DEVELOPMENT OF COMBINATION VACCINE FOR PROPHYLACTIC IMMUNIZATION OF DOMESTIC RUMINANTS AGAINST RIFT VALLEY FEVER AND PULPY KIDNEY

By

Matome Selina Matsiela

A research dissertation presented in fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the discipline Biochemistry

School of Life Sciences, College of Agriculture, Engineering and Science at theUniversity of KwaZulu-Natal, Pietermaritzburg

July 2024

Supervisors: Dr T. Khoza and Dr N. Mokoena

PREFACE

The research data presented in this dissertation was collected by the candidate at Onderstepoort Biological Products from 2020-2023, while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Drs. Thandeka Khoza and Nobalanda Mokoena. The Research was financially funded by the National Research Foundation (NRF), under grant number 127779 and Onderstepoort Biological Products, grant number 7920.

This dissertation submitted for the degree of Doctor of Philosophy in the College of Agriculture, Engineering and Science, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, represents original work by the author and has not been submitted in any form for a degree or diploma at any other university.

Signed: Matome Selina Matsiela (Student)

Date: July 2024

We certify that the above statement is correct and as the candidate's supervisors we have approved this dissertation for submission.

Signed: Dr Thandeka Khoza (Supervisor)

Date: July 2024

Signed: Dr N Mokoena (Supervisor)

Date: July 2024

DECLARATION 1: PLAGIARISM

I, Matome Selina Matsiela (218088225) declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

4. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a. Their words have been re-written, but the general information attributed to them has been referenced.

b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.

5. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: Matome Selina Matsiela

Date: July 2024

DECLARATION 2: RESEARCH OUTPUTS

Research outputs for this project comprised of publications and conference proceedings. My role in each research output was indicated, and the corresponding authors indicated by * in the publication.

Publications

1. Matsiela M S, Naicker L, Khoza T * and Mokoena N *, 2023. Safety and immunogenicity of inactivated Rift Valley Fever Smithburn viral vaccine in sheep, Virology journal: 2023. 20: Article number 221.

Conference proceedings

2. Poster Presentation: 5th Annual BioAfrica convention, 27-31st August 2022, International Convention Centre, Durban, South Africa.
3. Oral Presentation: 20th Annual SASVEPM Congress, 23-25th August 2023, Avani Gaborone Resort & Casino, Gaborone Botswana, South Africa.

Signed: Matome Selina Matsiela

Date: July 2024

ACKNOWLEDGEMENTS

Though it will not be enough to express my gratitude in words to all those who helped me, I would still like to give my sincere thanks to all these people:

Firstly, I would like to express my heartfelt gratitude to my esteemed mentor/supervisor, Dr. N. Mokoena, for unhesitatingly accepting the role of supervising my PhD project and helping me shape my career as a Resaerch Scientist. Beyond serving as a supervisor, Dr. Mokoena played the role of a life coach and director, imparting invaluable guidance and support. She has shown so much patience even in those days when I did not deserve it. Her assistance has been instrumental in my success, and I am confident that without her help, I would not have been able to meet the expectations required to complete this project. The most valuable advice she imparted was to identify the strategies that work best for me to maintain my strength and focus at all times.

I also would like to extend my sincere gratitude and thanks to Dr T Khoza for being willing to guide me through every step of this project, and for the support and words of encouragement towards the success and completion of this project.

I would also like to express my gratitude to Dr L. Naicker, Dr B Moetlhoa for the words of encouragement and support towards the progress of this project.

I also appreciate the continued support I received from the Onderstepoort Biological Products (OBP) (Research & Development, Virus and Bacterial Production, Raw Materials, Packaging, Quality Control and the Clinical departments).

To Mr Hector Ngobeni, thank you very much Sir for your unwavering support, prayers and constant words of encouragement throughout this journey.

Last but not least, I am deeply grateful to my family, "*Bakone ba ntshi dikgolo"*. My late grandparents for their unwavering love, support, and patient guidance throughout my academic journey. I am deeply indebted to them for their prayers and love, which have provided me with strength and resilience to overcome any obstacles that may have arisen during my studies. My aunt, my mom, and my uncles, thank you very much guys for your support. To my baby, Mashudu Mudau, thank you my love for being such a good little Nano for mommy, I love you.

Above all I give thanks to God through his son Jesus Christ who has carried me through this journey. His grace and mercy are sufficient and have managed to sustain me until this far. All my strength, peace, joy and power are found in him and I will forever be grateful.

ABSTRACT

Rift Valley Fever (RVF) and pulpy kidney (PK) are two severe veterinary diseases of domestic ruminants that have a negative impact on the sheep industry. Control and prevention of these diseases depend on strict adherence to vaccination programs. However, epidemics of RVF occur at irregular intervals, leading to periodic vaccination programs that are implemented following emergency warnings of extensive heavy rainfalls and floods. The present vaccination strategy has been consistently reported to result in low vaccination of RVF coverage across Africa and the Arabian peninsula. This approach delay the vaccination of livestock and result in a significant number of animals not developing protective immunity before the onset of infection. These challenges highlight the urgent need for an alternative effective vaccination strategy for livestock in endemic areas to improve the RVF vaccination coverage. In contrast, the epsilon toxoid has been widely used to immunise livestock against PK. The current research aimed to develop a safe and immunogenic RVF/PK combination vaccine as a strategy to improve vaccination coverage against RVF in endemic regions. The first objective of the study was to evaluate safety and efficacy of the inactivated RVF Smithburn (SB) vaccine as one of the oldest products widely used for immunisation of ruminants against RVF. The SB vaccine has a residual pathogenic effect, which limits its use to non-pregnant animals. The live-attenuated RVFV SB strain was utilized for developing an inactivated vaccine against RVF. The BEI-inactivated RVFV SB vaccine candidate was proven to be safe and elicited an immune response in merino sheep. Consequently, the inactivated RVFV SB was used for formulation of a combination vaccine product with epsilon toxoid at predetermined concentrations. The RVF/PK combination vaccine was evaluated for safety and immunogenicity in a clinical trial with OBP-commercial inactivated RVFV and alum-formulated PK vaccines serving as positive controls. The results demonstrated that the RVF/PK combination vaccine was safe for use in sheep, and no clinical signs against RVF or PK infections were observed following primary and secondary injections. The RVF/PK combination vaccine elicited neutralizing antibodies at levels comparable to those of commercial monovalent products for RVF and PK. These antibody levels remained high throughout the duration of the study. These findings suggest that the RVF/PK combination vaccine may be a possible alternative strategy for improving vaccination coverage against RVF.

Keywords: Rift Valley Fever, Pulpy kidney, Epsilon toxin, virus inactivation, aluminium hydroxide adjuvant

LIST OF TABLES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

LIST OF FIGURES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

LIST OF ABBREVIATIONS

ACEA: The European Automobile Manufacturers' Association

ACN: Acetonitrile

Al $(OH)_3$: Aluminium hydroxide gel adjuvant

APC: Antigen presenting cells

ATV: Trypsin versene solution

BEA: Bromoethylammonium bromide

BEI: Bromoethyleneimine

BHI: Brain Heart Infusion

BSL3: Biological safety level 3

BTA: Broth tryptose agarose

CI: Cell index

CO_2: Carbon dioxide

cm: centimeter

CPE: Cytopathic effect

CPE: Enterotoxin

CSIR: Council for Scientific and Industrial Research

DALRRD: Department of Agriculture Land Reform and Rural Development

DIVA: Differentiating infected from vaccinated animals

DNA: Deoxyribonucleic acid

DPV: Days post vaccination

ECACC: European collection of authenticated cell cultures

ELISA: Enzyme linked immunosorbent assay

IgG: Immunoglobulin-G

IgM: Immunoglobulin-M

kDa: kilo Daltons

KEVEVAPI: Kenya Veterinary Vaccine Producing Institute

LC-MS: Liquid chromatography-mass spectrometry

MCI: Multi-Chemical Industry

MLD: Mouse lethal dose

MOI: Multiplicity of infection

MSD: Merck Sharp & Dohme

mRNA: messenger ribonucleic acid

NetB: necrotic enteritis B-like toxin

NFF: Normal Flow Filtration

OBP: Onderstepoort Biological Products Pty (Ltd)

PYP: Peptone Yeast and potassium hydrogen phosphate

PBS: Phosphate buffered saline

PFU: Plaque forming unit

PK: Pulpy kidney

PK-ETX: Epsilon toxoid pulpy kidney vaccine

RID: Radial immunodifussion

RNA: Ribonucleic acid

RVFV: Rift valley fever virus

RVF: Rift valley fever

RVFV-SB: Rift valley fever virus Smithburn strain

RVF-SB: Rift valley fever Smithburn vaccine

RTCA: Real Time cell Analysis

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SA: South Africa

TCID50: Tissue culture infectious dose

TPP: Target Product Profile

TYG: Tryptone yeast and glucose

TFF: Tangential Flow Filtration

S/C: Subcutaneous

SDS/PAGE: Sodium dodecyl-sulfate polyacrylamide gel

SEM: Standard error of the mean

SNT: Serum Neutralisation Test

USA: United States of America

USAMRIID: United States Army Medical Research Institute of Infectious Diseases

VLPs: Virus like particles

VSVRI: Veterinary Serum and Vaccine Research Institute

WFI: Water for injection

WHO: World Health Organisation

WOAH: World Organization for Animal Health

ZH501: Zagazig Hospital RVFV strain 501

1 CHAPTER ONE: RESEARCH STUDY BACKGROUND

1.1 Introduction to the study

Livestock production is a significant component of the agricultural sector and accounts for 30 to 80% Gross Domestic Products in Africa (Rust and Rust, 2013, Erdaw, 2023). The sector not only provide a source of food security and family income, but also plays a crucial role in the social and ritual activities of African communities (Bettencourt *et al.*, 2015, Oduniyi *et al.*, 2020). In South Africa, the livestock sector contributes 40% to the total agricultural output per annum, of which approximately 53% was accounted by cattle, goat and sheep farming (Maltou and Bahta, 2019, Nyam *et al.*, 2022). According to a report by the Department of Agriculture Land Reform and Rural Development (2021), the cattle, goat and sheep industries made significant contributions to the economy over the past decade. The cattle and goat industries alone generated an average annual gross value of R9.8 million and R530 million, respectively. The sheep industry is the most important subsection of the livestock production sector and accounted for an average value of R5.7 billion per annum during the same period (Department of Agriculture, 2021). However, statistics in South Africa indicate that over the past decade a decline in number of cattle, goats and sheep has occurred by 17.4%, 25.7% and 12.2%, respectively. Several factors associated with the decline in livestock production worldwide, including: climate change, genetics and breed, nutrition, and disease outbreaks. Disease outbreaks present the most significant constraint, as they result in a reduction of animal numbers, leading to decreased milk production and hindering the export of livestock and animal products, thereby substantially impacting the economy of affected regions (Godde *et al.*, 2021).

