanzania was characterized by outbreaks that originated from north eastern province of Kenya in November 1997 and spread to the north-central region of Tanzania in June 1998 (WHO, 2007; CDC, 2007). Another wave of RVF epidemic occurred in 2006–2007 in these three countries, with cases first reported in the north eastern province of Kenya and later in Tanzania, where the last livestock and human cases were reported in June 2007 (Mohamed *et al.*, 2010, Munyua *et al*., 2010). Recent outbreaks in East Africa were reported in 2018 in Kenya, Rwanda Uganda and in South Sudan, with severe cases in livestock and humans (Anyamba *et al.*, 2018; CDC, 2018).

Historically, the emergence of RVFV in new areas has been unpredictable, but it has frequently been linked to animal trade (Salem *et al.*, 2010). As "a transmissible disease that has the potential for very serious and rapid spread, without regards for national borders, and with serious socio-economic and public health consequence as well as major importance in the international trade of animals and animal products," the World Organization for Animal Health (WOAH) recognizes RVF as a notifiable animal disease of concern. Similarly, the World Health Organization's research and development blueprint named RVFV a priority pathogen due to its "epidemic potential" (Mehand *et al.,* 2018). A better understanding of the eco-epidemiology of RVF could help inform intervention and surveillance strategies to reduce the burden of disease and minimize pathogen range expansion.

2.2 Structure of Rift Valley Fever virus

The Rift Valley Fever Virus has a single-stranded, tripartite RNA genome, composed of L, M, and S segments (Figure 2.1). The large negative-sense L RNA segment encodes a single-polypeptide L protein, and the negative-sense M RNA segment encodes the glycoprotein precursor. The M segment encodes at least four proteins: the structural glycoproteins Gn and Gc, the non-structural protein Nsm, and a large 78-kDa glycoprotein (LGp) (Bian *et al.,* 2023). The structural glycoproteins mediate binding and entry via receptors on the target cell membrane. The function of the 78-kDa glycoprotein is unclear, although it is thought to be incorporated into virions matured in mosquito cells, where it may facilitate transition between different host species, and function during replication in the mosquito host (Weingartl *et al.*, 2014). However, due to its ability to form a complex with the Gc glycoprotein (Gerrard and Nichol, 2007), the 78-kDa glycoprotein may constitute a target for the immune system, and could potentially contribute to novel vaccine development. Additionally, the virus does not encode a matrix protein so the surface glycoproteins interact directly with the ribonucleoprotein (Mansfield *et al.*, 2015). The S segment adopts an ambisense strategy for expressing NSs and nucleoprotein (Terasaki *et al.*, 2011). The RVFV viral genome is wrapped by the nucleoprotein and the conserved 39 and 59 ends of the genomic RNA are bound to L protein, resembling the influenza virus (Flug *et al.*, 2014). The L protein is a multifunctional enzyme that catalyses genome replication and viral gene transcription, which are initiated de novo by prime-and-realign and cap-snatching mechanisms, respectively (Gogrefe *et al.*, 2019).

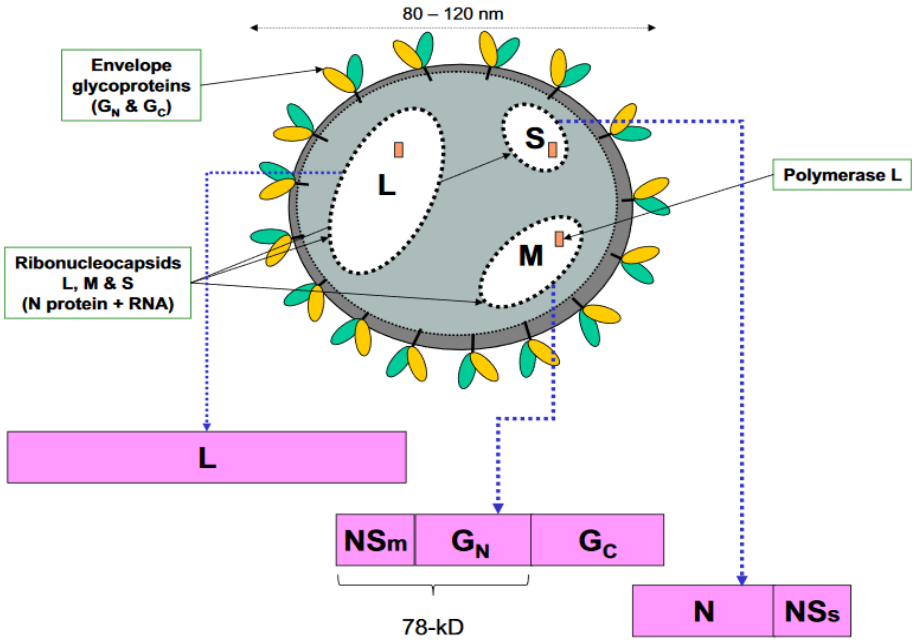

Figure: 2.1 Schematic diagram of Rift Valley Fever virus Genome
On the Right is the viron containing small (S), medium (M) and large; NSm= non-structural protein M; glycoproteins Gn and Gc; N= nucleoprotein; NSs= non-structural protein S. On the Left shows the representation of the (L) RNA genome segments, coding strategy of the RNA genomesegments; L = L protein (Pepin *et al.*, 2010).

2.3 Transmission and pathogenesis

Rift Valley Fever Virus is mostly transmitted by bites from *Culex* and *Aedes* spp. mosquitoes or by direct contact with body fluids of viraemic animals or through ingestion of unpasteurized or uncooked by-products of infected animals, or also through inhalation of aerosols produced during the slaughter of infected animals (Balenghien *et al.*, 2013). To date, no human-to-human transmission of RVFV has been documented (Javelle *et al.*, 2020).

Once the virus gains entry, it typically infects local dendritic cells and macrophages at the inoculation site. The infected cells then transport the virus to nearby lymph nodes, where initial replication occurs, leading to a period of viremia (Ikegami and Makino, 2011). During viremia, RVFV disseminates through the bloodstream to several target organs, most notably the liver. In the liver, the virus directly infects hepatocytes, leading to cellular injury and necrosis. This hepatocellular damage can result in elevated liver enzymes and, in severe cases, fulminant hepatitis accompanied by haemorrhagic manifestations (Nair *et al.*, 2023).

In some patients, the virus may also breach the blood brain barrier, contributing to encephalitis, while involvement of ocular tissues can lead to retinitis and vision problems. The clinical course of RVFV infection in humans ranges from a mild, self-limiting febrile illness to severe disease forms characterized by haemorrhagic fever, neurological complications, and multi-organ failure (Connors and Hartman, 2022).

Underlying these clinical manifestations is a complex interplay between direct viral cytopathic effects and the host's immune response. The innate immune system responds by releasing cytokines and interferons to control the infection; however, an excessive or dysregulated immune response may further contribute to tissue damage (Quellec *et al.*, 2023). Additionally, RVFV has developed mechanisms to evade these antiviral defences, thereby facilitating its replication and spread within the host (Javelle *et al.*, 2020).

After being infected with RVFV, humans may experience generalized fatigue, low-grade fever, headache, photophobia, and joint pains. Some patients infected with RVFV will develop encephalitis, retinopathy, or disseminated intravascular coagulation leading to haemorrhage and even death, with a case fatality rate of approximately 20% (Zhao *et al.*, 2020).

2.4 Climate Change and Rift Valley fever outbreaks

The results of the greenhouse effect include changes in average daily temperature, precipitation, flooding, droughts and other climate-related disasters (Métras *et al.,* 2013; Oyas *et al*., 2018). Other major catastrophic livelihood challenges are reported in many geographical areas following disease outbreaks, spread and increase of disease vectors (Braack *et al.,* 2018; Bett *et al.,* 2019).

The amount of rainfall is considered to be the main factor initiating RVF outbreaks as they are preceded by heavy rains associated with flooding (Vizcaíno *et al.,* 2013). The wide distribution of competent vectors in non-endemic areas coupled with global climate change poses a significant threat of the transboundary spread of RVF (Bett *et al.,* 2019). The rainfall which causes increased flooded dambos and booming of dambo mosquitoes make prediction of disease occurrence, vector increase and disease spread difficult (Pepin *et al.,* 2010). It is assumed that RVFV adopts both vertical and horizontal strategies to promote survival under a range of ecological conditions and pressures (Mwaengo *et al.,* 2012).

In the face of climate change, range expansions and redistributions of mosquito-borne viruses are expected (Ryan *et al.,* 2019). Recent outbreaks in a French overseas department and some

seropositive and imported cases in Turkey, Libya, and China have raised concerns that RVF could extend to other parts of the world, which poses a serious threat to global health and economics (Vloet *et al.*, 2017).

With this climate change background, update on RVF epidemiological information in countries like Zambia that has in the recent years experienced heavy rains and dambo flooding is a prerequisite for proper control and preventive strategies in sub-Saharan Africa.

2.5. Hosts and vectors of rift valley fever

There are a wide range of hosts for RVFV, ranging from cattle, sheep, goats, pigs, camels, wild ruminants to humans and bats. However, the primary amplifying hosts are domestic ruminants (Saeed *et al.,* 2021); Pepin *et al*., 2010; Gregor *et al.,* 2021). It has been assumed that wildlife are reservoir hosts of RVFV. However due to little published data on RVFV pathogenicity on various wildlife species, validation of diagnostic tests for wildlife exposure to RVFV and understanding the virus dynamics during the endemic periods in wild ruminants has not been carried out (Rosta *et al.,* 2017).

Rift Valley fever virus (RVFV) is primarily transmitted by mosquitoes, notably those in the *Aedes* and *Culex* genera. Field studies have identified over 50 mosquito species across eight genera within the *Culicidae* family as RVFV-positive, with more than 65 species considered potential vectors. *Aedes* mosquitoes serve as primary reservoir vectors, capable of maintaining the virus through vertical transmission to their offspring, thus sustaining the virus during inter-epidemic periods. Secondary vectors, including *Culex*, *Anopheles*, and *Mansonia* species, contribute to the amplification and spread of the virus during outbreaks by feeding on infected animals or humans. This complex vector ecology underscores the importance of comprehensive mosquito control strategies in managing RVF outbreaks (Linthicum and Anyamba, 2016; Hartman, 2017). *Culex* are associated with more permanent fresh water bodies while *Aedes* species are associated with freshly flooded temporary or semi-permanent fresh-water bodies (Pepin *et al.,* 2010; Pachka *et al.,* 2016). The population of these two species is highly dependent on the environmental factors at play in an area, where conditions such as unavailability of permanent water can prevent the desiccation of eggs hatching in the case of the *Aedes* spp (Pachka *et al*., 2016). Infected *Aedes* spp lay eggs/progeny that contain the RVFV (transovarial transmission of the virus) therefore enabling survival of the virus during periods of drought or the inter-epidemic period and infected eggs can hatch upon flooding , therefore associating RVF to heavy rains or irrigation(Gardela *et al.*, 2024).

Other biting arthropods, such as midges (Culicoids), ticks and sandflies can become infected with the virus and could potentially act as mechanical vectors (Lee, 1979; Davies and Highton, 1980; Linthicum *et al.,* 2016; Klimentov *et al*., 2020).

Aedes mosquitoes are known for their ability to maintain the virus during inter-epidemic periods through transovarial transmission, where the virus is passed from female mosquitoes to their offspring. Culex mosquitoes can act as secondary vectors, facilitating the spread of the virus during outbreaks (Chambaro *et al.*, 2022).

Studies have indicated that Aedes and Culex species are prevalent in Zambia, particularly in regions prone to flooding and areas with abundant vegetation. The majority of mosquito-borne viruses, including RVFV, have been reported in the Western and North-Western provinces of Zambia. These areas provide suitable breeding habitats for these mosquito species, increasing the risk of RVF transmission (Kainga *et al.,* 2022).