Rift Valley Fever (RVF) is a mosquito-borne zoonosis caused by a negative sense RNA virus, which belongs to the order Bunyavirales, the family *Phenuiviridae,* and the genus *Phlebovirus* (Daubney *et al.*, 1931, Hartman, 2017). The disease primarily affects domestic animals such as cattle, sheep, goats and dromedary camels. The Rift Valley Fever Virus (RVFV) may also infect wild buffaloes, giraffe and springboks, which manifest a mild to no symptoms following infection. Domestic ruminants, mainly sheep and goats, present more severe clinical signs of the disease, associated with high mortality rates in newborn lambs (more than 95%), reduced milk production, foetal malformations (nearly 100%) and abortions in pregnant animals (Swanepoel, 2004, Bird and Nichol, 2012, Lorenzo *et al.*, 2015, Kwaśnik *et al.*, 2021). Human infections with RVFV may result from direct contact with body fluids and tissues

of infected animals (Kwaśnik *et al.*, 2021). Infected individuals usually develop mild symptoms such as fever, headache, anorexia, backache, and diarrhoea. However, up to 8% of patients present more severe symptoms of the disease, including: haemorrhagic syndrome, hepatitis, encephalitis, retinitis, and blindness (Madani *et al.*, 2003, Alkan *et al.*, 2023). The RVF disease is classified as notifiable by the World Organisation for Animal Health (WOAH), based on its potential to spread globally causing substantial socio-economic losses, and negative impact on public health(Mcelwain and Thumbi, 2017).

The RVF disease was first detected in sheep during an outbreak in the Rift Valley region of Kenya in 1931 (Daubney and Hudson, 1932). Since then, the disease has spread through African countries into Madagascar and the Arabian Peninsula (Bird *et al.*, 2009, Samy *et al.*, 2017, Fawzy and Helmy, 2019). RVF outbreaks are linked with periods of heavy rainfalls and floods, which creates favourable conditions for increased breeding of mosquito vectors (mostly from the *Aedes* and *Culex* species) (Rostal *et al.*, 2010, Murithi *et al.*, 2011). It has also been reported that movement of animals to low-lying areas prone to flooding during heavy rainfalls, as well as animal trade from African countries into middle east represent a constant risk of the RVF incursions in disease free regions (Ibrahim *et al.*, 2008, Chengula *et al.*, 2013, Nielsen *et al.*, 2020b).

The first epidemic of RVF in South Africa was documented between 1950-1951, and resulted in high mortalities of 100 000 in sheep and 500 000 abortions among pregnant ewes (Gerdes, 2004, Swanepoel *et al.*, 2004). The largest epidemic occurred in 1973-1974, which resulted in a high mortality rate of 95% in newborn animals, and widespread abortions accompanied by death of the infected adult animals (Mcintosh and Barnard, 1980). In 2010, South Africa recorded another major outbreak where Free State, Eastern Cape and Northern Cape provinces experienced severe effects of the disease (Pienaar and Thompson, 2013). During this period the 13000 of the 14 000 livestock cases reported were sheep (Pienaar and Thompson, 2013). This outbreak negatively affected the national livestock production with an estimated total loss of R213.6 million (Ntombimbini and Klein, 2015). Furthermore, in the 2011 outbreak, a total of 135 livestock cases were reported where sheep accounted for 90% of the 4 000 animals affected (Pienaar and Thompson, 2013). Since 2011, no major outbreak of RVF has been recorded in the country, until 2018, when an isolated outbreak was documented from the sheep farm in Free State province, with the human cases confirmed (Pienaar and Thompson, 2013, Jansen Van Vuren *et al.*, 2018). Following the 2018 outbreak, extensive prevention measures were implemented during seasons of heavy rainfalls (Jansen Van Vuren *et al.*, 2018).

Several preventive strategies are currently being utilised in RVF endemic regions to break cycle and avoid disease outbreaks. These include the following: (i) control of animal movement, (ii) vector control (iii) public education (iv) and vaccination of susceptible animals (Sindato *et al.*, 2012). Animal vaccination is widely regarded as the most effective control mechanism against RVF, as it enables animals to establish immunity against infection (Faburay *et al.*, 2017). Veterinary RVF vaccines are commercially available in both live attenuated and inactivated forms. These vaccines have been successfully utilised for immunisation of livestock in endemic regions such as Kenya, South Africa, Egypt, Tanzania, Saudi Arabia and Sudan (Geering *et al.*, 2002, Davies and Martin, 2003).

Live-attenuated vaccines are produced from a multiple passage of virulent RVFV isolates in mammalian cell culture (Lauring *et al.*, 2010). These vaccines include: live attenuated Smithburn (Onderstepoort Biological Products, South Africa; KEVEVAPI, Kenya), Clone-13 (OBP, SA), and thermostable Clone-13 (MCI Sante Animale, Morocco), and have the ability to confer long-term protective immunity in ruminants with a single dose (Coackley *et al.*, 1967, Faburay *et al.*, 2017). The live attenuated vaccines are not recommended for use in pregnant animals as they induce abortions and fetal malformations (Kamal, 2009, Makoschey *et al.*, 2016). The live attenuated vaccines are also associated with the risk of reversion to virulence, as well as possible genetic reassortment with virulent RVFV (Botros *et al.*, 2006, Kamal, 2009, Grobbelaar *et al.*, 2011, Dungu *et al.*, 2013). Due to the pathological effects associated with utilising the live attenuated vaccines, farmers are hesitant to use the live attenuated RVF vaccines and prefer the inactivated form of the product (Mdlulwa, 2015). They are non-replicating and may be used during outbreaks of RVF and are considered safe for use during pregnancy (Dungu *et al.*, 2013, Mdlulwa, 2015).

The inactivated RVFV vaccines are manufactured from virulent strains of RVFV, chemically treated to obtain a killed and non-infectious pathogen. Inactivated RVFV vaccines includes the formalin inactivated RVF (OBP, SA); formalin inactivated RVFV-Menya/sheep/258 strain (VACSERA, Egypt) and the BEI inactivated ZH501 strain (Veterinary Serum and Vaccine Research Institute, Egypt) (Barnard *et al.*, 1977, Davies and Martin, 2003, Ahmed Kamal, 2011). The inactivated RVFV vaccines are considered safer options for use in pregnant animals and cannot revert to virulence or have a risk of reassortment (Alhaj, 2016, Matsiela *et al.*, 2022). The major concern associated with this formulation is that it is manufactured using virulent virus strain, which poses safety hazard for production technologists and laboratory personnel handling and processing the antigen. A safer antigen for handling during production is therefore required for formulation of the safe and efficacious inactivated RVFV vaccine.

Vaccination programmes of livestock have been developed and implemented in RVF endemic countries such as Kenya, Egypt, Tanzania, Saudi Arabia, Sudan and South Africa (Geering *et al.*, 2002, Davies and Martin, 2003). These programmes rely on weather prediction methods and animal surveillance in RVF endemic regions to predict the next season of heavy rainfall and floods associated with high possibilities for the occurrence of RVF outbreaks. During these periods, an early warning system is used to enable authorities to implement effective control measures in affected regions. A report from the field survey of 150 South African farmers (Free State, Northern Cape and Eastern Cape), who have experienced large RVF outbreaks between 2008-2011 have indicated that only 77% of livestock was vaccinated following the early warning systems (Ntombimbini and Klein, 2015). It has also been discovered that most livestock farmers do not immunize their animals as recommended, due to limited knowledge about the disease (Mdlulwa, 2015, Faburay *et al.*, 2017, Habiyaremye *et al.*, 2017). In addition, a 40% low vaccination coverage was achieved in livestock following the 2000 outbreak in Saudi Arabia after the vaccination programmes. Therefore, an alternative vaccination strategy is required to effectively eradicate RVFV in endemic regions.

According to a socio-economic study, farmers prefer using the RVF vaccine as a combination product for effective prophylaxis of animals against RVFV infections. This could be an efficient strategy to overcome the non-sustained vaccination regime of RVF, while providing immunity against a common disease for which vaccination is mandatory (Decker, 2001, Dodd, 2003, Wallace *et al.*, 2020). The research and development of combination vaccines, particularly at the product and clinical development stage, has been documented in recent publications, such as LSD-RVF, LSD-BEF, and BTV-RVF, to name a few (Moreno *et al.*, 2020, Wallace *et al.*, 2020, Douglass *et al.*, 2021). A suitable vaccine candidate for use in combination with RVF should; (i) be inactivated, (ii) used in small ruminants at young age (3-4 months old), (iii) be of high demand and regularly utilised in the field by farmers. There are a number of commercially available combination vaccines, including the combined botulism/black quarter (OBP, SA), Blanthrax and Botuthrax (MSD Animal Health, USA) and the combination vaccines consisting of *Clostridial* antigens (Clostrivax B/B+, Clostrivax O, Enterotect P) as well as Ovipast manufactured by Design Biologix in South Africa. Pulpy kidney (PK) disease, is also one of the widely common conditions that affect small ruminants, and farmers practice regular vaccination to protect herds against the infection.

PK is caused by *Clostridium perfringens* type D, a gram positive, obligate anaerobic bacterium which resides in the intestinal canal of ruminants as normal flora (Sumithra *et al.*, 2013, Uzal *et al.*, 2016). Conditions that result in the manifestation of PK include a sudden change of diet and overeating which

leads to the uncontrolled replication of the bacteria, and results in the production of epsilon toxin that induces clinical signs of the disease (Jemal *et al.*, 2016). The disease is often clinically manifested by diarrhoea, chronic neurological condition, convulsions, blindness and sudden death of infected animals with mortality rate of up to 30% (Uzal and Songer, 2008, Mokoena *et al.*, 2017). PK is one of the most common diseases that affects farmers in South Africa. The only effective control is achieved through vaccination of livestock at young age of 3-4 months. Currently available epsilon toxoid vaccines for PK include Pulpyvax® 1-Shot (MSD, Animal health, SA), Clostrivax O (Design Biologix, SA), and Enterotoxaemia vaccines adjuvanted with oil emulsion or Alum-precipitated (OBP, SA). The OBP vaccines has successfully been used for systematic vaccination of herds against PK disease in sheep and goats for the past 20 years. Over the past decade, approximately 100 billion doses were sold in the South African market. The animals are primarily immunised with the oil-emulsion and booster inoculated with the Alum-precipitated formulation following 3-4 weeks of primary immunisation. Farmers regularly vaccinate livestock to provide long term immunity against pulpy kidney disease (Uzal *et al.*, 2016). The frequent use of the PK vaccine in small ruminants, makes it an ideal vaccine candidate for combination with RVFV.

Developing a single vaccine formulation that combines RVFV and PK has the potential to create a competitive product in the market. Combination vaccines offer several advantages, including cost savings, streamlined manufacturing processes, and enhanced immunisation schedules (Ma *et al.*, 2022). By administering two immunogens in one shot, the new RVF/PK combination vaccine will be cost-efficient for farmers and will offer enhanced immune protection of small ruminants against RVFV and PK. Additionally, incorporating the highly demanded PK vaccine into the RVF product may encourage farmers to vaccinate against the RVF disease, thereby increasing vaccination coverage. This study focuses on the development of a new combination RVF-PK vaccine for the prophylaxis immunization of sheep against both diseases. This is a novel combination vaccine that is not currently available on the market. It is expected that this study will contribute to the commercialization and subsequent adoption of the vaccine by farmers, who will benefit from the economic advantages of the product.

1.2 Aims and Objectives

The aim of the study was to develop a safe and efficacious combination vaccine consisting of the inactivated RVFV and PK (*Clostridium perfringens* type D epsilon toxoid).

1.2.1 Study objectives

1.2.1.1 Evaluate safety and immunogenicity of the inactivated RVF Smithburn vaccine

- Determine the inactivation kinetics of RVFV Smithburn
- Selection of a suitable adjuvant for formulation of the inactivated RVF Smithburn vaccine.
- Assess the safety and immunogenicity of the inactivated RVF Smithburn vaccine in sheep.

1.2.1.2 Optimizing the anaerobic growth conditions for Clostridium perfringens type D (ET663) culture

- Determine suitable growth conditions of the *Clostridium perfringens* type D (ET663).

1.2.1.3 Identify the optimal formulation ratio of the RVF/PK combination vaccine.

- Identify the optimal formulation ratio for the RVF/PK combination vaccine.
- Assess the safety and immunogenicity of the RVF/PK combination vaccine in sheep.

1.3 References

Ahmed Kamal, S. (2011). Observations on rift valley fever virus and vaccines in Egypt. *Virology Journal,* 8, 1-9.

Alhaj, M. (2016). Safety and efficacy profile of commercial veterinary vaccines against Rift Valley fever: a review study. *Journal of Immunology Research,* 2016.

Alkan, C., Jurado-Cobena, E. & Ikegami, T. (2023). Advancements in Rift Valley fever vaccines: a historical overview and prospects for next generation candidates. *npj Vaccines,* 8, 171.

Barnard, B. J. H., Amp & Botha, M. J. (1977). An inactivated Rift Valley fever vaccine. *Journal of the South African Veterinary Association,* 48, 45-48.

Bettencourt, E. M. V., Tilman, M., Narciso, V., Carvalho, M. L. D. S. & Henriques, P. D. D. S. (2015). The livestock roles in the wellbeing of rural communities of Timor-Leste. *Revista de Economia e Sociologia Rural,* 53, 63-80.

Bird, B. H., Ksiazek, T. G., Nichol, S. T. & Maclachlan, N. J. (2009). Rift Valley fever virus. *Journal of the American Veterinary Medical Association,* 234, 883-893.

Bird, B. H. & Nichol, S. T. (2012). Breaking the chain: Rift Valley fever virus control via livestock vaccination. *Current Opinion in Virology,* 2, 315-323.

Botros, B., Omar, A., Elian, K., Mohamed, G., Soliman, A., Salib, A., Salman, D., Saad, M. & Earhart, K. (2006). Adverse response of non-indigenous cattle of European breeds to live attenuated Smithburn Rift Valley fever vaccine. *Journal of Medical Virology,* 78, 787-791.

Chengula, A. A., Mdegela, R. H. & Kasanga, C. J. (2013). Socio-economic impact of Rift Valley fever to pastoralists and agro pastoralists in Arusha, Manyara and Morogoro regions in Tanzania. *Springerplus,* 2, 1-14.

Coackley, W., Pini, A. & Gosden, D. (1967). The immunity induced in cattle and sheep by inoculation of neurotropic or pantropic Rift Valley fever viruses. *Research in Veterinary Science,* 8, 406-414.

Daubney, R. & Hudson, J. (1932). Rift Valley Fever. *Lancet*, 611-12.

Daubney, R., Hudson, J. & Garnham, P. (1931). Enzootic hepatitis or Rift Valley fever. An undescribed virus disease of sheep cattle and man from East Africa. *The Journal of Pathology and Bacteriology,* 34, 545-579.

Davies, F. G. & Martin, V. (2003). *Recognizing rift valley fever*, Food & Agriculture Org.

Decker, M. D. (2001). Combination vaccines. *Primary Care: Clinics in Office Practice,* 28, 739-761.

Department of Agriculture, L. R. a. R. D. (2021). A Profile of the South African Mutton Market Value Chain.

Dodd, D. (2003). Benefits of combination vaccines: effective vaccination on a simplified schedule. *American Journal of Managed Care,* 9, S6-S12.

Douglass, N., Omar, R., Munyanduki, H., Suzuki, A., De Moor, W., Mutowembwa, P. & Williamson, A. (2021). The Development of Dual Vaccines against Lumpy Skin Disease (LSD) and Bovine Ephemeral Fever (BEF). *Vaccines*, 9, 1215.

Dungu, B., Donadeu, M. & Bouloy, M. 2013. Vaccination for the control of Rift Valley fever in enzootic and epizootic situations. *Vaccines and Diagnostics for Transboundary Animal Diseases*. Karger Publishers, 135, 61-72

Erdaw, M. M. (2023). Contribution, prospects and trends of livestock production in sub-Saharan Africa: a review. *International Journal of Agricultural Sustainability*, 21, 2247776.

Faburay, B., Labeaud, A. D., Mcvey, D. S., Wilson, W. C. & Richt, J. A. (2017). Current status of Rift Valley fever vaccine development. *Vaccines*, 5, 29.

Fawzy, M. & Helmy, Y. A. (2019). The one health approach is necessary for the control of Rift Valley fever infections in Egypt: A comprehensive review. *Viruses*, 11, 139.

Geering, W. A., Davies, F. G. & Martin, V. (2002). *Preparation of Rift Valley fever contingency plans*, Food & Agriculture Org.

Gerdes, G. (2004). Rift valley fever. *Revue scientifique et technique (International Office of Epizootics)*, 23, 613-623.

Godde, C. M., Mason-D'croz, D., Mayberry, D., Thornton, P. K. & Herrero, M. (2021). Impacts of climate change on the livestock food supply chain; a review of the evidence. *Global Food Security*, 28, 100488.

Grobbelaar, A. A., Weyer, J., Leman, P. A., Kemp, A., Paweska, J. T. & Swanepoel, R. (2011). Molecular epidemiology of Rift Valley fever virus. *Emerging Infectious Diseases*, 17, 2270.

Habiyaremye, A. D., Maziya, M., Chaminuka, P. D. & Mdlulwa, Z. (2017). Smallholder livestock farmers' knowledge, attitudes, practices and perceptions towards vaccinations: The case of five provinces in South Africa. *Human Sciences Research Council, https://idl-bnc-idrc.dspacedirect.org/*

Hartman, A. (2017). Rift valley fever. *Clinics in laboratory medicine*, 37, 285-301.

Ibrahim, M., Aziz, A. & Eid-Ul-Adha, H. (2008). A post card from uk rift valley fever: the story unfolds to family in Sudan. *Sudan J Public Heal*, 3, 5-10.

Jansen Van Vuren, P., Kgaladi, J., Patharoo, V., Ohaebosim, P., Msimang, V., Nyokong, B. & Paweska, J. T. (2018). Human cases of Rift Valley fever in South Africa, 2018. *Vector-Borne and Zoonotic Diseases*, 18, 713-715.

Jemal, D., Shifa, M. & Kebede, B. (2016). Review on pulpy kidney disease. *Journal of Veterinary Science and Technology*, 7, 361.

Kamal, S. A. (2009). Pathological studies on postvaccinal reactions of Rift Valley fever in goats. *Virology Journal*, 6, 94.

Kwaśnik, M., Rożek, W. & Rola, J. (2021). Rift Valley fever–a growing threat to humans and animals. *Journal of Veterinary Research,* 65, 7-14.

Lauring, A. S., Jones, J. O. & Andino, R. (2010). Rationalizing the development of live attenuated virus vaccines. *Nature Biotechnology,* 28, 573-579.

Lorenzo, G., López-Gil, E., Warimwe, G. M. & Brun, A. (2015). Understanding Rift Valley fever: contributions of animal models to disease characterization and control. *Molecular Immunology,* 66, 78-88.

Ma, J., Li, Z., Sun, Y., Liu, Z., Dang, Y. & Huang, Y. (2022). Improving innovation and access to combination vaccines for childhood immunization in China. *International Journal of Environmental Research and Public Health,* 19, 15557.

Madani, T. A., Al-Mazrou, Y. Y., Al-Jeffri, M. H., Mishkhas, A. A., Al-Rabeah, A. M., Turkistani, A. M., Al-Sayed, M. O., Abodahish, A. A., Khan, A. S. & Ksiazek, T. G. (2003). Rift Valley fever epidemic in Saudi Arabia: epidemiological, clinical, and laboratory characteristics. *Clinical Infectious Diseases,* 37, 1084-1092.

Makoschey, B., Van Kilsdonk, E., Hubers, W. R., Vrijenhoek, M. P., Smit, M., Wichgers Schreur, P. J., Kortekaas, J. & Moulin, V. (2016). Rift Valley fever vaccine virus clone 13 is able to cross the ovine placental barrier associated with foetal infections, malformations, and stillbirths. *PLOS Neglected Tropical Diseases,* 10, e0004550.

Maltou, R. & Bahta, Y. T. (2019). Factors influencing the resilience of smallholder livestock farmers to agricultural drought in South Africa: Implication for adaptive capabilities. *Jàmbá: Journal of Disaster Risk Studies,* 11, 1-7.

Matsiela, M. S., Naicker, L., Dibakwane, V. S., Ntombela, N., Khoza, T. & Mokoena, N. (2022). Improved safety profile of inactivated Neethling strain of the lumpy skin disease vaccine. *Vaccine: X,* 12, 100209.

Mcelwain, T. F. & Thumbi, S. (2017). Animal pathogens and their impact on animal health, the economy, food security, food safety and public health. *Revue Scientifique et Technique (International Office of Epizootics),* 36, 423.

Mcintosh, B., * Jupp, Pg,* Dos Santos, I.* & Barnard, B. (1980). Vector studies on Rift Valley fever virus in South Africa. *South African Medical Journal,* 58, 127-132.

Mdlulwa, N. Z. (2015). The socio-economic impact of the 2008-2010 Rift Valley fever outbreak on livestock farmers in South Africa. University of Pretoria, 2015, 30714621.

Mokoena, T., Chakauya, E., Crampton, M., Weyers, B., Tselanyane, M., Tsekoa, T. & Chikwamba, R. (2017). Evaluation of plant-produced Clostridium perfringens type D epsilon toxoid in a vaccine against enterotoxaemia in sheep. *Onderstepoort Journal of Veterinary Research,* 84, 1-7.

Moreno, S., Calvo-Pinilla, E., Devignot, S., Weber, F., Ortego, J. & Brun, A. (2020). Recombinant Rift Valley fever viruses encoding bluetongue virus (BTV) antigens: Immunity and efficacy studies upon a BTV-4 challenge. *PLOS Neglected Tropical Diseases,* 14, e0008942.

Murithi, R., Munyua, P., Ithondeka, P., Macharia, J., Hightower, A., Luman, E., Breiman, R. & Njenga, M. K. (2011). Rift Valley fever in Kenya: history of epizootics and identification of vulnerable districts. *Epidemiology & Infection,* 139, 372-380.

Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Depner, K., Drewe, J. A., Garin-Bastuji, B., Rojas, J. L. G., Schmidt, C. G. & Michel, V. (2020). Rift Valley Fever–epidemiological update and risk of introduction into Europe. *EFSA Journal,* 18, e06041.

Ntombimbini, Z. M. & Klein, K. (2015). Socio-economic impacts of lumpy skin disease and rift valley fever on the South African livestock economy. ARC. LNR.