Zambia has experienced RVF documented cases in areas such as Chisamba district in central Zambia. Recent serological studies have revealed evidence of silent circulation of RVFV during inter-epidemic periods, particularly in districts like Sesheke in the western part of Zambia, where a seroprevalence rate of 19.23% was observed. This suggests that the virus persists in certain regions, maintaining the potential for future outbreaks (Kasongamulilo *et al.*, 2025).

Environmental factors, such as heavy and persistent rainfall leading to flooding, create ideal conditions for mosquito breeding and can trigger RVF epidemics (Chemison *et al.*, 2024). Retrospective analyses have shown a positive correlation between anomalous precipitation events, like La Niña, and the emergence of RVF in Zambia. These climatic conditions facilitate the hatching of infected Aedes mosquito eggs and the subsequent spread of the virus (Chambaro *et al.*, 2022).

2.6 Diagnosis of Rift Valley Fever virus

Timely detection of suspected cases is crucial for implementing rapid control measures to minimize the significant disease burden. An RVF epidemic is typically marked by the sudden occurrence of widespread abortions ("abortion storms") and high mortality rates among young livestock, alongside the emergence of the disease in humans (Gerdes, 2004). In humans, the clinical

recognition of acute haemorrhagic fever cases generally triggers an outbreak investigation in endemic regions (Mangat and Louie, 2024).

Reference laboratories are often responsible for the coordination of field sampling and testing but with heterogeneous laboratory coverage in some endemic regions, delays in diagnosing the disease commonly occur. Laboratory diagnosis would ideally rely upon a combination of serological and molecular approaches. The usefulness of the chosen assay is dictated by the disease kinetics i.e. the windows in which particular virological markers (e.g. virus, viral RNA, IgG, IgM, hepatic lesions) are likely to be detected. There is likely to be some variability in disease kinetics between humans and animal species, due to variation in susceptibility between species, and even within species (Bird and Nichol , 2012).

Rift valley fever Virus can be detected by classic virological methods which include virus isolation; this method involves culturing the virus in permissive cell lines, such as Vero cells, or using intracerebral inoculation of new born mice. The presence of RVFV is confirmed by observing cytopathic effects (CPE) in infected cell cultures, followed by additional confirmatory tests such as immunofluorescence assays (IFA) or reverse transcription-polymerase chain reaction (RT-PCR) (Woods *et al.,* 2002). Histopathology examination of tissue samples from infected animals or humans can provide valuable diagnostic insights. The characteristic lesions of RVFV infection include hepatocellular necrosis, haemorrhages, and neuronal degeneration, particularly in the liver, brain, and spleen (Coetzer , 1982). Antigen detection assays, such as ELISA and IFA, are useful for detecting RVFV proteins in clinical and environmental samples. These methods utilize monoclonal or polyclonal antibodies to target specific RVFV antigens, such as the Nucleocapsid (N) protein or glycoproteins (Fukushi *et al.*, 2012). Antibody detection, including immunoglobulin M (IgM) and immunoglobulin G (IgG) ELISAs, virus neutralization tests (VNT), and hemagglutination inhibition assays, are widely used for detecting RVFV-specific antibodies in infected individuals and livestock (vanderwal *et al.*, 2012) and nucleic acid based assays , such as RT-PCR, loop-mediated isothermal amplification (LAMP), and next-generation sequencing (NGS), have revolutionized RVFV detection by providing highly sensitive and rapid results (Roux *et al.*, 2009). For reporting RVFV in animals, the World Organisation for Animal Health (WOAH), require laboratory confirmation by at least two positive results from a combination of different diagnostic approaches preferably for the same specimen i.e. either positive for virus/viral RNA and

antibodies or positive for IgM and IgG with demonstration of rising titres between paired sera samples collected 2 – 4 weeks apart (Mangat and Louie, 2024).

2.6.1 Serodiagnosis of Rift Valley Fever virus

Serological diagnosis, usually by IFA, virus neutralisation or ELISA, is commonly used to confirm RVFV infection in an affected individual (animal or human), during outbreak management and also to determine the prevalence of exposure to RVFV in a susceptible population (surveillance) (Lapa *et al.,* 2024). Several assays are available for the detection of anti-RVFV antibodies in a variety of animal species. Both the Virus Neutralisation Assay and ELISAs are highly specific, although there is the potential that they could be hindered by cross-reactivity between RVFV and other phleboviruses (Pepin *et al*., 2010).

ELISAs can be employed to confirm the presence of either specific IgM antibodies, which appear transiently from 4 days after infection or specific IgG antibodies, which appear from 8 days after infection and may persist for several years (Paweska *et al.,* 2005). ELISAs are developed and widely applied by OIE Reference Laboratories (Williams *et al.,* 2011), and several have been developed using either whole cell lysate derived from infected cells or purified Nucleocapsid protein as antigen (Zhan *et al.*, 2017). However, the commercially available ELISAs (IgG and IgM) are based upon recombinant RVFV Nucleocapsid protein, despite the potential for background issues with this antigen (Faburay *et al.*, 2013). An indirect ELISA based on the recombinant Nucleocapsid protein of RVFV has been developed for the detection of specific antibodies in human and animal sera (McElroy *et al.*, 2009).

The IFA is widely used for identifying antibodies, such as IgG and IgM, in serological tests, autoimmune diagnostics, and research. Its high sensitivity allows for the detection of very low antigen or antibody concentrations, making it especially useful for early disease diagnosis even if the cost of this method is a bit expensive compared to ELISA. Moreover, because IFA depends on the precise binding of antibodies to their specific antigens, it offers high specificity and helps reduce false-positive results (Medical Lab, 2023). In one study evaluating the diagnostic value and cost-effectiveness of ELISA versus IFA for autoimmune disorders, statistical comparisons of antinuclear antibody (ANA) profiles revealed that ELISA had considerably lower sensitivity than IFA-51% compared to 78% in one set of criteria-matched cases and 55% versus 79% in cases meeting connective tissue disease criteria. Notably, ELISA failed to detect antibodies in some rare

cases, whereas IFA achieved 100% sensitivity. However, both methods demonstrated similar specificity, ranging from 70% to 72% in cases that met the established criteria (Karumanchi and Oommen, 2018).

Another investigation, which employed a Bayesian validation approach to compare IFA with ELISA and the complement fixation test (CFT) for detecting antibodies against *Coxiella burnetii* in goat serum, found that IFA had a significantly higher diagnostic sensitivity (Wood *et al.*, 2019). For detecting IgG antibodies, IFA reached 94.8% (95% CI: 80.3 – 99.6), outperforming ELISA at 70.1% (95% CI: 52.7 – 91.0) and CFT at 29.8% (95% CI: 17.0 – 44.8). While all three tests were highly specific for goat IgG, IFA also showed superior sensitivity for IgM antibodies (88.8%; 95% CI: 58.2 – 99.5) compared to ELISA (71.7%; 95% CI: 46.3 – 92.8). These results emphasize IFA's enhanced capability for detecting both IgG and IgM antibodies in goat serum, with potential applicability to other ruminants as well (Muleme *et al.*, 2016).

2.6.2. Molecular Methods for Rift Valley Fever Virus detection

Rift Valley fever virus (RVFV) detection has been significantly enhanced through various molecular methods, which offer rapid and accurate diagnosis. These techniques are crucial for timely outbreak response and effective disease management. Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Grolla *et al.*, 2012) is a widely used molecular technique for detecting RVFV. It amplifies specific viral genetic sequences, providing high sensitivity and specificity. Studies have demonstrated that RVFV is more consistently detected in whole blood than in plasma samples, likely due to the association of the virus with blood cells. This highlights the importance of selecting appropriate sample types for accurate diagnosis. Advancements in technology have led to the development of portable PCR machines. These devices enable rapid and sensitive RVFV detection in field settings, facilitating immediate deployment during suspected outbreaks. Field-deployable detection methods provide reliable results, which are essential for prompt epidemiological assessments and containment measures (Trujillo *et al.*, 2024). Recombinase Polymerase Amplification (RPA) is an isothermal amplification technique that operates at constant temperatures (37–42°C), eliminating the need for thermal cycling. By adding a reverse transcriptase enzyme, RPA can detect both RNA and DNA, making it suitable for RVFV detection. Its simplicity and rapid turnaround time make RPA an excellent candidate for developing low-cost, point-of-care diagnostics (Piepenburg *et al.*, 2020). The performance of molecular detection methods for RVFV

has been evaluated through international external quality assessments. These evaluations ensure the reliability and accuracy of diagnostic techniques across different laboratories, highlighting the importance of standardized protocols in RVFV detection (Escadafal *et al.*, 2018).

2.7 Prevention and Control of Rift Valley Fever Virus

Considering RVF is mosquito-borne disease (Paweska , 2015), preventing RVFV transmission involves using physical barriers like long clothing and bed nets, along with chemical repellents and insecticides. These measures should continue for at least a week after illness onset. There is no specific treatment for RVF, so management focuses on infection prevention and supportive care while avoiding hepatotoxic drugs.

Importantly, haemorrhagic complications require high cautious infection control measures, following the CDC guidance on infection control precautions for haemorrhagic viral fevers (HVFs), while waiting for the exclusion of other HVFs such as Ebola virus disease or Crimean Congo haemorrhagic fever (Nsengimana *et al.*, 2024). Standard precautions with Personal Protective Equipment (PPE) were reported sufficient to prevent from nosocomial transmission of RVFV during the outbreak in Arabian Peninsula (Hamdan *et al.*, 2015), and must be implemented according the WHO checklist (WHO, 2019), to care any suspected case regarding the theoretic risk of RVFV transmission through contact with infected blood, tissues, or other body fluids, secretions and excretions.

Control measures include proper animal disposal, active surveillance, vaccination, livestock movement restrictions, public awareness campaigns, epidemiological investigations, and mosquito control programs, including targeted spraying, to limit transmission. (Pepin *et al.*, 2010; Himeidan, 2016; Oyas *et al.*, 2018). These strategies are robust to limit the effect of the outbreak and contain the disease from spreading to other areas.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was carried out from Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, Kalabo and Lukulu District Hospitals in Western Province, Zambia (Figure. 3.1). The selection of study sites was based on geographical, epidemiological, and diagnostic considerations to assess Rift Valley Fever Virus (RVFV) exposure across different population groups. Choma, Kalabo, and Lukulu are rural or semi-rural districts where livestock farming is prevalent, increasing human exposure to RVFV among farmers, herders, and abattoir workers who frequently handle cattle, goats, and sheep. In contrast, Kanyama Hospital, located in an urban setting, serves a peri-urban population with potential indirect exposure to livestock through food trade, transportation, or travel between rural and urban areas. Including both rural and urban sites allowed the study to determine whether RVFV is confined to livestock-rearing communities or spreading beyond traditional hotspots. This inclusion helped to assess potential spillover of RVF infections.