Nyam, Y., Bahta, Y., Oduniyi, O. & Matthews, N. (2022). Smallholder sheep farmers' perception of production constraints and competitiveness strategies in South Africa. *Scientific African,* 16, e01192.

Oduniyi, O. S., Rubhara, T. T. & Antwi, M. A. (2020). Sustainability of Livestock Farming in South Africa. Outlook on Production Constraints, Climate-Related Events and Upshot on Adaptive Capacity. *Sustainability,* 12, 2582.

Pienaar, N. J. & Thompson, P. N. (2013). Temporal and spatial history of Rift Valley fever in South Africa: 1950 to 2011. *Onderstepoort Journal of Veterinary Research,* 80, 1-13.

Rostal, M. K., Evans, A. L., Sang, R., Gikundi, S., Wakhule, L., Munyua, P., Macharia, J., Feikin, D. R., Breiman, R. F. & Njenga, M. K. (2010). Identification of potential vectors of and detection of antibodies against Rift Valley fever virus in livestock during interepizootic periods. *American Journal of Veterinary Research,* 71, 522-526.

Rust, J. & Rust, T. (2013). Climate change and livestock production: A review with emphasis on Africa. *South African Journal of Animal Science,* 43, 255-267.

Samy, A. M., Peterson, A. T. & Hall, M. (2017). Phylogeography of Rift Valley fever virus in Africa and the Arabian Peninsula. *PLOS Neglected Tropical Diseases,* 11, e0005226.

Sindato, C., Karimuribo, E. & Mboera, E. (2012). The epidemiology and socio-economic impact of Rift Valley fever epidemics in Tanzania: A review. *The Onderstepoort Journal of Veterinary Research,* 79, 1.

Sumithra, T., Chaturvedi, V., Siju, S., Susan, C., Rawat, M., Rai, A. & Sunita, S. (2013). Enterotoxaemia in goats—A review of current knowledge. *Small Ruminant Research,* 114, 1-9.

Swanepoel, R. (2004). Rift Valley fever, In. Infectious diseases of livestock, Edited by JAW Coetzer & RC Tustin. *Oxford University Press,* Cape Town, South Africa.

Swanepoel, R., Coetzer, J. & Tustin, R. (2004). Infectious diseases of livestock with special reference to southern Africa. *Oxford University Press* Southern Africa, 2, 730-1605.

Uzal, F. A., Giannitti, F., Finnie, J. W. & García, J. P. (2016). Diseases produced by Clostridium perfringens type D. *Clostridial Diseases of Animals*, 157.

Uzal, F. A. & Songer, J. G. (2008). Diagnosis of Clostridium perfringens intestinal infections in sheep and goats. *Journal of Veterinary Diagnostic Investigation,* 20, 253-265.

Wallace, D. B., Mather, A., Kara, P., Naicker, L., Mokoena, N. B., Pretorius, A., Nefefe, T., Thema, N. & Babiuk, S. (2020). Protection of cattle elicited using a bivalent lumpy skin disease virus-vectored recombinant Rift Valley fever vaccine. *Frontiers in Veterinary Science,* 7.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Livestock diseases pose a significant threat to the health and welfare of animals globally. According to the World Organisation for Animal Health (WOAH), there are currently 207 notifiable veterinary diseases that have the potential to spread rapidly and cause substantial socioeconomic losses, as well as harmful consequences for public health. Livestock diseases are endemic in specific regions around the world and they are attributed to agricultural practices and climate conditions that facilitate the spread of these vector-borne diseases into new geographical areas (Tomley and Shirley, 2009).

Rift Valley Fever (RVF) is a notifiable veterinary disease of concern and is endemic in Africa and the Arabian Peninsula. The disease is caused by zoonotic Rift Valley Fever Virus (RVFV), which belongs to the *Phenuiviridae* family and *Phlebovirus* genus (Adams *et al.*, 2017, Gaudreault *et al.*, 2019). The RVFV infects livestock, including sheep, goats, and cattle, and can result in severe outbreaks that lead to high mortality rates especially in newborn and young animals, abortions in pregnant ewes, as well as severe clinical signs (Ikegami and Makino, 2011). These outbreaks cause significant economic losses in the livestock industry and pose a threat to public health (Sindato *et al.*, 2012, Godde *et al.*, 2021). The outbreaks of the disease occur periodically, and are linked to seasons of exceptionally heavy rainfall and floods. The *Aedes* mosquitoes, which were first identified as *Aedes lineatopennis* before 1985, and later reidentified as *Aedes* (*Neomelaniconion*) *mcintoshi*, serve as both the vector and reservoir of the RVFV (Huang, 1985). During the outbreak seasons, the RVFV circulates in nature through horizontal transmission between mosquito vectors and livestock, as well as wild ruminants (such as springbok, giraffe, wildebeest, and black-faced impala) which serve as reservoirs of the virus (Dondona *et al.*, 2016, Lumley *et al.*, 2017). Additionally, vertical transmission of the virus from *Aedes* mosquitoes into their eggs has been widely reported (Linthicum *et al.*, 1985, Lumley *et al.*, 2017). The RVFV exists as a single serotype with different strains (Maluleke *et al.*, 2019, Bob *et al.*, 2022). There are several methods available for diagnosis of the RVFV infection in hosts. These methods involve detecting the virus RNA in plasma or serum using molecular test techniques such as the reverse transcription polymerase chain reaction (RT-PCR) and sequencing platforms (Garcia *et al.*, 2001, Drosten *et al.*, 2002, Bird *et al.*, 2007, Le Roux *et al.*, 2009). Serological test analysis for detection of anti-RVFV IgM/IgG or neutralising antibodies using enzyme linked immunosorbent assay or serum neutralisation tests may also be applied for diagnosis of virus infection (Ellis *et al*., 2014, Alkan 2023).

Molecular techniques are mostly preferred for detecting the virus at an early stage, which enables timely interventions to control the spread of the virus and prevent outbreaks in affected regions. Serological testing, on the other hand, is useful for evaluating ongoing infections by detecting the presence of the circulating antigen or pre-exposure to the virus as indicated by the presence of specific IgM and IgG antibodies (Ellis *et al.*, 2014, Petrova *et al.*, 2020). The virus has been controlled through vaccination of livestock, vector control, banning of animal movement, and public education in endemic regions (Sindato *et al.*, 2012, Mpeshe *et al.*, 2014).

Several commercial livestock vaccines are currently available in live attenuated and inactivated forms, which have traditionally been effective in preventing RVF disease outbreaks in endemic regions. However, these vaccines have several drawbacks and do not completely meet the required specifications according to the World Health Organisation (WHO). Live attenuated vaccines have safety concerns related to the possibility of reversion to virulence, either through genetic reassortment with wild-type strains or mutations (Gaudreault *et al.*, 2019). The production of inactivated RVFV vaccines using a virulent strain of the virus poses a safety risk during manufacturing. To address these challenges, various vaccine development platforms, such as recombinant, DNA-vectored, and subunit vaccines, have been developed as alternative potential vaccine candidates for the prevention of RVF disease (Faburay *et al.*, 2017, Alkan *et al.*, 2023). This chapter reviewed the RVF disease, virus structure and genome, as well as the strategies used for the prevention and control of the disease in livestock, and the new generation of vaccines developed as potential candidates for prophylactic prevention of both animals and humans against RVFV.

2.2 Classification of the RVFV

The RVFV was initially classified under the family *Bunyaviridae*, until the International Committee on Taxonomy of Viruses created the new order Bunyavirales in 2016 and renamed *Bunyaviridae* to family *Phenuiviridae* (Adams *et al.*, 2017, Gaudreault *et al.*, 2019). The order Bunyavirales consists of nine new families, related with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes (refer to Table 2.1) (Maes *et al.*, 2019). The order Bunyavirales also consists of four recognized genera: *Goukovirus*, *Phasivirus*, *Phlebovirus* and *Tenuivirus* (Adams *et al.*, 2017). The RVFV belongs to the *Phlebovirus* genus and family *Phenuiviridae*, grouped with other pathogenic viruses causing diseases such as haemorrhagic fevers, influenza, respiratory tract infections, encephalitis, and a range of other illnesses affecting livestock and humans worldwide (Table 2.1) (Strauss and Strauss, 2008, Käfer *et al.*, 2019). Viruses belonging to the *Phenuiviridae* family are characterised by three segmented RNA molecules with complementary genes containing 3’ and 5’ termini.

Table 2. 1: Classification of the negative sense RNA viruses in the order Bunyavirales.

Family	Common names	Virion naked/enveloped	Genome	Hosts	References
Wupedeviridae	Wuhan millipede virus-2	Enveloped	Tripartite (3 segments)	Myriapods (millipedes)	(Kuhn *et al.*, 2023c)
Fimoviridae	Fig mosaic virus	Enveloped	Multipartite (4-10 segments)	Plants	(Elbeaino *et al.*, 2018)
Hantavirus	Hantavirus pulmonary syndrome	Enveloped	Tripartite (3 segments)	Human	(Vaheri *et al.*, 2013)
Mypoviridae	Hubei Myriapoda virus-5	Enveloped	Tripartite (3 segments)	Myriapods (centipedes/millipedes)	(Kuhn *et al.*, 2023b)
Nairoviridae	(i)Crimean-Congo Hemorrhagic fever virus (ii)Nairobi-sheep disease virus	Enveloped	Tripartite (3 segments)	Birds, bats, ruminants, humans	(Garrison *et al.*, 2020)
Peribunyaviridae	Bunyamwera virus	Enveloped	Tripartite (3 segments)	Humans, ruminants, birds, midges, mosquitoes	(Hughes *et al.*, 2020)
Phasmaviridae	Ferak feravirus	Enveloped	Tripartite (3 segments)	Insect (Phantom midges)	(Walker *et al.*, 2021)
Phenuiviridae	Rift Valley Fever Virus, rice stripe tenuivirus, Dabie bandavirus	Enveloped	Multipartite (2–8 segments)	Livestock, humans, birds, plants, fungi, arthropods	(Sun *et al.*, 2022)
Cruliviridae	Crustacean virus 9	Enveloped	Tripartite (3 segments)	Crabs (Portunid crustaceans)	(Kuhn *et al.*, 2023a)

2.3 Genome and structure of the RVFV

RVFV is a negative sense RNA virus and consists of three negative-stranded RNA genome, with a small (S), medium (M) and large (L) segments (Figure 2.1). These genomic segments consist of the conserved untranslated regions at the 3' and 5' ends, creating circular panhandle structures (Figure 2.1) (Pardigon *et al.*, 1982, Kolakofsky and Hacker, 1991, Bouloy and Weber, 2010). The genome segments form ribonucleoprotein complexes with the viral nucleocapsid protein and the viral RNA-dependent RNA polymerase (Figure 2.1). The S segment of RVFV encodes the nucleocapsid (N) protein in the negative-sense (viral-sense), and the non-structural (NSs) gene in the positive-sense (antiviral-sense) genome in an ambisense manner (Figure 2.1) (Giorgi *et al.*, 1991, Bouloy *et al.*, 2001). The N protein encloses viral genome and is critical for RNA synthesis and packaging of viral RNA into virions (Hayashi *et al.*, 2021). The NSs protein has been identified as the major virulence factor of RVFV, which is unessential for viral replication, yet necessary for evasion of host innate immune responses (Lihoradova and Ikegami, 2014). The M segment encodes a single open reading frame, while the M mRNA can transcribe at least four different proteins (NSm1, NSm2, Gn and Gc) (Collett, 1986, Kakach *et al.*, 1988, Kakach *et al.*, 1989, Gerrard and Nichol, 2007). The role of NSm1 protein remains unknown. The Gn and Gc are the viral structural glycoproteins projected on the lipid bilayer (viral envelope) enclosing the viral genome. The viral envelope consists of approximately 122 glycoproteins, arranged in an icosahedral lattice, and located 2.2 nm apart (Figure 2.1) (Bouloy and Weber, 2010, Pepin *et al.*, 2010). These proteins are projected as hollow cylinders from the virus surface, and measures 5 to 8 nm in length and 5 nm diameter (Figure 2.1). The Gn plays a role in receptor binding and Gc serves as a class II fusion protein. The Gn and Gc proteins are composed of protective epitopes that induces the production of neutralising antibodies (Allen *et al*., 2018). The overall structure of RVFV particles were determined to have an icosahedral symmetry, with an average particle diameter of 90 to 110 nm when viewed under electron microscopy and negative staining (Ellis *et al.*, 1979, Freiberg *et al.*, 2008). The L segment encodes the RNA-dependent RNA polymerase (L-protein), which serves a role in genome replication and viral mRNA transcription (Lopez *et al.*, 1995, Ikegami *et al.*, 2005).

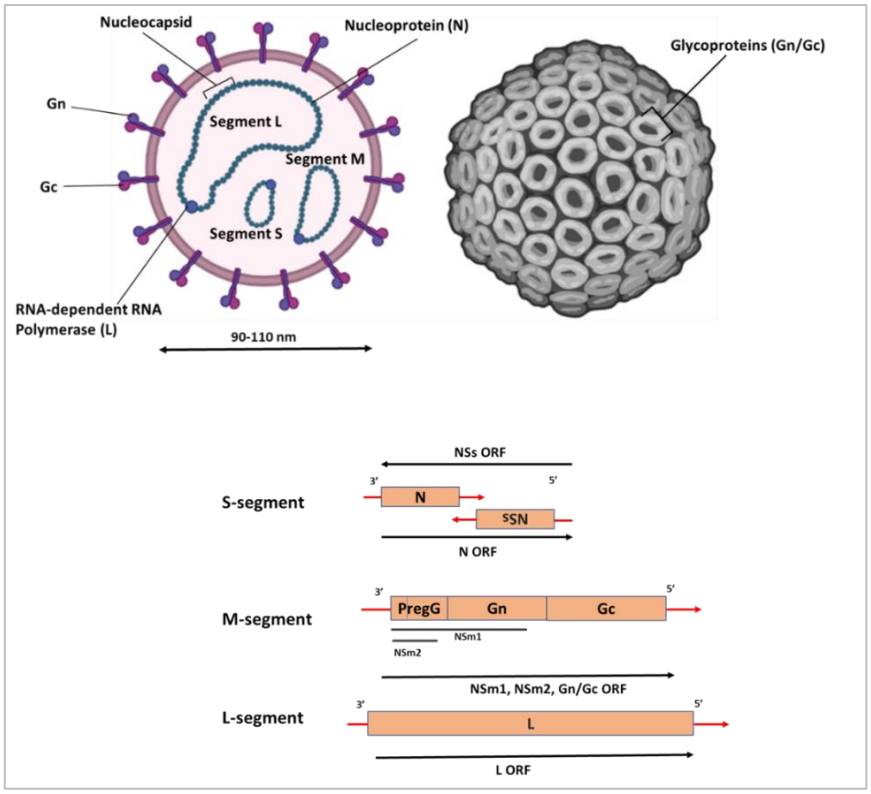


Figure 2. 1: The RVFV structure and the tri-segmented negative sense RNA genome. The S-segment encodes for nucleoprotein (N) in the negative-sense and a non-structural protein (NSs) in an ambisense manner. M-segment encodes two structural glycoproteins (Gn and Gc) and two NSm proteins (NSm1 and NSm2). L-segment encodes L protein. Modified from (Fawzy and Helmy, 2019).

2.4 Replication cycle of RVFV

The mechanisms underlying the transcription and replication of RVFV are similar to those of other negative-stranded RNA viruses. The replication cycle of RVFV occurs within the host cell's cytoplasm, with the process taking approximately 10-12 hours to complete a single cycle (Gaudreault *et al.*, 2019). The replication cycle commences with the virus attaching to the host cell surface through interactions with host cell receptors, such as the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), L-SIGN, heparan sulfates (HS), and the virus's Gn/Gc glycoproteins (Figure 2.2) (De Boer *et al.*, 2012a, Hofmann *et al.*, 2013, Léger *et al.*, 2016). The interaction between virus-glycoproteins and host cell receptors results in receptor clustering and subsequent endocytosis, which allows virus entry mediated by caveolin into the host cell (Filone *et al.*, 2010, Lozach *et al.*, 2011, Harmon *et al.*, 2012). Following entry, the virus-membrane fusion occurs due to a change in pH (from 7.4 to 4.5), and the viral ribonucleoproteins are released into the cytosol (Figure 2.2). Each virus-RNA segment is then transcribed into messenger RNA and replicated to produce original copies of the viral

genome, known as complementary RNA (cRNA), as indicated in Figure 2.2 (De Boer *et al.*, 2012b, Willensky *et al.*, 2016). The cRNA of the S segment serves as the template for the synthesis of the NSs mRNA. The glycoproteins (Gn/Gc) heterodimerize in the endoplasmic reticulum lumen before being transported to the Golgi for post-translational modifications (Bouloy and Weber, 2010). Additionally, the virus reportedly exploits the autophagy machinery by increasing the levels of protein LC3-II, which facilitates the assembly and release of nascent virions (Charlton *et al.*, 2019). The newly replicated viral genomic segments and proteins are transported to the Golgi for assembly and packaging of virus particles, as depicted in Figure 2.2. Finally, mature virions are released from the infected cell through a process known as budding (Gaudreault *et al.*, 2019).

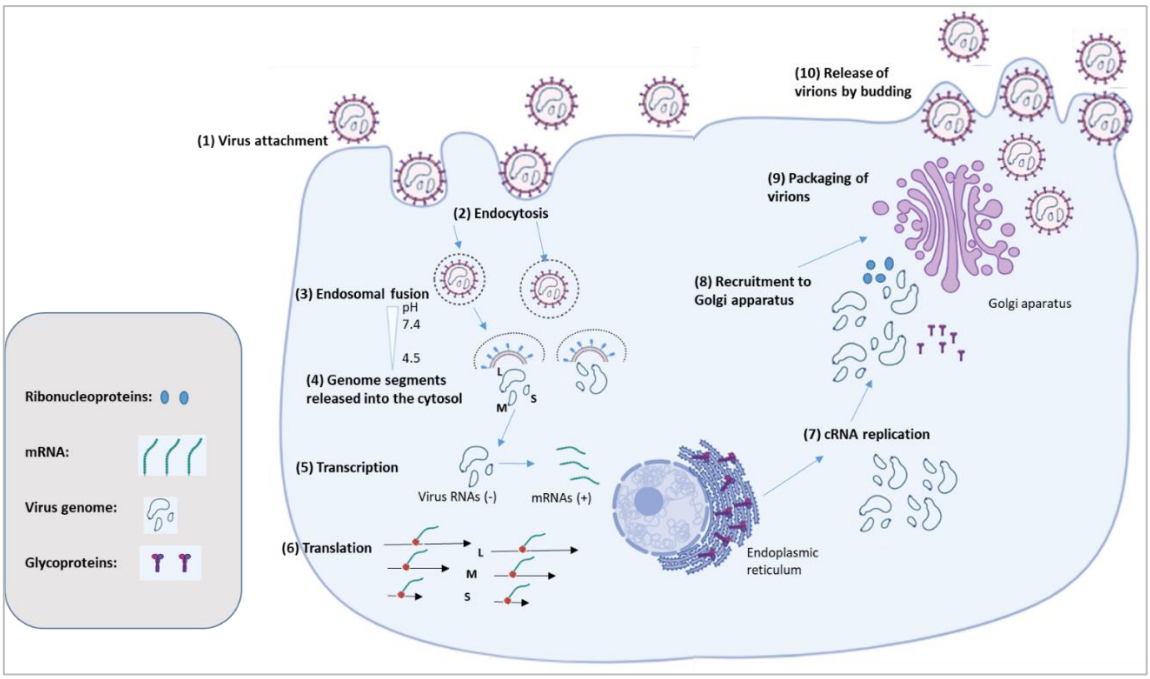


Figure 2. 2: Replication cycle of RVFV. (1) Attachment of the virus to the host cell surface. (2) Virus entry by endocytosis. (3) Virus uncoating by low pH-dependent membrane fusion. (4) Release of genome segments into the cytosol. (5) Transcription of the virus genome segments into mRNA. (6) Translation of the mRNA into the viral cRNA using the viral coded RdRP. (7) Replication of the cRNA, and heterodimerisation of the viral glycoproteins at the endoplasmic reticulum. (8) Recruitment of the virus genome segments, glycoproteins and ribonucleoproteins to Golgi apparatus. (9) Packaging of virions. (10) Release of virions by budding through plasma membrane. *Created using Biorender.com, and modified from (Gaudreault et al., 2019).*

2.5 The transmission cycle of RVFV

RVF has interepizootic periods ranging from 5 to 15 years, and virus circulation occurs during periods of heavy rainfall and floods associated with the El Niño Southern Oscillation climatic conditions (Anyamba *et al.*, 2002, Nielsen *et al.*, 2020a, De Glanville *et al.*, 2022). These conditions create an ideal environment for the breeding of vectors that are responsible for transmitting the RVFV to susceptible hosts. Various species of Aedes mosquitoes, which are classified into the subgenera Neomelaniconion, Aedimorphus, Ochlerotatus and Catageiomyia, act as primary vectors for the transmission of RVFV (Alomar *et al.*, 2023). The mosquitoes are capable of transmitting the virus vertically to their offspring through eggs, which are laid in temporary ponds that become pools of water after heavy rainfall. The virus in these eggs remains infectious for several years even during the dry conditions of the interepizootic periods (Figure 2.3). The RVFV transmission cycle begins with the hatching of numerous infected Aedes mosquito eggs, into a multitude of infected mosquito vectors during heavy rainfall or flooding (Figure 2.3) (Linthicum *et al.*, 1985, Lumley *et al.*, 2017). Infected Aedes mosquitoes transmit the virus by biting susceptible animals for a blood meal (Figure 2.3) (Wright *et al.*, 2019). The virus's incubation period in infected animals is typically 24-36 hours. Once the virus has multiplied within the host, other vector species, such as Culex, as well as arthropods (midges, sandflies, and ticks), can serve as potential mechanical vectors from viraemic livestock to susceptible healthy hosts through bites. (Figure 2.3) (Lumley *et al.*, 2017). The rate of virus transmission among the susceptible hosts may differ due to the different types of vectors involved for facilitating the process (Pepin *et al.*, 2010). The clinical signs exhibited by adult livestock animals infected with RVF include high fever, ocular or nasal discharge, vomiting, abdominal colic, haemorrhagic diarrhoea, lymphadenitis, jaundice, dysgalactia, and lasting prostration (Ikegami and Makino, 2011). The virus also causes high mortalities in newborn ruminants, particularly sheep and goats, and a significant percentage of abortions (approximately 100%) in pregnant animals. Under these outbreak conditions which result in increased handling of the virus infected animals, present the high risk of human exposure. Virus infection in humans occur through direct or indirect contact with body fluids or organs of infected animals, acquired during slaughtering, assisting with animal births, conducting veterinary procedures, or from the disposal of carcasses or foetuses (Figure 2.3). The occupational groups that are at risk of RVFV infection includes slaughterhouse workers, herders, farmers, veterinarians, and laboratory personnel. Transmission of RVFV to humans through mosquito bites is less common (Pendell *et al.*, 2016, Mcmillen and Hartman, 2018, Kainga *et al.*, 2022). Infected mosquitoes may also transmit the virus to wild life including; African elephants and buffaloes, giraffe, impalas, black rhino, and Asian monkeys which mainly act as carriers of the RVFV, without developing clinical signs of infection (Evans *et al.*, 2008, Murithi *et al.*,

2011). It is only the specific species of buffaloes and giraffe which develop signs of the RVF infection (Rostal *et al.*, 2017). Furthermore, the infected wild life may also serve as the RVFV amplifying hosts and allow transmission to the mosquito vectors (Wright *et al.*, 2019). The direct transmission of the virus from infected livestock to the naïve one has not been successfully demonstrated.