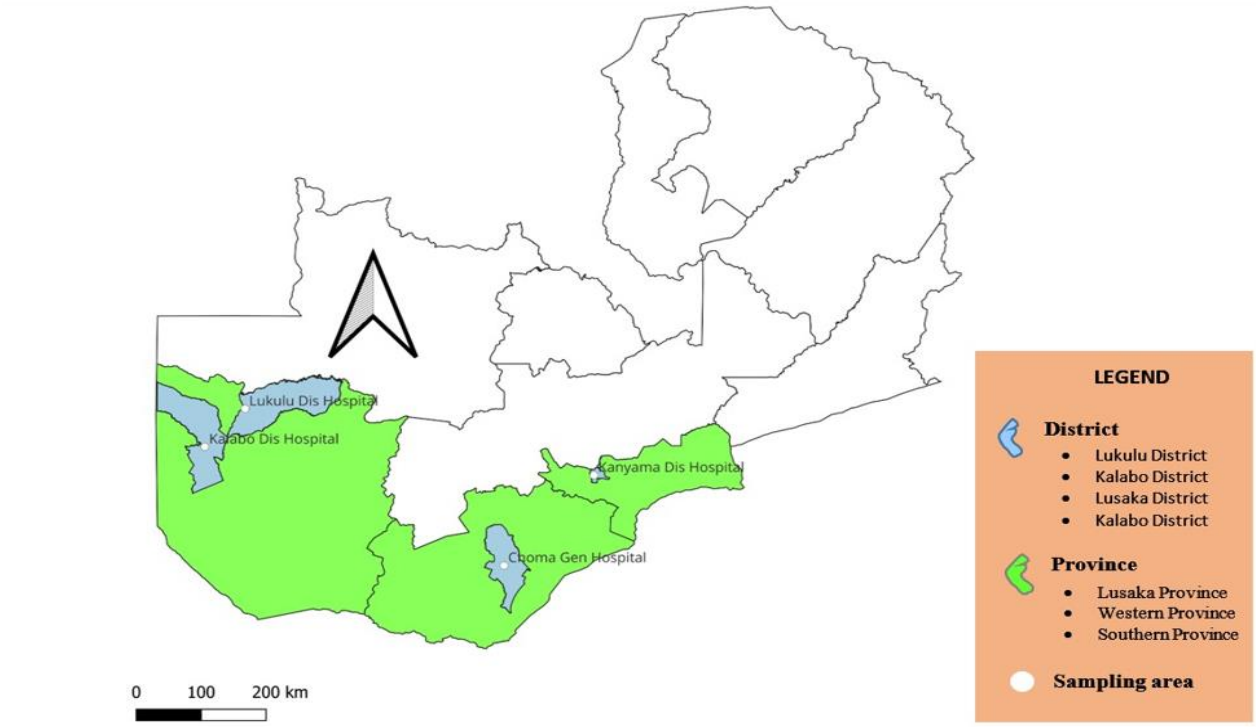

Figure 3.1: Map of Zambia highlights RVF study areas, with Lusaka, Western, and Southern Provinces in green. Sampling districts in blue include Lukulu, Kalabo, Lusaka, and Choma, with hospitals marked by white circles. A scale (0–200 km) and a north arrow provide spatial reference.

3.2 Study Design

This was a retrospective cross-sectional study, targeting archived patient samples collected between March 2024 to September 2024 from Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, and Kalabo and Lukulu District Hospitals in Western Province, Zambia.

3.3 Sample Size Determination

The number of samples analysed was estimated using a specific statistical formula to ensure accurate representation. (Robert V. Krejcie university of minnesota, Duluth, 1970) below:

$$\boldsymbol{n = Z^2 \frac{P(1-P)}{E^2}}$$

Where;

n = estimated sample size

Z = table value of the chi squire of 1 degree freedom at the desired confidence level (95% = 1.96)

P = the prevalence of 50% was used in the calculation of sample size because we could not find the prevalence of RVF in literature particularly focusing on Hospital patients in Zambia; p was set at 0.5 to get the maximum sample size.

E = is the margin of error mostly estimated based on the desired confidence level (5%, 0.05).

$$\boldsymbol{n = Z^2 \frac{P(1-P)}{E^2}}$$

$$n = \frac{1.96^2 \times 0.5\ (1-0.5)}{0.05^2}$$

$$n = \frac{1.96^2 X 0.5 \times 0.5}{0.0025}$$

$$n = \frac{0.9604}{0.0025}$$

n=384.16

n=384.

Therefore, the estimated number of samples was **384**.

3.4 Inclusion Criteria

All archived human serum samples that were previously collected and stored at -30°C in the laboratory for routine serological investigations were included in the study.

3.5 Exclusion Criteria

Human serum samples without patient clinical information were excluded from the study

3.6 Sampling Technique

This study utilized convenience sampling, obtaining archived human serum samples from selected hospitals, including Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, and Kalabo and Lukulu District Hospitals in Western Province, Zambia. Sample selection was based on availability, patient records, and storage conditions at -30°C. No new samples were collected; only previously stored specimens were used for analysis.

3.7 Sample preparation

Human serum samples were aseptically retrieved from archived specimens obtained from patients at Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, Kalabo and Lukulu District Hospitals in Western Province, Zambia. The total volume of serum collected was approximately 2 to 3 mL. The samples were transported on ice in cooler boxes to the Virology Laboratory, Disease Control Department, at the University of Zambia, School of Veterinary Medicine and stored at -80°C until analysis.

Before analysis, serum samples were thawed at room temperature, vortexed and spun down. Approximately 60 µL of each sample was transferred into a 96-well plate where they were heat-inactivated at 56°C for 30 minutes in a thermocycler. A 10X working serum was prepared by adding 50 µL of the sample into a cryovial tube containing 450 µL of sterile PBS. The serum samples for analysis were diluted to 1:50 by adding 10 µL of the working stock into 40 µL of PBS.

3.8 pCAGGS-Rift Valley Fever Virus - NP Cloning

3.8.1 RNA Extraction from Rift Valley Fever Virus

The Rift Valley fever virus RNA used for amplification and cloning was obtained from a positive sample stored in the UNZAVET biobank. Viral nucleic acid was extracted using the QIAamp®

Viral RNA Mini Kit, and its concentration and purity were assessed using a Nanodrop spectrophotometer.

3.8.2 cDNA Synthesis of Rift Valley Fever

The extracted RNA served as a template for complementary DNA (cDNA) synthesis through reverse transcription using Superscript IV Reverse Transcriptase (RT). The reaction included 50 µM random primers, with 5 µL of extracted viral RNA added to 15 µL of SuperScript master mix, resulting in a total reaction volume of 20 µL. The thermal cycling conditions consisted of an initial denaturation at 98°C, followed by 30 cycles of denaturation at 98°C, annealing at 55°C, and extension at 72°C. A final extension step was carried out at 72°C for five minutes, followed by a 4°C holding temperature. Successful amplification was confirmed through electrophoresis on a 1% agarose gel. The target DNA band was then excised and purified using a gel extraction kit.

3.8.3 Amplification of the Rift Valley Fever-N Gene

Rift Valley Fever Virus-specific primers, incorporating the restriction sites EcoRI and NheI, were used to amplify the N gene under optimized thermal cycling conditions. The PCR reaction was performed with an initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at an optimized temperature 54°C) for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The amplified products were then visualized using 1% agarose gel electrophoresis and examined under UV illumination to confirm successful amplification. The target DNA band was then excised and purified using a gel extraction kit.

3.8.4 Linearization of the cloning Vector (pCAGGS +)

The linearization of the cloning vector pCAGGS (+) was performed using restriction enzyme digestion. A reaction mixture was prepared by adding 3 µL of pCAGGS (+) (1 mg/mL), 1 µL each of EcoRI and NheI restriction enzymes, 5 µL of digestion buffer and 40 µL of nuclease-free water to achieve the desired reaction volume of 50 µL. The mixture was vortexed briefly to ensure proper mixing, followed by a quick spin down to collect the contents at the bottom of the tube. The reaction was incubated at 37°C for two hours to allow complete digestion of the plasmid, resulting in the linearized vector ready for subsequent cloning steps.

3.8.5 Agarose Gel Electrophoresis and DNA Purification

To verify successful linearization of the cloning plasmid, a 1% agarose gel was prepared, and the digested plasmid DNA was subjected to electrophoresis. The digestion product corresponding to the linearized plasmid was carefully excised using a sterile scalpel and purified using NucleoSpin kit (Takara Bio, Japan, 2024).

3.8.6 Ligation using infusion Enzyme

The purified template was ligated with the linearized and purified vector using an infusion enzyme-based cloning strategy. A 10 µL reaction mixture was prepared by combining 2.3 µL of the insert, 1.5 µL of the vector, 2 µL of the infusion enzyme, and 4.2 µL of nuclease-free water. The mixture was thoroughly mixed and incubated at 50°C for 15 minutes to facilitate the ligation process. Following incubation, the reaction was immediately placed on ice to halt the enzymatic activity. The ligated product was stored at 30°C for subsequent transformation steps.

3.8.7 Transformation of Stellar Cells

To initiate the transformation, 1 µL of plasmid DNA was mixed with 20 µL of Stellar™ Competent Cells (Takara Bio, Japan, 2024). The mixture was subjected to heat shock at 42°C for 45 seconds to facilitate DNA uptake, followed by immediate cooling on ice for 2 minutes to stabilize the cells. Subsequently, 180 µL of Super Optimal Broth (SOC) medium was added to the mixture, and the suspension was incubated at 37°C with shaking at 180 rpm for 1 hour to allow cell recovery and plasmid expression. After incubation, 40 µL of the transformed mixture was plated onto LB agar supplemented with ampicillin and incubated overnight at 37°C to promote the growth of successfully transformed bacterial colonies.

3.8.8 pCAGGS-Rift Valley Fever Virus - NP colony confirmation

To screen for successful RVF-N constructs after a cloning experiment, colony PCR (Polymerase Chain Reaction) was performed using KOD polymerase™ (Takara Bio, Japan, 2024) following the manufacturer's instructions. The PCR master mix was prepared by combining 5 µL of KOD Master Mix, 0.3 µL of (10 µM) pCAGGS vector specific forward primer; (5'-CATCATTTTGGCAAAGAATTCGCCACCATGGACAACTATCAAGAGCTTGCG –3') and

pCAGGS reverse, 0.3 µL of (10 µM) pCAGGS vector specific reverse primer: (5'-GGCAGAGGGAAAAAGATCTGCTAGCTTAGGCTGCTGTCTTGTAAGCCTGAGCGGC – 3') and 4.4 µL of nuclease-free water, resulting in a total reaction volume of 10 µL.

The PCR was carried out under the following thermal cycling conditions: an initial denaturation step at 98°C for 2 minutes, followed by 40 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 5 seconds, and extension at 68°C for 8 seconds. A final hold at 4°C was maintained until the completion of the reaction. The amplified products were then visualized using 1% agarose gel electrophoresis for successful RVF-N constructs.

3.8.9 pCAGGS-Rift Valley Fever Virus - NP plasmid constructs purification

Plasmid from the stellar culture cells was purified using the Miniprep protocol, following the manufacturer's guidelines. (Takara Bio, Japan, 2024). Transformed Steller colonies were collected, centrifugation, and the resulting cell pellets were lysed using lysis buffer. The lysate was further neutralized and centrifuged to remove cellular debris. The plasmid DNA was subsequently captured on a silica column, thoroughly washed to remove contaminants, and ultimately eluted in nuclease-free water.

3.8.10 Sanger sequencing of pCAGGS-Rift Valley Fever Virus - NP construct

The purified plasmid was subjected to Sanger sequencing to confirm the presence of the NP insert. The sequencing reaction included 1.0µL BigDye, primer forward 1µL (3.2µm), 3.5µL (5x) sequencing buffer, 3µL template and 11.5µL of Nuclease Free Water resulting in a total reaction volume of 20µL. The BigDye cycle sequencing was run using the standard conditions; an initial denaturation step at 96°C for 2 minutes, followed by 25 cycles of denaturation at 96°C for 30 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 2 minutes. A final hold at 4°C was maintained until the completion of the reaction. The sequences were verified by performing a BLAST search in the National Centre for Biotechnology Information (NCBI) database.

3.8.11 Amplification of Plasmid construct for transfection

After sequence verification, the plasmid was amplified by bacterial culture to obtain the optimal concentration for transfection (> 1000 ng/µL). To achieve this, colonies with the correct construct

was cultured in 50-100 mL LB broth overnight at 37°C with shaking at 180 rpm. The plasmid was purified following the Midiprep protocol (Takara Bio, Japan, 2024) according to the manufacturer's instructions. The cells were pelleted by centrifugation, followed by lysis using lysis buffer. The lysate was neutralized and centrifuged to remove cellular debris. Plasmid DNA was purified using a silica-based column, eluted, precipitated, and resuspended in nuclease-free water. The final purified plasmid was quantified using Nanodrop spectrophotometry. The purity ratio (2.0) was determined to ensure minimal contamination from proteins or RNA.