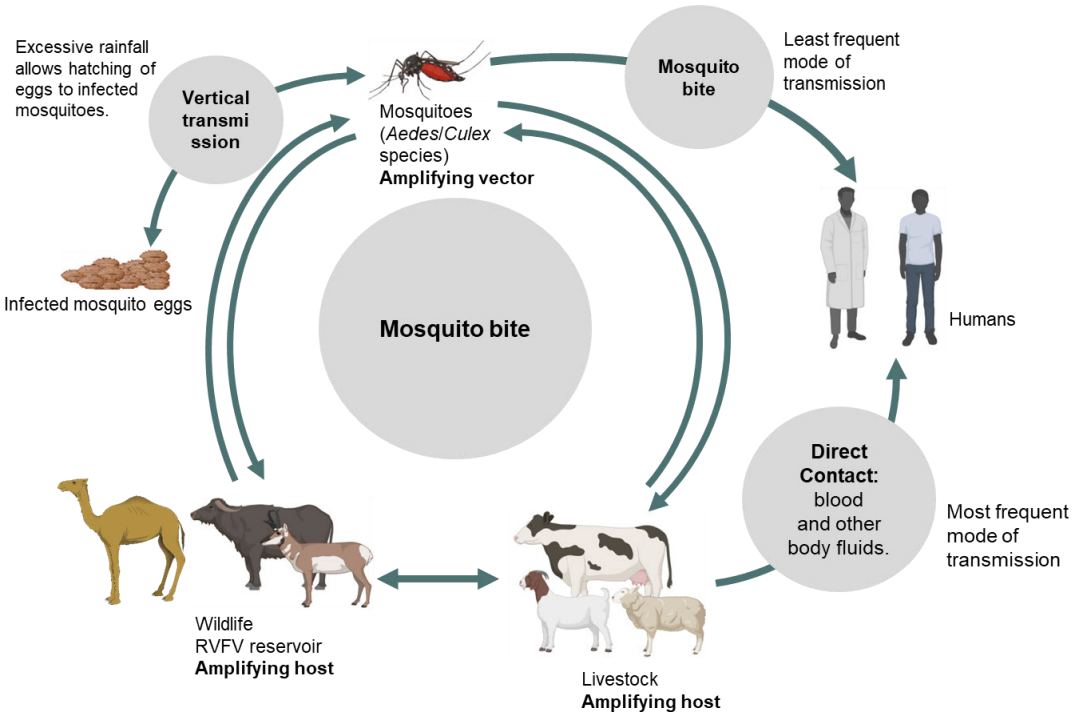


Figure 2. 3: An overview of the transmission cycle of RVFV within susceptible hosts. ***Created using Biorender.com. Modified from the WOAH website.***

2.6 Epidemiology of the RVF

RVF disease was first recognised in the Rift Valley of Kenya in 1931 (Daubney *et al.*, 1931, Gaff *et al.*, 2007, Nielsen *et al.*, 2020b). Since its first discovery, significant outbreaks of the disease impacting both livestock and humans have occurred in several African countries, including eastern Africa (Kenya, Tanzania, Comoros, Madagascar, Sudan, and Somalia); western Africa (Senegal, Mali, Niger, Gambia, and Mauritania); Northern Africa (Egypt) and Southern Africa (South Africa, Mozambique, Botswana, Namibia and Zimbabwe) (Bird *et al.*, 2009, Samy *et al.*, 2017, Fawzy and Helmy, 2019). In 1977, a major epidemic occurred in Egypt, resulting in approximately 200 000 human cases and 598 deaths (Laughlin *et al.*, 1979). Subsequently, RVF was identified in West Africa (Senegal and Mauritania) which resulted

in high human mortalities. The disease was later reported outside African countries into Saudi Arabia (2000) after the 1997–1998 East African outbreak (Jupp *et al.*, 2002, Gerdes, 2004). A significant number of livestock death and approximately 10 000 abortions were reported in infected sheep, cattle, goats, and camels, as well as 884 human cases confirmed with 124 death cases in Saudi Arabia (Shoemaker *et al.*, 2002). In 2001, the disease was reported in Yemen with 1328 human cases and 166 deaths recorded (Shoemaker *et al.*, 2002). The occurrence of the outbreaks of RVF in the Arabian Peninsula triggered concerns that the RVFV would spread to other regions of Asia and Europe (Rahman *et al.*, 2023). Currently, the RVF is endemic in Sub-Saharan Africa and the Arabian Peninsula, as illustrated on Figure 2.4. The most recent outbreak of the disease was recorded in Mauritania between August 30th and October 17th, 2022, with a total of 47 confirmed human cases and 23 fatalities (Mohapatra *et al.*, 2023). In December 2022, 102 suspected RVF human cases were identified in Uganda, Mbarara district. In addition, one probable death case and 24 suspected cases were subsequently identified in the same area from January-March 2023, with four reported death cases (Kabami, 2023). Eight RVF cases were further identified in Uganda, Kimotozi village, district of Nakaseke in June-July 2023. Of the eight cases, two were confirmed and six were suspected cases in male patients who presented typical clinical signs of RVF including fever, abdominal pain, headache, joint pain, and nose bleeding (Komugisha *et al.*, 2023). The cases were linked to the disease outbreak in livestock reported in the same area during the same time interval, which resulted in 42 deaths in young animals, abortions in 36 pregnant animals and death in 8 adult animals (Komugisha *et al.*, 2023). Furthermore, a recent study had confirmed seropositivity of the IgG anti-RVFV antibodies tested against the N-protein of RVFV in animals from Algeria, Libya and Turkey (Zouaghi *et al.*, 2021). Therefore, there is an increased concern that outbreaks of RVF may reach the Asian countries (Mohapatra *et al.*, 2023).

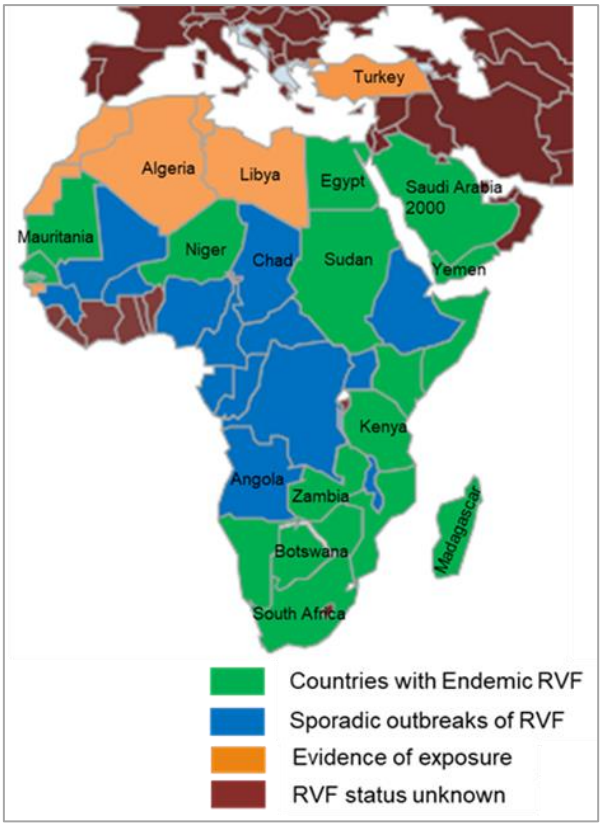


Figure 2. 4: Distribution map of the Rift Valley Fever.

2.7 Diagnosis of RVF

The RVFV infections may be confirmed using various molecular procedures that can be used for detection of the viral nucleic acid. Firstly, blood samples are ought to be collected from suspected infected hosts, as early as 3-6 days post infection for virus isolation (Kroeker *et al.*, 2020, Petrova *et al.*, 2020). Detection of the nucleic acid may be established through PCR (Kwaśnik *et al.*, 2021). Molecular detection of RVFV through conventional and RT-PCR tests are currently the most sensitive and rapidly used for evaluation of the RVFV exposure and quantification during outbreaks (Garcia *et al.*, 2001). Tests performed include: quantitative RT-PCR, multiplex PCR-based microarray assay, nested RT-PCR, recombinase polymerase amplification, and RT loop-mediated isothermal amplification (Garcia *et al.*, 2001, Drosten *et al.*, 2002, Bird *et al.*, 2007, Le Roux *et al.*, 2009, Euler *et al.*, 2012, Venter *et al.*, 2014). Isolation of RVFV is performed using the whole blood or serum samples collected from the infected host during the early phase of infection. The virus may also be extracted from numerous organs such as the brain, spleen, liver, and aborted foetuses of the infected host during post-mortem analysis (Kwaśnik *et al.*, 2021). Recent methods developed for evaluating

exposure of RVFV are focused on the next generation sequencing, colorimetry, or TaqMan array cards (Liu *et al.*, 2016, Brinkmann *et al.*, 2017, Zaher *et al.*, 2018). In addition, a lateral flow immuno-chromatographic strip test was established for detection of the N-protein of RVFV, which can better the management of the diagnosis of the RVFV and control of the disease in endemic regions (Cêtre-Sossah *et al.*, 2019).

2.8 Immunology of RVFV

Antibody detection against the RVFV is required to analyse the type and level of immune response the infected host possess after infection with the virus. Infection with RVFV causes the population of IgM and IgG antibodies induced against the N protein of the virus (Fafetine *et al.*, 2007, Mcelroy *et al.*, 2009, Pepin *et al.*, 2010, Wright *et al.*, 2019). The RVFV-specific IgM antibodies are detected from day 4 to 6 and following which positive response for specific IgG accumulates from day 7 or 14 post infection (Daubney *et al.*, 1931). Confirmation of the IgG antibody capture by neutralisation assay may be required due to the unrecognised cross reactions of RVFV with other phleboviruses. The levels of neutralising antibodies are known to be equivalent to protection of animals against infection with the wild type RVFV (Daubney *et al.*, 1931, Pepin *et al.*, 2010). Therefore, vaccine development programmes aim at eliciting high neutralising antibody titres against RVFV. Neutralising antibodies can be detected within the first week of infection. These antibodies target the virus envelope glycoproteins (Besselaar and Blackburn, 1991, Besselaar and Blackburn, 1992, Wallace *et al.*, 2006, Lagerqvist *et al.*, 2009, Warimwe *et al.*, 2016). Studies suggested that the RVFV N-protein does not elicit neutralising antibodies, since it is not a surface protein (Wright *et al.*, 2020, Doyle *et al.*, 2022). This was evident with the vaccination of animals with the DNA construct encoding the N-protein that had afforded partial protection, suspected to have been from cellular immune response (Lorenzo *et al.*, 2010). On the other hand, specific antibody immune response was detected in rabbits immunized with Gn glycoprotein. B cells extracted from the immunised rabbits were used to produce hybridoma cells that induced specific neutralising antibodies against RVFV which protected naïve recipient mice against challenge with the wild type RVFV (Allen *et al.*, 2018). The study conducted by Chrun et al. (2018) demonstrated that neutralising antibody immune response induced in sheep immunised with the expression plasmid encoding the ectodomain of the RVFV glycoprotein Gn protected animals against challenge with virulent RVFV (Chrun *et al.*, 2018). The RVFV recombinant vaccine expressing Gn and Gc glycoproteins elicited neutralising antibody response against the virus in immunised sheep (Said *et al.*, 2017). Animals pre-exposed to the virus, develop immunity and become completely resistant to infection upon re-exposure (Daubney *et al.*, 1931). Protection against challenge with the

RVFV may also be acquired from administration of sera containing antibodies into naïve animals. This technique was shown to confer protection in lambs vaccinated with human sera against RVFV (Daubney *et al.*, 1931, Smithburn, 1949, Niklasson *et al.*, 1984, Peters *et al.*, 1988). More research is required to investigate the humoral immune response of RVF, in order to determine epitopes on the virus proteins responsible for inducing neutralising antibodies against RVFV (Oscherwitz, 2016).

Development of vaccine products are guided by the type of immune response induced through natural infection of the virus. Despite the antibody immune response, the virus can also induce cellular immunity. The first immune cells encountered after infection with the RVFV are macrophages and dendritic cells (Lozach *et al.*, 2011, Terasaki and Makino, 2015). Doyle et al. (2022) had demonstrated the sufficiency of splenocytes transferred to naïve recipient mice to control RVFV-infection after challenge, suggesting that cellular immune response can prevent the infection of RVF (Doyle *et al.*, 2022). Other studies have also established work that demonstrated critical role of T cell immune response (such as the CD4+ T cells, CD8+ T cells and monocytes) inhibiting virus infection following challenge with the wild type virulent RVFV (Dodd *et al.*, 2013, Harmon *et al.*, 2018). A recent study had also demonstrated the replication of the RVFV in the brain of mice lacking B cells, T cells, macrophages and natural killer cells, which concluded that CD4+ T cells protects mice against developing encephalitis (Michaely *et al.*, 2022). However, the specific function of cellular immune response for management and prevention of RVFV infection is less understood (Doyle *et al.*, 2022).

2.9 Prevention and control of RVFV

The management of RVF outbreaks in livestock involves systematic surveillance, vector control, restrictions or banning of animal movement, and animal vaccination. Implementation of these measures is critical in preventing the spread of RVFV and minimizing its impact on the affected population. In Kenya, systematic surveillance of livestock herds for early detection of RVF infections during rainy seasons, which favour increased mosquito breeding, has been used as an effective early warning system. Additionally, the monitoring of climatic conditions is crucial in predicting the next season of floods and enhancing control measures to prevent RVF outbreaks (Anyamba *et al.*, 2010). The one health approach is also being used to control RVF outbreaks through improving the education system to the public, administrative structures and legislation law (Fawzy and Helmy, 2019). Vaccination is considered as the most effective method to prevent the spread of the disease in both endemic and non-endemic regions among all the methods being used to control RVF infections (Kwaśnik *et al.*, 2021). A sustainable vaccination programme of livestock may be developed to prevent RVF outbreaks in animals. There are no registered vaccines available for use in humans, while the live

attenuated and inactivated RVFV vaccines have been developed for veterinary use and were successfully used in endemic regions (Kitandwe *et al.*, 2022).

In 2019, a drafted guideline of the RVF vaccines Target Product Profile (TPP) was published by WHO, and categorised them according to their intended purposes (Who, 2019). These categories are comprised of: (a) RVF vaccines for human, intended for use during emergencies in populations experiencing an outbreak, (b) RVF vaccines for use in human at high risk of exposure to infection of RVF, and (c) RVF vaccines for use in ruminants including sheep, goats, cattle, and dromedary camels. These vaccines are required to reach a minimum shelf life of 12 months after storage at −20 °C or acquire stability of 6 months at 2–8 °C (Who, 2019). TPP draft for RVF vaccines highlights that during outbreaks of RVF, the use of live-attenuated RVF vaccines should be avoided in both livestock and humans to prevent the reassortment of the virus with the virulent field strain. Additionally, the TPP draft stipulates that live-attenuated RVFV vaccines should be safe for use during pregnancy, without causing abortion or teratogenesis in pregnant animals, and should not be vertically transmitted to the foetus (Who, 2019). Therefore, it is important to assess the current characteristics of the commercially available RVFV vaccines, following criteria: (a) cell lines used for vaccine production, (b) vaccine doses, (c) spread to mosquitoes, (d) safety during pregnancy in ewes, (e) potential for generating pathogenic reassortants, (f) temperature stability, and (g) compliance for differentiating infected from vaccinated animals (DIVA), in order to develop a vaccine formulation that is safe, efficacious and compliant with the WHO TPP for eradication of the virus in endemic regions.

2.9.1 Live attenuated vaccines

There are three types of live attenuated vaccines, namely Smithburn, Clone-13, and thermostable Clone-13 strains that have been licensed for use in livestock across South Africa and other endemic regions (Dungu *et al.*, 2018, Alkan *et al.*, 2023). Additionally, the live attenuated MP-12, has been conditionally licensed for veterinary use in the United States in 2013 (Fawzy and Helmy, 2019, Alkan *et al.*, 2023). The RVFV Clone-13 strain is a plaque-isolated clone, derived from a 74HB59 RVFV strain obtained from infected immunocompetent patients in the Central Republic (Muller *et al.*, 1995, Kortekaas *et al.*, 2011). The strain encodes 70% of the natural deletion of 549 nucleotides of the NSs gene in the S-segment (Muller *et al*., 1995; Kwaśnik *et al.*, 2021). The Clone-13 strain lacks a functional NSs gene, which enables it to evade suppression of the host's innate immunity, including the upregulation of type-I IFNs and IFN-stimulated genes. This contributes to its attenuation phenotype in healthy animals (Alkan *et al.*, 2023). However, it is important to note that the attenuation of the Clone-13 strain is solely attributed to the deletion of the NSs gene, and not to the upregulation of type-I IFNs

or IFN-stimulated genes. Therefore, acute lethal disease can be reproduced in mice that are knockout for type I IFN receptors (Ifnar-/-) (Bouloy *et al.*, 2001). The Clone-13 strain has been used in several African countries, including South Africa, Botswana, Kenya, Namibia, Zambia, and Mozambique (Faburay *et al.*, 2017). The vaccine was shown to be highly immunogenic and considered safe for administration in pregnant animals, with a single dose being sufficient to induce protection. Furthermore, no detectable viremia in vaccinated ruminants was observed, which reduces the risk of virus transmission to the foetus or to mosquito vectors (Dungu *et al.*, 2010). However, the Clone-13 vaccine was evaluated at an overdose (1×10^6 to 1×10^7 $TCID_{50}$/dose) in the first trimester of pregnant animals, and the virus was detected in the placenta of vaccinated ewes, also causing stillbirths and fetal malformations. The Clone-13 was further assessed for its stability at -20 °C, and the vaccine was found to be stable for a period of less than twelve months (Makoschey *et al.*, 2016). According to the TPP draft, the Clone-13 vaccine strain does not meet the total requirements of a good RVFV vaccine for use in the field, since it may be a potential concern regarding vaccination at early gestation (Alkan *et al.*, 2023).

To improve stability of the RVFV clone-13 vaccine, the thermostable Clone-13 was developed from isolating the RVFV Clone-13 cultured on Vero cells at 56 °C. Virus isolation was achieved following a series of heating and selection cycles which resulted into a heat-stable variant strain (Daouam *et al.*, 2015, Daouam *et al.*, 2020). The vaccine developed from this virus isolate was demonstrated to be safe and immunogenic in sheep, goats, cattle, and pregnant camels (Daouam *et al.*, 2014, Daouam *et al.*, 2015). The thermostable Clone-13 virus has shown an improved thermostability at both 37 °C and 45 °C when compared to the original Clone-13 virus. The virus further demonstrated an improved shelf life of over 18 months when stored at 4 °C in a lyophilized form (Daouam *et al.*, 2020). The thermostable Clone-13 vaccine was registered under in Morocco under the names RIFTOVAX-LR and RIFTOVAX-SR. In addition, the vaccine was evaluated for its immunogenicity in ruminants in Senegal and Mali (Dungu *et al.*, 2018).

The live attenuated RVFV Smithburn is one of the oldest and widely used vaccine strain. It was first isolated in 1944 from a mosquito *Eretmapodites* specie in Uganda, initially named the Entebbe strain (Smithburn *et al.*, 1948, Grobbelaar *et al.*, 2011). Between 1953 and 1985, Smithburn and his colleagues developed the neurotropic Smithburn strain from 87 serial passages of the Entebbe strain in mouse brain (intracerebral passages), 54 passages in embryonated chicken egg and additional 16 passages in mouse brain for vaccine development. The vaccine strain was later modified in 1971 by amplification in Baby Hamster Kidney (BHK-21) cell culture, resulting in the live attenuated vaccine for

vaccination of livestock in endemic countries (Ikegami and Makino, 2009). The RVFV Smithburn vaccine has successfully been used in susceptible animals in countries such as South Africa, Kenya Saudi Arabia and Egypt (Ikegami and Makino, 2009). The Smithburn strain of RVFV had also been used to propagate the live attenuated vaccines in Kenya and Egypt in the year 1960 and 1994, respectively. Though the vaccine is efficacious and cost-effective, it is only recommended for non-pregnant animals, as it has been known to induce residual pathogenicity, abortions, fetal malformations, as well as potential for reversion to virulence. As a result, the live attenuated vaccine is prohibited in pregnant animals and restricted to non-endemic countries. Additionally, during outbreaks, the use of the vaccine can lead to reassortment, resulting in increased diversity of the virus (Ikegami, 2012). This vaccine has been associated with pathological changes in the liver of newborn animals and abortions in pregnant ewes. The live attenuated Smithburn vaccine does not comply to the TPP RVFV vaccine requirements based on its safety profile in vaccinated animals, can be transmitted to mosquitoes and is not DIVA compliant. Nevertheless, the RVF Smithburn vaccine induces protective immune response following a single dose of administration and provides a long duration of immunity against the virus (Fawzy and Helmy, 2019). The vaccine has been widely distributed and have recorded over one million doses utilised during the sereve outbreaks reported in in South Africa and Kenya (1951–1968), 3 million doses in Zimbabwe (1978–1979), 4.2 million doses in South Africa, six million doses in Zimbabwe (1969–1970), 10 million doses in Saudi Arabia (2001), and 22 million doses in Namibia and South Africa (1974–1976) (Dungu *et al.*, 2013).