3.8.12 Optimization of Transfection and IFA for Gene Expression in Vero E6 Cells

The efficiency of DNA plasmid transfection can vary depending on the choice of transfection reagent. To optimize this process, a comparative analysis was conducted between TransIT-LT1 and X-tremeGENE HP DNA transfection reagents. Vero E6 cells were seeded in a 12-well plate, reaching approximately 60-70% confluency at the time of transfection. A 100 µl transfection mixture was prepared for each well using different reagent conditions, including TransIT-LT1 at ratios of 1:3 and 1:5, and X-tremeGENE HP DNA at ratios of 1:1 and 1:3. Each mixture contained 100 µl of Opti-MEM, 1.08 µl of pCAGGS-RVFV-NP, and the respective transfection reagent in specified volumes. The mixture was incubated at room temperature for 30 minutes before being added dropwise to the cells, which were gently rocked and incubated at 37°C for 48 hours to allow gene expression.

The spent medium in 6-well cell culture plates was removed, and the cells were washed twice with PBS. A 0.025% trypsin solution with EDTA was then added followed by incubation at 37°C for 1-3 minutes. 2 mL of Dulbecco's Modified Eagle Medium (DMEM) / 2% FBS was added to re-suspend the cells. A cell count was performed to adjust the cell concentration to 1.5×10^5 cells/ml. The cells were seeded onto a 24-well slide at 20 µL per well (Figure: 3). The slides were placed into a cell culture dish, and incubated in a 5% CO_2 incubator at 37°C overnight.

Antigen Slide Preparation

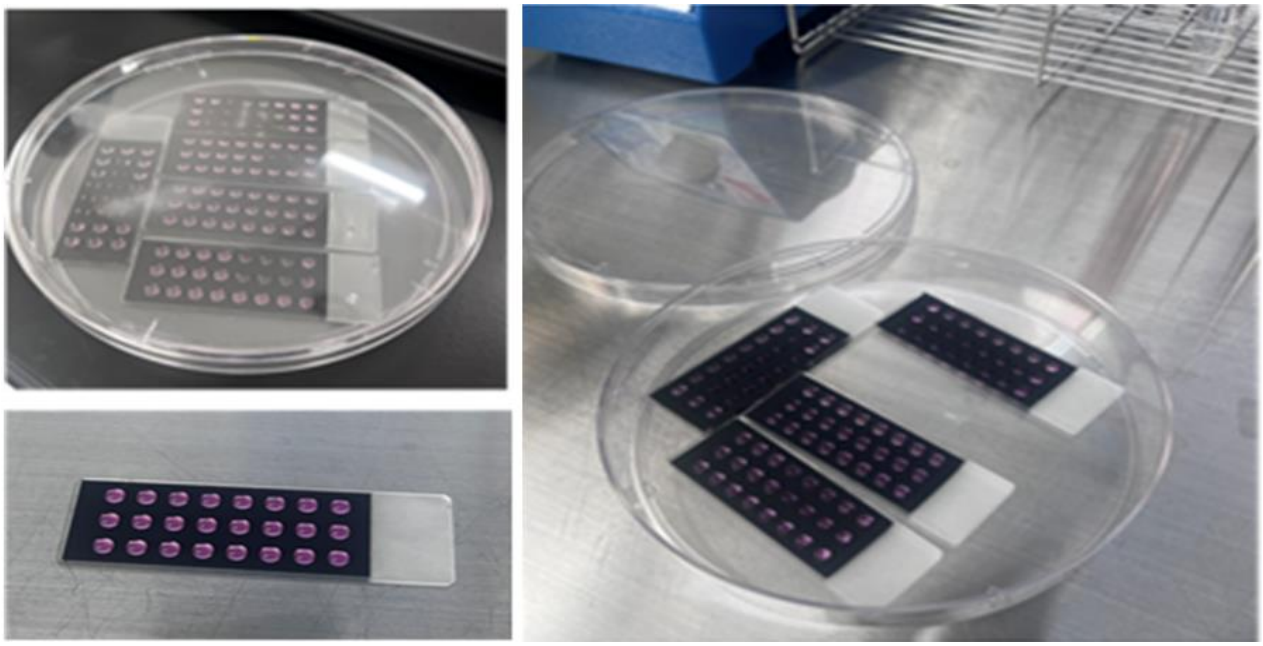

Figure: 3.2 Antigen slide preparation showing the black IFA slides containing multiple wells where transfected Vero E 6 cells were pre-fixed (seeded) and placed inside a petri dish with a lid to protect them from contamination and drying out prior to incubation.

Following incubation, IFA staining was performed to assess RVFV-NP expression. The cell culture medium was discarded, and the cells were fixed by adding ice-cold absolute methanol (200-300 µl per well) and incubating at -30°C for 10 minutes. The cells were washed three times with PBS, dried, and blocked with 200 µl of 50% Block ACE (Biorad, USA) in PBS for one hour at room temperature. After blocking, the primary antibody, rabbit anti-RVFV-NP, was added at dilutions of 1:300 and 1:3000, followed by overnight incubation at 4°C. The next day, cells were washed and incubated with the secondary antibody, Alexa Fluor-488 goat anti-rabbit IgG (1:1000) (Thermo Fisher Scientific), along with Hoechst 33342 (1:100) for nuclear staining. After one hour of incubation in the dark, cells were washed and analyzed under a fluorescence microscope.

3.8.13 screening for Rift Valley Fever Virus antibody in human serum samples

A 20 µL of the serum samples (1:50) was added onto each well of a 24-well antigen slide and incubated at room temperature. After 1 hour, the serum was discarded into a disinfectant reagent

(sodium hypochlorite), and the slide was washed in PBS three times for 3 minutes each. The slide was allowed to air dry.

Subsequently, 20µL of a 1:1000 dilution of the secondary antibody (Alexa Fluor™ 488 goat anti-human IgG; Invitrogen) in PBS was added, and the slide was incubated in the dark at room temperature for 30 minutes. The secondary antibody was discarded, and the slide was washed in 0.1% Tween-20 in PBS three times for 5 minutes each. The glass cover slip was mounted on the slide with 90% glycerol in PBS. Excess fluid was discarded and the edges of the cover slip were sealed with transparent nail polish. Finally, the fluorescence signal was examined using a fluorescence microscope.

3.9 IgG competition multi-species ELISA

Samples that tested positive on IFA were subjected to further testing using the ID Screen® Rift Valley Fever Competition Multi-species ELISA**,** a commercial indirect competition ELISA kit, as a confirmatory test.

All reagents were allowed to come to room temperature and mixed by inversion before use. Serum samples were diluted in plates by adding 50 µL of Dilution Buffer-19 to each well of the dilution plates, followed by 50 µL of either control sera or test sera. A 50 µL volume of each test sample was transferred to pre-coated test plates using a multichannel pipette.

The plates were incubated for 1 hour at 37°C, followed by three washes with 300 µL per well of washing buffer. A 100 µL of the Anti-RVF-NP-HRP conjugate, diluted in Dilution Buffer 19 at a 1:10 ratio, was added to each well. The plates were incubated at room temperature (21ºC ± 5ºC) for 30 minutes. The wells were emptied and washed three times with 300µl of the Wash Solution. A 100 µL of ready-to-use TMB substrate was added to each well and incubated in the dark at room temperature for 15 minutes. Finally, 100 µL of stop solution was added to each well to stop the reaction. The resulting discolouration depended on the quantity of the specific anti-bodies present in the sample tested. Where there was absence of anti-bodies, a blue solution appeared and turned yellow upon addition of the stop solution while in the presence of anti-bodies no coloration occurred. The microplates were then read at 450nm Optical Density (OD) with a microplate Merilyzer EIAQuant Enzyme Immunoassay Plate reader (Meril Diagnostic Pvt. Supplies Limited, Gujarat, India) with an inference filter of 405 nm to determine the results.

All tests conducted were recorded on a plate map. The test was considered valid if the mean optical density of the positive control (ODPC) was less than 30% of the negative control (ODNC), represented as [ODPC/ODNC < 0.3], and if the mean optical density of the negative control (ODNC) was greater than 0.7, represented as [ODNC > 0.7]. All runs met these validity criteria. The results were interpreted by calculating percentage inhibition (competition) using the following formula:

Suspect or negative (S/N) = (OD Sample / OD Negative Control) ×100

An S/N value of ≤ 40% was considered positive, an S/N value between 40% and 50% (40% < S/N ≤ 50%) was classified as doubtful, and an S/N value greater than 50% was considered negative.

3.10 Sensitivity and Specificity of IFA

The sensitivity and specificity of the IFA were evaluated by comparing its results to a reference standard, ELISA. Sensitivity was determined by calculating the proportion of true positive samples correctly identified by IFA, reflecting its ability to detect individuals with antibodies against the target pathogen. Specificity was assessed by measuring the proportion of true negative samples accurately classified by IFA, indicating its effectiveness in excluding individuals without the antibodies. The results of both assays were organized in a 2×2 contingency table, categorizing samples as true positives, true negatives, false positives, and false negatives. Sensitivity and specificity were then computed using standard formulas, where sensitivity was calculated as (True Positives / [True Positives + False Negatives]) × 100, and specificity as (True Negatives / [True Negatives + False Positives]) × 100 (Table 3.1).

However, before concluding that IFA was an entirely flawless test, additional statistical validation methods were considered. One such method was the calculation of the confidence interval (CI) for sensitivity and specificity, which was determined using the Wilson score method (Lady Margaret, 2022) at a 95% confidence level.

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

Where:

$\boldsymbol{p}$ = proportion (sensitivity or specificity)

$\boldsymbol{n}$ = total number of positive or negative cases

1.96= corresponds to the 95% confidence level (standard normal distribution value).

Accuracy was determined using the formula: Accuracy = (TP+TN / TP+FP+FN+TN) × 100%. Additionally, Cohen's Kappa (Tan *et al.*, 2024) was employed to measure the level of agreement between IFA and ELISA while adjusting for chance agreement. The formula used was $\kappa = (Po - Pe) / (1 - Pe)$, where Po (Observed Agreement) was calculated as TP+TN / Total, and Pe (Expected Agreement by Chance) was determined using the formula: (TP+FP) (TP+FN) + (TN+FN) (TN+FP) / Total^2.

This analysis provided insights into the diagnostic performance of IFA in comparison to ELISA, ensuring the reliability of its use in serological surveillance.

Table 3.1: 2x2 contingency table

IFA Result	ELISA Positive (Reference Test Positive)	ELISA Negative (Reference Test Negative)	Total
IFA Positive	True Positives (TP)	False Positives (FP)	TP + FP
IFA Negative	False Negatives (FN)	True Negatives (TN)	FN + TN
Total	TP + FN	FP + TN	TP + FP + FN + TN

3.11 Data Management and Analysis

Data management and analysis were systematically performed to ensure accuracy and reliability in the results of both the IFA and ELISA. Immunofluorescence assay data were recorded qualitatively, with samples categorized as positive or negative based on fluorescence intensity or titre levels. Variations in fluorescence intensity among positive samples were assessed to compare IFA results.

ELISA data were documented as quantitative values, with OD readings used to determine antibody concentrations. The data were analysed by calculating mean OD values and standard deviations, allowing for a clear distinction between positive and negative samples based on predefined cut-off thresholds.

The data for sensitivity and specificity was managed by organizing IFA and ELISA results into a 2×2 contingency table. Data analysis was done by calculating sensitivity as the proportion of true positives correctly identified and specificity as the proportion of true negatives accurately classified. Standard formulas were applied, and confidence intervals were determined using the

Wilson score method at a 95% confidence level. Additional validation included accuracy calculations and Cohen's Kappa to measure agreement with ELISA.

Data cleaning for RVF seroprevalence and risk factor determination was performed using Microsoft Excel, and imported into STATA version 15 for analysis using predefined statistical commands. Descriptive statistics were used to summarize the data. For bivariate analysis, associations between potential risk factors and the presence of RVFV antibodies were assessed using the Chi-square test and Fisher's exact test, depending on each test assumptions (Hosmer & Lemeshow, 2000). Univariate logistic regression was performed to compute crude odds ratios (cOR) with their 95% confidence intervals (CI) for variables linked to RVF infection. Multivariate logistic regression to obtain adjusted odds ratios (aOR) was then conducted to determine independent risk factors for RVF seropositivity, including variables that were statistically significant in bivariate analysis.

To build the final multiple logistic regression models, an investigator-led stepwise regression approach was used. The final model was selected based on the Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC), with the model having the smallest AIC and BIC values chosen as the best fit. Both Crude (cOR) and adjusted odds ratios (aOR) with their 95% confidence intervals (CI) were reported. A p-value < 0.05 was considered statistically significant.

3.12 Ethical Consideration

Ethical approval was obtained from ERES Converge IRB, Ref. No. 2024-Nov-030 (Appendix 8.1.2) and from National Health Research Authority, Registration number NHRAR-R-14/04/2024 (Appendix 8.2) in Lusaka, Zambia. The permission letter to collect human serum samples was obtained from Ministry of health Ndeke House Lusaka, Zambia. Additionally, permission to proceed with the sample collection was made from the District Health Director's office and to the hospital in-charge upon presenting the permission letter obtained from the ministry of health. To maintain participant anonymity, sample identification numbers and not patients' names were utilized. Access to study results was restricted to authorized personnel only, and findings will be shared with relevant stakeholders, including health authorities and participating institutions, to support public health interventions and future research.

CHAPTER FOUR

RESULTS

4.1 Cloning the NP gene of Rift Valley Fever Virus in pCAGGS vector

The NP gene of RVFV was successfully cloned into the pCAGGS expression vector following a series of molecular techniques as shown below;

4.1.1 RNA Extraction and Amplification of the Rift Valley Fever-N Gene

The extracted viral RNA from the positive RVFV sample showed good yield and purity, with the ratio of 2.0, confirming minimal protein contamination. Reverse transcription of the extracted RNA successfully generated cDNA, which was amplified using PCR. Agarose gel electrophoresis revealed a distinct DNA band of ~738bp (Figure 4.1), corresponding to the expected size of the RVFV-N gene.

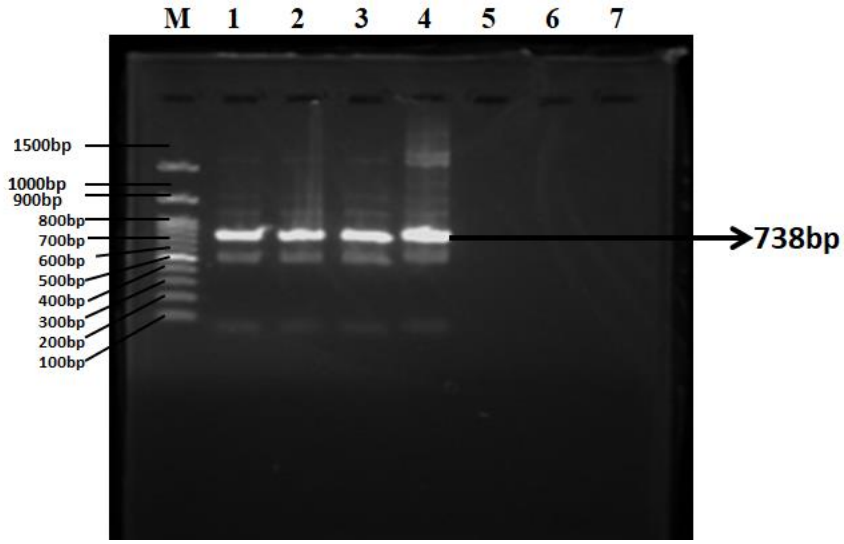

Figure 4.1: Agarose gel electrophoresis showing RVF N gene amplification Lane M: Ladder, lane 1 – 3: RVF-N plus primers, lane 4: RVF-N Positive control, Line 5: Negative control.

4.1.2 Linearization and purification of the Cloning Vector (pCAGGS +)

Restriction digestion of the pCAGGS (+) vector with EcoRI and NheI enzymes resulted in complete linearization, as confirmed by gel electrophoresis (Figure 4.2). It was observed that 3

reactions (Lane 1-3) which had the restriction enzymes had a short migration distance compared to the reaction that had no enzyme, indicating successful digestion.

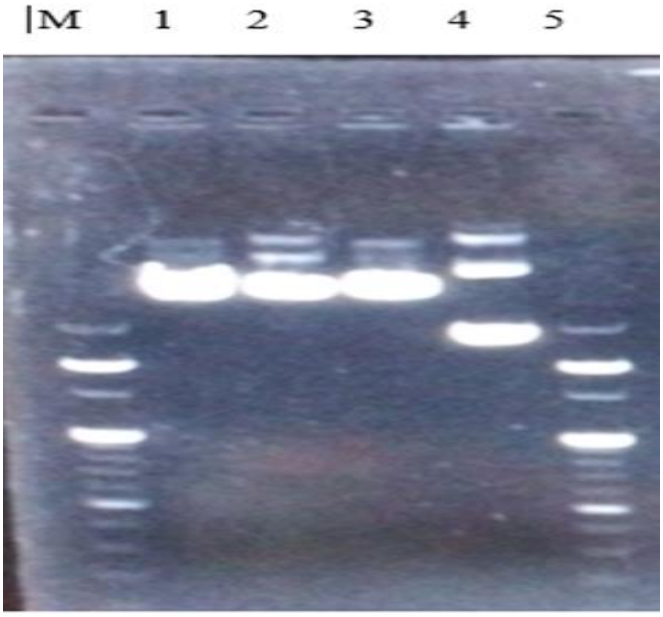

Figure 4.2: Agarose gel showing restriction enzyme digestion of pCAGGS. Marker (M), lane 1: pCAGGS- NheI, lane 2: pCAGGS- EcoRI + NheI, Lane 3: pCAGGS- EcoRI, Lane 4: pCAGGS-control, Lane 5: marker

Following electrophoresis, the linearized vector DNA was excised from the gel and purified. The purified DNA showed a concentration of 154 ng/µL and the ratio of ~2.0, confirming high purity suitable for ligation. The RVFV-NP gene was successfully ligated into the linearized pCAGGS (+) vector using the Infusion enzyme-based cloning strategy. The ligation reaction yielded a construct with high efficiency, as confirmed by subsequent transformation and colony screening.

4.1.3 Transformation of Stellar Cells

Following heat-shock transformation, bacterial colonies were observed on ampicillin-supplemented LB agar plates after overnight incubation at 37°C.

4.1.4 pCAGGS-Rift valley Fever Virus - NP Colony Confirmation

The PCR products were visualized using 1% agarose gel electrophoresis to verify the expected amplicon size of 738bp in positive colonies (Figure 4.3). Negative control colonies without insert (Lane 9) did not yield any amplification. Therefore this showed that the insert was in the correct orientation. Sequence alignment with the reference RVFV-NP gene, accession number KY926697 showed 97.7% identity, confirming the RVF-N gene with no detected mutations.

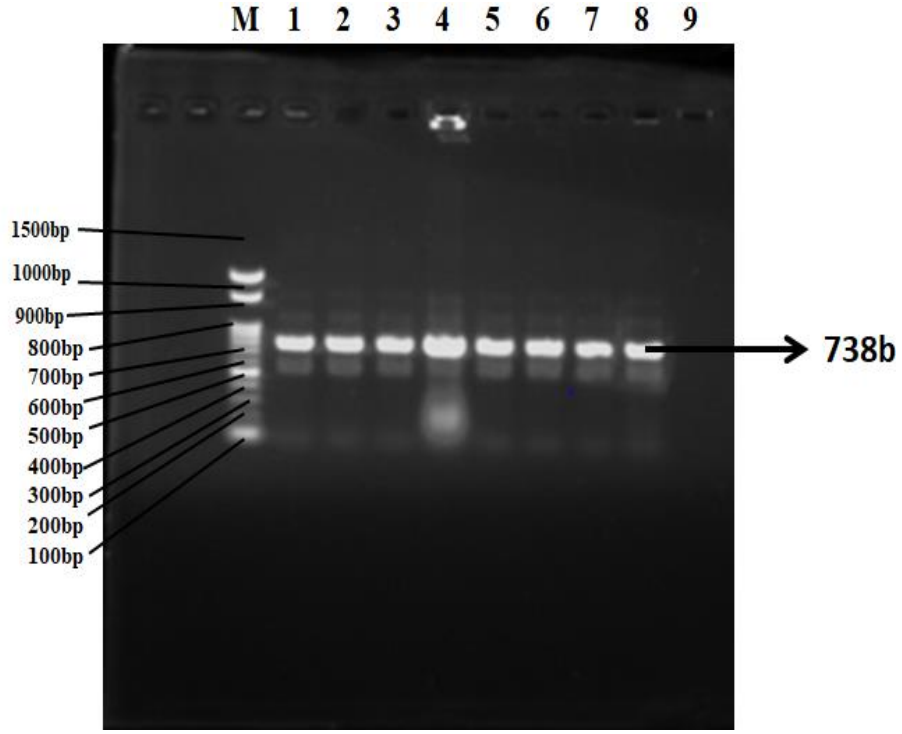

Figure: 4.3 Agarose gel electrophoresis showing RVF N gene amplification Lane M: Ladder, lane 1 – 8 Colony, Lane 9: Negative control.

4.1.5 Optimization of Transfection and IFA for Gene Expression in Vero E6 Cells

4.1.5.1 Optimization of Transfection Efficiency

The comparative analysis between TransIT-LT1 and X-tremeGENE HP transfection reagents was conducted to determine the most effective condition for gene expression (Figure 4.4).

The results indicate that increasing the transfection reagent did not significantly enhance transfection efficiency. Images C and D depict X-tremeGENE HP transfection at different ratios,

with Image C representing a 1:1 ratio and Image D a 1:3 ratio. Among all tested conditions, X-tremeGENE HP at a 1:3 ratio (Image D) demonstrated the highest transfection efficiency, achieving approximately 50% gene expression. This was observed through the presence of green fluorescence, indicating successful RVFV-NP expression. Blue fluorescence corresponds to nuclear staining, confirming cell viability. Based on these findings, the X-tremeGENE HP (1:3) condition was selected for antigen slide preparation due to its superior performance.

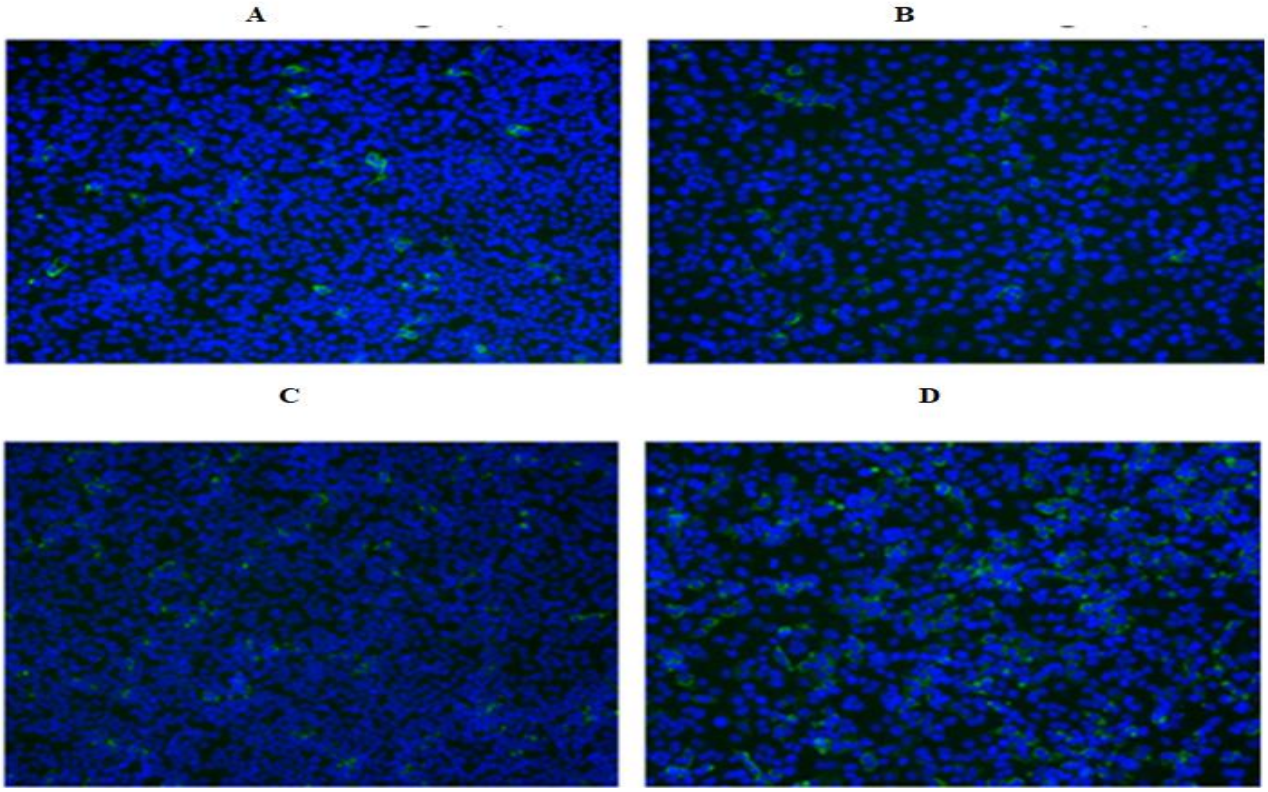

Figure 4.4: Immunofluorescence images showing RVFV-NP expression. Images A and B show TransIT-LT1 transfection at 1:3 (1 µg plasmid + 3 µl reagent) and 1:5 ratios, respectively, while Images C and D depict X-tremeGENE HP transfection at 1:1 and 1:3 ratios.

4.1.5.2 Immunofluorescence of Rift Valley Fever Virus -N Antibodies in Human Serum Samples

Out of the 593 tested serum samples, 10/593 (~1.7%) exhibited positive fluorescence signals, indicating the presence of RVFV-specific IgG antibodies. These positive results were further confirmed using a competitive ELISA (cELISA), reinforcing the reliability of both methods. Figure 4.5 presents representative immunofluorescence images to validate the findings. Image A serves as the negative control, where no serum was added, ensuring that any observed fluorescence in the

experiment resulted from specific RVFV antibody binding rather than non-specific interactions or autofluorescence. Image B represents the positive control, where a known reagent, Rabbit Anti-RVFV-NP, was used to bind specifically to the RVFV nucleocapsid protein, producing clear fluorescence and confirming the assay's performance. Image C shows a known negative human serum sample, which displayed minimal fluorescence due to the absence of RVFV antibodies. In contrast, Image D illustrates a known positive human serum sample, which exhibited strong fluorescence, indicating the presence of naturally occurring RVFV-specific antibodies. This differentiation between the synthetic rabbit antibody in the positive control and the naturally occurring human antibodies in the tested samples highlighted the effectiveness of IFA in detecting RVFV seropositivity.

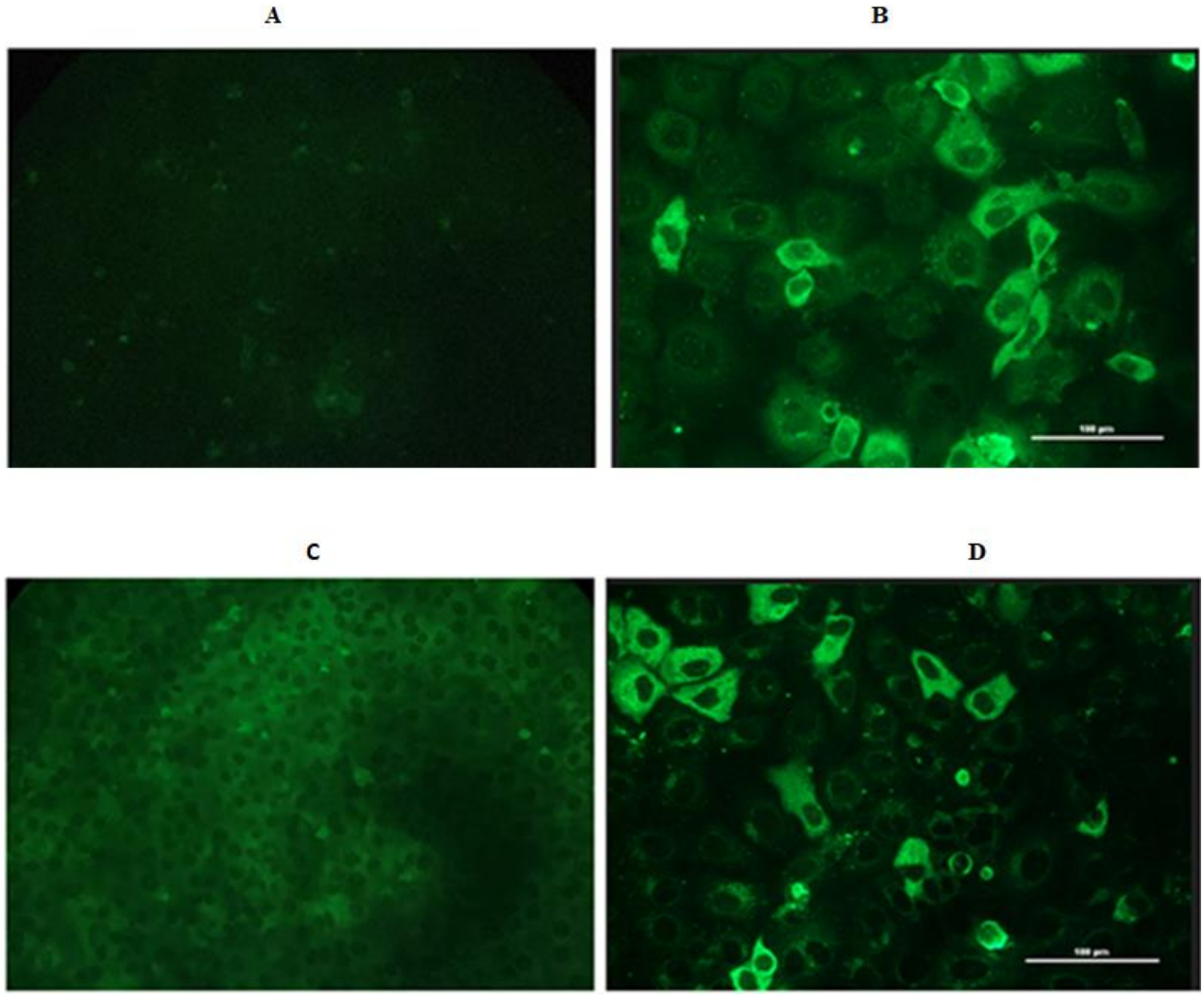

Figure 4.5: Immunofluorescence assay (IFA) images for RVFV antibody screening.

Image A Negative control, B positive control, C Negative serum sample and D Known positive serum sample

4.1.6 Sensitivity and Specificity of IFA results

The 2x2 contingency table illustrated the distribution of IFA and ELISA results. True Positives (TP) referred to cases where both IFA and ELISA yielded positive results. False Negatives (FN) indicated IFA negative, but ELISA positive. True Negatives (TN) encompassed cases where both IFA and ELISA results were negative (Table 2).

Table 4.1: 2×2 contingency table showing results for both IFA and ELISA

IFA Result	**cELISA (Reference Test)**		**Total**
	Positive	**Negative)**	
IFA Positive	10	0	10
IFA Negative	0	583	583
Total	**10**	**583**	**593**

The sensitivity result obtained was 100%, indicating that the IFA test correctly identified all individuals with antibodies detected by ELISA.

Similarly, specificity was determined and the results also yielded 100%, showing that the IFA test accurately excluded all individuals without antibodies, meaning no false positives were recorded. After the Wilson score determination, both sensitivity and specificity were found to be perfect at 100%. The 95% confidence intervals also remained at 100%. Further validation conducted by accuracy and Cohen's Kappa, showed 100% IFA detection while Cohen's Kappa (κ) demonstrated 1.0, indicating perfect agreement between the two tests.

4.2 Demographic characteristics

The total of 593 participants was derived from the archived human serum samples collected for the study. Of these, 46.9% (278) (were male, while 53.1% (315/593) were female. The majority of participants 63.6% (377/593) were from the Outpatient Department (OPD), indicating that most cases were managed in an outpatient setting without requiring prolonged hospitalization. A total of 19.6% (116/593) were admitted as in-patients.

Additionally, 16.9% (100/593) were from the unit, which primarily focuses on maternal, neonatal, and child health services. This indicates that a portion of the study population included pregnant women, postpartum mothers, and children. Regarding occupational distribution**,** employed 52.5% (311/593) formed the largest group, followed by unemployed 33.6% (119/593)**,** while farmers 14% (83/593) constituted the smallest group. This distribution suggests that the study population was more engaged in formal employment than in farming.

The most frequently reported symptoms were headache 88% (522/593)**,** joint pain 68.5% (406/593)**,** and general malaise 67.3% (399/593), all of which are non-specific symptoms that could be associated with various infections. Photophobia 4.4% (26/593) was the least reported symptom, followed by vomiting 18.9% (112/593)**.** Flu-like fever was present in 43.2% (256/593) of participants, while diarrhea 48.7% (256/59) was relatively common, and vomiting 18.9% (112/593) was less frequent.

4.2.1. Seroprevalence of Rift Valley Fever according to Demographic, study site and occupational characteristics

The analysis revealed several statistically significant associations in the seroprevalence of Rift Valley Fever (RVF) (Table 3). Geographic location played a crucial role in seropositivity, with Western Province having the highest number of positive cases 1.01% (6/593), while Lusaka and Southern Province, despite contributing the largest sample sizes, recorded the lowest proportion of seroprevalence 0.34% (2/593) each.

A similar trend was observed at the facility level, where Lukulu District Hospital recorded the highest number of seroprevalence 0.84% (5/593), followed by Choma General Hospital and Kanyama Hospital with 0.34% (2/593) each, and Kalabo District Hospital with 0.17% (1/593). Occupation emerged as a significant factor in RVF seroprevalence, with farmers being the most affected group, with 1.35% (8/593) seroprevalence. In contrast, employed and unemployed individuals exhibited considerably lower seroprevalence of 0.17% (1/593) each.

Among clinical symptoms, not having photophobia was significantly associated with RVF seropositivity ($p = 0.007$).

Table 4.2 Seroprevalence of Rift Valley Fever according to Demographic, study site and occupational characteristics

Variable	Number Sampled	RVF		p-value
		Negative	Positive	
Overall				
N= 593	593	583 (98.3%)	10 (1.7%)	
Age Group				
≤25	289	284 (47.9%)	5 (0.84%)	
26-35	185	183 (30.9%)	2 (0.34%)	0.401
36-45	75	74 (12.5 %)	1 (0.17%)	
≥46	44	42 (7.1%)	2 (0.34 %)	
Gender				
Male	278	272 (45.9%)	6 (1.01%)	0.527
Female	315	311 (52.4%)	4 (0.67%)	
Province/ City				
Lusaka	366	364 (61.4%)	2 (0.34%)	
Southern	152	150 (25.3%)	2 (0.34%)	**0.000**
Western	75	69 (11.6%)	6 (1.01%)	
Facility/ Address				
Choma General Hospital	152	150 (25.3%)	2 (0.34%)	
Kalabo District Hospital	313	30 (5.1 %)	1 (0.17%)	**0.000**
Kanyama Hospital	366	364 (61.4%)	2 (0.34%)	
Lukulu District Hospital	44	39 (6.6%)	5 (0.84%)	
Section/Unit				
In-patient	116	112 (18.9%)	4 (0.67%)	
MCH	100	98 (16.5%)	2 (0.34%)	0.158
OPD	377	373 (62.9%)	4 (0.67%)	
Occupation				
Employed	311	310 (52.3%)	1 (0.17%)	
Farmers	83	75 (12.6%)	8 (1.35%)	**0.000**
Unemployed	199	198 (33.4%)	1 (0.17%)	
Fever				
Yes	256	249 (41.9%)	7 (1.18%)	0.08
No	337	334 (56.3%)	3 (0.51%)	

Variable	Number Sampled	RVF		p-value
		Negative	Positive	
Headache				
Yes	522	513 (86.5%)	9 (1.52%)	1.000
No	71	70 (11.8%)	1 (0.17%)	
Joint pain				
Yes	409	397 (66.9%)	9 (1.52%)	0.183
No	187	186 (31.4%)	1 (0.17 %)	
General Malaise				
Yes	399	391 (65.9%)	8 (1.35%)	0.511
No	192	192 (32.4%)	2 (0.34%)	
Photophobia				
Yes	26	23 (3.9%)	3 (0.51%)	**0.007**
No	567	560 (94.4%)	7 (1.18%)	
Diarrhea				
Yes	289	282 (47.6%)	7 (1.18%)	
No	304	301 (50.8%)	3 (0.51%)	0.212
Vomiting				
Yes	112	110 (18.5%)	2 (0.34%)	1.000
No	481	473 (79.8%)	8 (1.35%)	

4.2.2. Risk Factors Associated with Rift Valley Fever Infection

The multivariate analysis revealed several statistically significant factors associated with Rift Valley Fever (RVF) seropositivity. Age was an important determinant, with participants aged ≥46 years having significantly lower likelihood of seropositivity after adjustment (OR = 0.04, p = 0.049) (Table 4.1). Geographical differences were evident, as participants from Western Province had significantly higher odds of RVFV seropositivity compared to those in Lusaka (cOR = 15.83, 95% CI: 3.13 - 80.04, p = 0.001). Participants from Lukulu District Hospital had significantly increased odds of being seropositive compared to those from Choma General Hospital (cOR = 9.62, 95% CI: 1.79 - 51.45, p = 0.008). This association remained significant after adjustment (aOR = 10.50, 95% CI: 1.23 - 97.97, p = 0.039), confirming that participants from Lukulu had a higher risk

of RVFV infection. The study sites, including Kanyama Hospital, Choma General Hospital, Kalabo District Hospital, and Lukulu District Hospital, did not exhibit significant associations.

Occupation also played a key role in RVFV seropositivity. Farmers were used as the reference group, and both unemployed (aOR = 0.02, 95% CI: 0.00 - 0.25, p = 0.003) and unemployed (aOR = 0.01, 95% CI: 0.00 - 0.23, p = 0.004) had a significantly lower risk of infection.

After adjusting for confounders, not having photophobia remained significant (aOR = 0.89, 95% CI: 0.01 - 0.98, p = 0.048). Other symptoms, including joint pain, diarrhea, flu-like fever, headache, and vomiting, were not significantly associated with seropositivity in both crude and adjusted analyses.

Table: 4.3 Determining the Risk Factors Associated with Hospital-Based Patients for Rift Valley Fever Infection

	Unadjusted		**Adjusted**	
Variables	**Odds ratios (95% CI)**	***P*-value**	**Odds ratios (95% CI)**	***P*-value**
Age Group				
≤25	Ref.			
26-35	0.62 (0.12 - 3.23)	0.571	0.21 (0.02 – 1.77)	0.151
36-45	0.77 (0.09 - 6.67)	0.810	0.11 (0.01 -0.98)	0.130
≥46	2.70 (0.51- 14.39)	**0.243**	**0.04** (0.00 - 0.98)	**0.049**
Gender				
Male	Ref.			
Female	0.58 (0.16 - 2.09)	0.407	Eliminated	
Province/ City				
Lusaka	Ref.			
Southern	2.43 (0.34 - 17.39)	0.378	Eliminated	
Western	15.83 (3.13 - 80.04)	0.001		
Facility/ Address				
Choma General Hospital	Ref.			
Kalabo District Hospital	2.5 (0.22 - 28.46)	0.460	1.29 (0.05 – 34.89)	0.876
Kanyama Hospital	0.41 (0.06 - 2.95)	0.378	3.88 (0.23 – 66.87)	0.350
Lukulu District Hospital	9.62 (1.79 - 51.45)	**0.008**	10.50 (1.23- 97.97)	**0.039**

Variables	Unadjusted		Adjusted	
	Odds ratios (95% CI)	***P*-value**	**Odds ratios (95% CI)**	***P*-value**
Section/Unit				
In-patient	Ref.			
MCH	0.57 (0.10 -3.19)	0.523	Eliminated	
OPD	0.30 (0.07 - 1.22)	0.994		
Occupation				
Farmers	Ref.			
Employed	0.03 (0.00 -0.25)	**0.001**	0.02 (0.00 - 0.25)	**0.003**
Unemployed	0.05 (0.01 - 0.39)	**0.004**	0.01 (0.00 - 0.23	**0.004**
Flu-Like Fever				
Yes	Ref.			
No	0.32 (0.08 - 1.25)	0.101	Eliminated	
Headache				
Yes	Ref.			
No	0.81 (0.10 - 6.52)	0.847	Eliminated	
Joint pain				
Yes	Ref.			
No	0.24 (0.03 - 1.89)	0.174	0.21 (0.01 – 2.99)	0.247
General Malaise				
Yes	Ref.			
No	0.51 (0.11 - 2.42)	0.396	Eliminated	
Photophobia				
Yes	Ref.			
No	0.09 (0.02 - 0.39)	**0.001**	0.89 (0.01 - 0.98)	**0.048**
Diarrhea				
Yes	Ref.			
No	0.40 (0.10 - 1.57)	0.189	0.16 (0.20 – 1.03)	0.054
Vomiting				
Yes	Ref.			
No	0.93 (0.19 - 4.44)	0.928	Eliminated	

CHAPTER FIVE

DISCUSSION

This study aimed to assess RVFV exposure among patients in selected hospitals in three provinces of Zambia: Lusaka (Kanyama Hospital), Southern (Choma General Hospital), and Western (Kalabo and Lukulu District Hospitals). Additionally, it sought to identify potential risk factors associated with RVFV exposure. To facilitate serological surveillance, the RVFV-NP gene was successfully cloned into the pCAGGS expression vector through a series of molecular techniques, including RNA extraction, reverse transcription, and PCR amplification, yielding a distinct 738bp band.

Restriction digestion using EcoRI and NheI confirmed complete linearization of the vector, followed by purification and ligation of the NP gene using an Infusion enzyme-based strategy. Transformation into Stellar cells was successful, and colony PCR confirmed correct insertion and was amplified for transfection, and Nanodrop analysis confirmed high purity.

Optimization of transfection efficiency in Vero E6 cells revealed that X-tremeGENE HP (1:3 ratios) yielded the highest efficiency (~50%) and was used for antigen slide preparation. IFA detected RVFV-NP expression, and of the 593 human serum samples screened, 1.7% (10/593) tested positive. IFA results were also 100% concordant with cELISA, confirming its reliability for RVFV serological surveillance. These findings highlight the successful cloning, expression, and application of rNP based- IFA for detecting RVFV antibodies in human serum, reinforcing its potential for epidemiological studies and targeted public health interventions (Takara Bio, Japan, 2024)

IFA was chosen as the primary method for detecting RVF antibodies in human serum samples due to its high specificity and ability to provide direct visualization of antigen-antibody interactions (Bio-Techne, 2014). The use of recombinant NP enhanced the assay's specificity by reducing cross-reactivity with other arboviruses, making it a reliable choice for hospital-based serological surveillance (Abraham *et al.,* 2010).

A separate study aimed at establishing an IFA for the detection of IgG antibodies against a new bunyavirus reported that, among 126 patients, 96 had paired serum specimens that tested positive, corresponding to an antibody-positive rate of 76.19%, while 30 patients tested negative. The

performance of IFA was similar to that of RT-PCR, which detected antibodies in 72.22% (91/126) of cases, with no significant difference between the two methods ($P > 0.05$). This study confirms that IFA offers high sensitivity and specificity along with operational simplicity (Xy *et al*., 2012).

A UK study optimized RVFV N protein gene insertion into pEAQ-HT vector for use in IFA (Sainsbury *et al.*, 2020). South African (Mbewana *et al.*, 2018) and Sierra Leonean (Borrega *et al.*, 2021) research contributed cost-effective RVFV diagnostics and optimized COVID-19 ELISA conditions, respectively. A Zambian study transfected RVFV NP gene into Vero E6 cells via plasmid, pursuing serosurveillance. Cells were cultivated on glass slides, fixed, stored for IFA. The advantage of this approach lies in its ability to produce a standardized and controlled antigen source for serosurveillance using Immunofluorescence Assay (IFA). By transfecting the RVFV NP gene into Vero E6 cells, the study ensured consistent expression of the Nucleocapsid protein, which is a key target for RVFV-specific antibodies in serum samples. Cultivating the cells on glass slides and fixing them allows for long-term storage and use in IFA, enabling rapid and reliable detection of RVFV antibodies. This method enhances serological surveillance efforts, particularly in resource-limited settings where access to live virus or commercially available antigens may be restricted (Saasa *et al.*, 2018).

The study found Lukulu District Hospital recording the highest seroprevalence 0.84% (5/593) followed by Kanyama Hospital and Choma General Hospital, both at 0.34% (2/593), while Kalabo District Hospital had the lowest prevalence 0.17% (1/593). The seroprevalence of RVF IgG antibodies observed at Lukulu District Hospital 0.84% (5/593) suggests a slightly higher exposure to RVFV compared to other study sites A Zambian study transfected RVFV NP gene into Vero E6 cells via plasmid, pursuing serosurveillance. Cells were cultivated on glass slides, fixed, stored for IFA. The advantage of this approach lies in its ability to produce a standardized and controlled antigen source for serosurveillance using Immunofluorescence Assay (IFA). By transfecting the RVFV NP gene into Vero E6 cells, the study ensured consistent expression of the Nucleocapsid protein, which is a key target for RVFV-specific antibodies in serum samples. Cultivating the cells on glass slides and fixing them allows for long-term storage and use in IFA, enabling rapid and reliable detection of RVFV antibodies. This method enhances serological surveillance efforts, particularly in resource-limited settings where access to live virus or commercially available antigens may be restricted (Saasa *et al.*, 2018). One possible explanation is that Lukulu's environmental conditions, including floodplains, wetlands, and livestock farming, create an ideal

habitat for mosquito vectors such as Aedes and *Culex* species, which are known to transmit RVFV (Velu *et al.*, 2023. However, it is important to note that the actual source of these cases remains uncertain, as there is no information on the travel history of the patients attended to at Lukulu Hospital. Some of these individuals may have been local residents, while others could have travelled from neighbouring towns or even from outside Zambia, potentially influencing the observed prevalence. Similar uncertainties exist for Kanyama and other study sites, as urban hospitals often serve diverse populations that include both residents and visitors. Additionally, while RVFV circulation in Zambia has not been well-established, more research is needed to assess vector presence and transmission dynamics in both rural and urban settings to better understand the epidemiology of the virus in the country. (Velu *et al.*, 2023).

Rift valley fever virus is primarily transmitted in livestock-human interfaces, where infected animals serve as an amplifying host, increasing occupational exposure among farmers (Kabali *et al.*, 2015)**.** Studies on vector competence in Zambia have shown that mosquito species in flood-prone areas are often infected with multiple arboviruses, including RVF, West Nile Virus, and Chikungunya Virus (Mubemba *et al.*, 2022). Additionally, livestock farming practices in Lukulu may play a role in the observed high seroprevalence**.**

The multivariate analysis in this study identified occupation as a significant factor, with farmers having an increased risk of RVF exposure. Given that Western Province has a substantial cattle population, and previous studies in Zambia have reported RVFV seroprevalence in animals (Dautu *et al.*, 2012), it is likely that RVF outbreaks in livestock have occurred unnoticed, leading to human exposure through direct contact with infected animal fluids or vector bites.

Comparing these findings to previous studies in Zambia, RVFV seroprevalence has been documented in both humans and animals, particularly in regions with livestock farming and wetland ecosystems (Kasongamulilo *et al.*, 2025). Past research on RVFV in Zambian cattle, goats, and sheep has shown seropositivity rates ranging from 3% to 30% (Saasa *et al.*, 2018), depending on the study site.

The observed seroprevalence rates of RVF antibodies at Kanyama Hospital and Choma General Hospital (both 0.34%) and Kalabo District Hospital (0.17%) highlighted regional variations in RVF exposure. These differences were likely influenced by several factors, including ecological conditions, vector presence. Kanyama Hospital, located in Lusaka, an urban area, was typically considered lower risk for RVF due to reduced livestock farming and fewer suitable vector habitats.

However, studies have identified urban risk factors such as the presence of goats near homes, consumption of raw milk, and animal slaughtering activities (Gerken *et al.*, 2022). These behaviours can facilitate RVF transmission even in city environments (Nicholas *et al*, 2014). Kanyama represents a high-density urban catchment area in Lusaka**,** serving a large and diverse population (2016 Central Statistical Office, Zambia), including both permanent residents and transient individuals from other towns or even neighbouring country. Given its socioeconomic and environmental conditions, including poor drainage, unplanned settlements, and proximity to livestock markets, the area could be at risk for vector-borne diseases like Rift Valley Fever. However, the circulation of RVFV in Zambia has not been well established, and there is limited data on past outbreaks or endemic transmission (Madzingira *et al.*, 2024).

Choma General Hospital, situated in a rural region with substantial livestock farming, also recorded a 0.34% seroprevalence. Rural areas with extensive livestock and favourable ecological conditions for mosquito vectors were generally more susceptible to RVF outbreaks. The high seroprevalence observed in this region aligned with findings from other African regions where livestock farming was prevalent (Msimang *et al.*, 2019). Kalabo District Hospital's lower seroprevalence of 0.17% may have been attributed to less favourable ecological conditions for mosquito vectors or differences in livestock farming practices, as most people in this area were primarily engaged in fishing activities. Comparing these findings to other studies in Zambia and Africa revealed that RVF seroprevalence varied widely, influenced by ecological zones, livestock practices, and vector presence. For instance, a study in Malawi reported seroprevalence rates ranging from 6.22% to 25.68% across different districts, with higher rates in areas with favourable ecological conditions for vectors (Kainga *et al.*, 2022).

Comparatively, a recent study in Central and Western Zambia reported an overall seroprevalence of 9.9%, with Sesheke district in Western Province having the highest seroprevalence at 19.23% and Chisamba district in Central Province showing the lowest at 1.41% (C. C. Kasongamulilo *et al.*, 2025a) . These findings suggest that RVF exposure varies significantly across regions, likely influenced by ecological differences, vector distribution, and human activities. Similarly, earlier studies conducted in Mazabuka and Lusaka reported a prevalence of 9.2%, indicating persistent RVF exposure in some parts of Zambia over time (Morita, 1988).

These variations in seroprevalence may be attributed to several key factors, including differences in study populations and methodologies. Unlike previous studies that focused on high-risk groups

such as livestock farmers, veterinarians, and individuals with direct exposure to livestock (Ndengu *et al.*, 2020), this study was hospital-based and included a broader patient population. Many participants may not have had direct contact with livestock or mosquito vectors, and some sought medical care for reasons unrelated to RVF, which may have diluted the proportion of exposed individuals.

The lower seroprevalence observed suggests that the general hospital population may not face the same level of RVF exposure as those in community-based studies targeting high-risk groups.

Furthermore, the occupational risk variations observed in previous studies, where slaughterhouse workers and farmers had the highest seropositivity, highlight the role of direct exposure to infected animal tissues, blood, and aerosols in RVFV transmission. Individuals involved in livestock handling, butchering, and veterinary services are at an increased risk due to their frequent contact with potentially infected animals, particularly during outbreaks (Nyamota *et al.*, 2023). These findings align with this study, where farmers exhibited the highest seropositivity, consistent with previous research highlighting the occupational risk associated with frequent animal contact. (Ngoshe *et al.*, 2019). These results collectively suggest that RVF may be endemic in multiple regions across Zambia, with varying degrees of exposure influenced by geographical and occupational factors.

The findings demonstrated that IFA exhibited perfect sensitivity and specificity, both at 100%, correctly identifying all individuals with antibodies and accurately excluding those without (Table 2). The absence of false positives and false negatives underscores the reliability of IFA in serological testing (Smith *et al*., 2020). Further validation using accuracy and Cohen's Kappa (κ = 1.0) confirmed its perfect agreement with ELISA, reinforcing its diagnostic precision (Jones & Brown, 2021). The Wilson score method also supported these results with 95% confidence intervals remaining at 100%, highlighting IFA's robustness in infectious disease surveillance (Miller *et al*., 2019).

While IFA is a reliable method, certain limitations were observed. The IFA process has a lower throughput compared to ELISA, making large-scale screening more time-consuming. In comparison, ELISA is more cost-effective as it uses enzyme-labelled antibodies, which are generally more affordable than fluorescent markers. Despite these limitations, IFA provides a more direct visualization of antigen-antibody interactions, reducing the risk of false positives or negatives

due to non-specific binding, which can sometimes occur in ELISA. It does not require enzyme-substrate reactions, which can be affected by environmental factors, ensuring greater stability and reliability in field or resource-limited settings. IFA enables antigen slide preparation using recombinant proteins, which enhances standardization and reproducibility, making it a viable replacement for ELISA in RVFV serological surveillance.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study focused on the application of the rNP-IFA for hospital-based serological surveillance of RVFV in humans. RVF is a zoonotic disease that poses a significant public health threat, particularly in regions where livestock farming and environmental conditions favour the transmission of the virus. Serological surveillance plays a critical role in understanding the extent of RVFV circulation in human populations, identifying individuals with past or recent exposure, and informing public health interventions.

This study aimed to assess the serological status of RVFV infection in humans and to identify potential risk factors associated with virus exposure in selected hospitals across three provinces of Zambia, Lusaka (Kanyama Hospital), Southern (Choma General Hospital), and Western (Kalabo and Lukulu District Hospitals). The use of rNP-IFA provided a sensitive and specific approach for detecting RVFV antibodies in human serum, offering an alternative to conventional ELISA-based assays. The findings contribute to a better understanding of RVFV seroprevalence in Zambia and support the integration of rNP-IFA into routine hospital-based surveillance systems to enhance early detection and response efforts.

The study demonstrated that IFA exhibited perfect sensitivity and specificity, accurately detecting all individuals with RVFV antibodies and excluding those without. Further validation using accuracy and Cohen's Kappa confirmed its strong agreement with ELISA, reinforcing its diagnostic precision and robustness for infectious disease surveillance.

The feasibility of IFA as a diagnostic tool in hospital-based serological surveillance of RVF in humans was successfully demonstrated. The study successfully applied IFA for the detection of RVF-specific antibodies in human serum samples, demonstrating high sensitivity and specificity in identifying seropositive cases. These findings highlight the potential of IFA as a valuable method for hospital-based surveillance.

6.2. Recommendations

- Considering the successful cloning into the pCAGGS vector, this expression system appears to be a robust tool for future research. It could be useful for scaling up the production of recombinant proteins in mammalian cell lines, and the vector could be further optimized for other RVFV protein expressions.
- The highest transfection efficiency was achieved with X-tremeGENE HP at a 1:3 ratio. Future experiments should replicate this condition to ensure maximum gene expression in Vero E6 cells. It may be worthwhile to further optimize the ratio and evaluate its reproducibility across different cell lines to confirm generalizability.
- While X-tremeGENE HP provided good results, testing other commercial transfection reagents in parallel could yield even higher transfection efficiencies, which is important for large-scale studies. Further development of rapid, point-of-care diagnostic tests (such as lateral flow assays) based on the recombinant RVFV-NP protein could improve early detection in rural or resource-limited areas.
- The study highlights that occupation, particularly farming, significantly increases the likelihood of RVFV infection. Public health interventions should target farmers and agricultural workers with educational campaigns on protective measures, such as vaccination, protective clothing, and avoiding exposure to infected animals. Given the higher seroprevalence in Western Province and Lukulu District Hospital, targeted interventions in these areas should be prioritized.

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APPENDICES

Appendix I. Ethical Approval Letter

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Ref. No. 2024-Nov-030

The Principal Investigator
Mr. Peter Chibale Mwansa
The University of Zambia
School of Veterinary Medicine
P. O Box 32379,
Lusaka, Zambia

Dear Mr. Mwansa

RE: APPLICATION OF RECOMBINANT NUCLEOCAPSID PROTEIN-BASED IMMUNOFLUORESCENCE ASSAY (IFA) IN HOSPITAL-BASED SEROLOGICAL SURVEILLANCE OF RIFT VALLEY FEVER IN HUMANS

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2024 -Nov-030**
Approval and Expiry Date	Approval Date: 09th December, 2024	Expiry Date: 08th December, 2025
Protocol Version and Date	Version - Nil.	08th December, 2025
Information Sheet, Consent Forms and Dates	• English, Tonga, Lozi, Nyanja.	08th December, 2025
Consent form ID and Date	Version - Nil	08th December, 2025
Recruitment Materials	Nil	08th December, 2025
Other Study Documents	Data Collection Sheet.	08th December, 2025
Number of participants approved for study	-	08th December, 2025

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered

to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

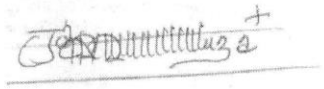
Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB



Dr. Jason Mwanza
Dip. Clin. Med. Sc., BA., M.Sc., PhD
CHAIRPERSON

Appendix II. Ethical Approval Certificate of Registration as Health Researcher

NATIONAL HEALTH RESEARCH AUTHORITY

The Health Research Act

(Act No. 2 of 2013)

MINISTRY OF HEALTH

CERTIFICATE OF REGISTRATION

THIS IS TO CERTIFY THAT

Peter Chibale Mwansa

has been registered as a Health Researcher

Dated this 17th April 2024

Registration number NHRAR-R-1492/14/04/2024

A/DIRECTOR AND CHIEF EXECUTIVE OFFICER

PROF. VICTOR CHALWE

Appendix III. Permission Letter to conduct research (Ministry of Health)

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 211 253040/5
Fax: +260 211 253344

In reply please quote
No .....

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

NDEKE HOUSE
P.O. BOX 30205
LUSAKA

12th December, 2024

Mr. Peter Chibale Mwansa
Zambia National Public Health
Stand 1186, Addis Ababa Drive & Chiholi Road
Lusaka, 10101, Zambia

RE: APPLICATION FOR PERMISSION TO CONDUCT RESEARCH

Reference is made to the above stated subject matter.

The ministry wishes to acknowledge receipt of your letter requesting for permission to conduct research titled" Application of Recombinant Nucleocapsid Protein-Based Immunofluorescence Assay (IFA) in Hospital-Based Serological Surveillance of Rift Valley Fever in Humans". The research is expected to be conducted from Kanyama Hospital in Lusaka, Choma General Hospital in Southern Province, Kalabo and Lukulu District Hospitals in Western Province, Zambia.

Following the review of the attached documents, the ministry has no objection to the application and you are further advised to submit your research protocol to the Ministry. Once in the respective Hospitals of your study, ensure you engage the District Health Offices for further guidance.

Dr. Kennedy Lishimpi
Permanent Secretary (T)
MINISTRY OF HEALTH

Cc: PHD – Lusaka Province
Cc: PHD - Southern Province
Cc: PHD – Western Province

Appendix IV. Data Collection Tool

ID	Age	Sex	Facility	Province /City	Sample Type	Collection Date	Section /Unit	Occupation	Signs and Symptoms							Results	
									Fever	Headache	Joint Pain	General Malaise	Photophobia	Diarrhoea	Vomiting	IFA	ELISA