The MP-12 vaccine strain was developed by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) in the 1980s (Caplen *et al.*, 1985). The strain was generated from the virulent ZH548 strain isolated from an Egyptian patient, serially passaged 12x in MRC-5 cells supplemented with a chemical mutagen 5-fluorouracil (Caplen *et al.*, 1985, Ikegami, 2017). The vaccine was developed for use in both livestock and humans. Though the MP-12 vaccine was found to be temperature sensitive, it was also shown to be safe for use in pregnant animals as it did not induce abortions, fetal malformation or virus shedding in milk when administered after 3 months of gestation. It is only when the vaccine was administered at an early stage of pregnancy that about 4% of lambs were aborted (Morrill and Peters, 2011). Nevertheless, the vaccine showed a potential beneficial effect of inducing protection against RVF in pregnant animals to also transfer immunity in newborn lambs. Though the MP-12 vaccine strain induces abortions and fetal malformations, it was also shown to be safe, immunogenic, and tolerable when administered at an adequate dose. Meanwhile, the vaccine was shown to induce protection in vaccinated individuals after challenge with ZH501 virulent strain (Pittman *et al.*, 2016).

2.9.2 Inactivated RVFV vaccines

Inactivated vaccines are produced using chemically treated pathogens to obtain non-infectious antigens, which then stimulate the immune response (Sanders *et al.*, 2015). These antigens are primarily composed of killed viruses that are enhanced with adjuvants to improve the cell-mediated immune response (Didierlaurent *et al*., 2009; Verma *et al.*, 2023). The first platform used for the development and production of the RVFV vaccines used formalin inactivated Entebbe strain isolated from mosquitoes in Uganda (Swanepoel *et al.*, 1994, Faburay *et al.*, 2017). The Entebbe strain underwent 176 intraperitoneal or intravenous passages in mice and was then propagated in African Green Monkey cells before being inactivated using formalin for the production of the NDBR103 vaccine (Randall *et al.*, 1964). This vaccine was successfully used for immunisation of 500 human volunteers, including 963 United Nations soldiers on a three-dose regime in 1977 (Niklasson *et al.*, 1985). The strain was further utilised for developing an inactivated TSI GSD 200 vaccine by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) (Faburay *et al.*, 2017). Humans immunised with this vaccine seroconverted after a primary three dose vaccination, followed a boost after six months in a 12-month trial (Pittman *et al.*, 1999).

Currently, there are three inactivated RVF vaccines developed specifically for use in livestock in endemic regions (Alkan *et al.*, 2023). The first vaccine was manufactured by the Onderstepoort Biological Products (OBP, South Africa) from the virulent field strain (HB74) of the RVFV isolated from a cattle in South Africa, propagated on BHK-21 cell culture, formalin inactivated and adjuvanted with aluminium hydroxide gel (Barnard and Botha, 1977). The vaccine is recommended for use in cattle, sheep, and goats at any age regardless of the stage of pregnancy or lactation, as well as the calves and lambs above six months old of age (Alkan *et al.*, 2023). The second inactivated RVFV vaccine was produced by the Egyptian Holding Company for Biological Products and Vaccines (VACSERA) in Egypt, using the formalin inactivated RVFV-Menya/sheep/258 strain adjuvanted with alum (Ahmed Kamal, 2011). Another vaccine produced in Egypt was by Veterinary Serum and Vaccine Research Institute (VSVRI, Egypt) from binary ethylenimine (BEI)-inactivated ZH501 strain adjuvanted with alum (Ahmed Kamal, 2011, Alhaj, 2016). Unlike the live attenuated vaccines that induces a desired level of protection after a single dose of administration, the inactivated RVF vaccines require two initial doses and annual booster doses to maintain a long-lasting protective immunity (Minke *et al.*, 2004, Alkan *et al.*, 2023). Nevertheless, the inactivated RVFV vaccines are considered safer options for use in pregnant animals and cannot revert to virulence or have a risk of reassortment nor vector transmission (Monath *et al.*, 2020). However, the vaccine manufacturing may pose safety concerns to personnel handling the virulent strain of the virus for processing (Petrova *et al.*, 2020). Therefore, a RVFV strain

that provides safety for handling during vaccine manufacturing may serve a safer option to consider for vaccine development that meet the key requirements proposed by the WHO.

2.9.3 New technologies for RVFV vaccine development approach

2.9.3.1 Recombinant RVFV vaccines

Many virus vectors were developed and used as vaccine candidates for controlling the spread of RVFV, including: the heterologous vectors expressing Gn and/or Gc glycoproteins and non-structural proteins of RVFV. These vectors include the lumpy skin disease virus (LSDV), used to experimentally express the RVFV glycoproteins to generate neutralising antibodies against LSDV, RVFV and sheep poxvirus (Wallace *et al.*, 2006, Wallace *et al.*, 2020). The second vector was developed from the vaccinia virus Copenhagen strain (Vco) lacking the virulent gene. This vector is used to express the RVFV structural glycoproteins (Gn and Gc) which were shown to be safe for use in mice, primates and ruminants, and induces protective antibody titres (Papin *et al.*, 2011). The third RVFV vectored vaccine was generated from the alphavirus (Sindbis) replicon vaccine which expresses the RVFV glycoproteins and the non-structural NSm protein. The vaccine was shown to induce neutralising antibody immune response in sheep, and 100% protection against RVFV in mice (Heise *et al.*, 2009). The fourth vectored RVFV vaccine was developed from modified vaccinia virus ankara (MVA) vector which was developed to express RVFV glycoproteins and showed efficacy in murine models (López-Gil *et al.*, 2013). The other vaccine was developed from recombinant new castle disease virus (NDV) constructed as a vector to express the RVFV Gn glycoprotein, which was shown to induce neutralising antibodies against RVFV in sheep and calves following 2 doses of administration (Kortekaas *et al.*, 2010, Kortekaas *et al.*, 2011). The other recombinant RVFV vaccine was developed from chimpanzee adenovirus construct vector (ChAdOx1-GnGc) incorporated with the RVFV glycoproteins, and had also shown to induce humoral and cell-mediated immunity with complete protection against RVFV infection in ruminants (Warimwe *et al.*, 2013, Warimwe *et al.*, 2016). Lastly, the equine herpesvirus type 1 (EHV-1) vector vaccine that expresses the RVFV glycoproteins, and was shown to effectively induce RVFV neutralizing antibody titres in sheep (Said *et al.*, 2017). The recombinant vaccine products were demonstrated to possess improved safety and efficacy in both pregnant and non-pregnant sheep (Warimwe *et al*., 2013; Said *et al*., 2017). However, the vaccines were experimentally tested and still require to be evaluated through the final stages of full product development including the stability data of the products, challenge studies in target animal species, as well as duration of immunity in vaccinated animals.

2.9.3.2 The RVF Plasmid DNA vaccines

A number of RVFV DNA vaccines were developed and evaluated for safety and immunogenicity in experimental mice. Spik et al. (2006) reported the RVFV vaccine developed from DNA plasmid (pWRG7077) encoding the RVFV glycoproteins, induced protective antibody immune response in mice when used as a monovalent or in combination with other DNA plasmid vaccines (Spik *et al.*, 2006). Another DNA vaccine encoding nucleoprotein N (RVFV cDNA N) or Gn and Gc (RVFV cDNA GnGc) induced neutralising antibody titres of >25 and >75 PRNT50, respectively (Lagerqvist *et al.*, 2009). A high neutralising antibody titres were induced in mice immunised with a DNA plasmid PTR600 expressing RVFV Gn coupled to 3 copies of the complement protein C3d. These antibody titres were comparable to those induced by the MP-12 vaccine and offered protection in vaccinated mice against the wild type RVFV strain (Bhardwaj *et al.*, 2010). Most recently, the phRVF/Gn and phRVF/Gc DNA vaccines were produced using plasmid phMGFP vector (Promega) which codes for a Monster Green® Fluorescent Protein (mGFP), and was cloned with the full-length of the RVFV Gn and Gc genes (Selina *et al.*, 2020). The DNA vaccines still require to be further investigated and evaluated in target hosts for full product development.

2.9.3.3 Virus-like particles based RVF vaccines

The virus-like particles (VLPs) of RVFV were generated from viral glycoproteins, or a combination of glycoproteins and nucleoproteins for vaccine development (Habjan *et al.*, 2008, Liu *et al.*, 2008, Pichlmair *et al.*, 2010). The positive aspect with developing vaccines using VLPs is that the particles are capable of stimulating both humoral and cellular immune responses against RVFV (Beyer *et al.*, 2001). Furthermore, the particles have similar morphological aspect to the virulent virus and can easily be adsorbed and digested in endolysosomes. VLP vaccines are non-replicating and do not require inactivation, and the particles are capable of stimulating both MHC class I and II responses (Beyer *et al.*, 2001, Roldão *et al.*, 2010). RVF VLPs are also stable and immunogenic producing high neutralising antibody titres in vaccinated mice, as well as offering protection against RVF challenge with ZH548 strain (Habjan *et al.*, 2008, Näslund *et al.*, 2009, Pichlmair *et al.*, 2010). The main disadvantages of the VLPs vaccine platform is that production of the vaccine at high scale is expensive and more than two subsequent vaccinations may be required to obtain a desired immune response from the immunised animals (Näslund *et al.*, 2009, Fawzy and Helmy, 2019).

2.10 Adjuvants used in formulation of vaccines

Adjuvants are substances or biomolecules incorporated into vaccine formulations to improve specific immune responses and increase vaccine efficacy (Sivakumar *et al.*, 2011). They have been widely utilized in the formulation of various inactivated vaccines, as well as the new generation recombinant and plasmid-DNA vaccines (Barnard and Botha, 1977, Salama, 2006, Smith, 2009, Chandran *et al.*, 2010, Hotez and Bottazzi, 2022). According to regulatory agencies including the U.S. Food and Drug Administration and the European Medicines Agency, selecting a suitable adjuvant with the qualities of enhancing vaccine safety and efficacy is required when formulating vaccines (Teixeira *et al.*, 2020). Qualities of effective adjuvants after vaccine formulation include the ability to induce a specific immune response, increase the levels of antibodies produced, prolong the duration of immunity, and induce a cytotoxic T lymphocyte response (Moni *et al.*, 2023). Numerous types of adjuvants with distinct mechanisms of action have been developed over the past nine decades to enhance the effective delivery of antigens to immune cells without modifying them. These adjuvants include the immune-stimulating complexes, biomolecules obtained from bacteria, emulsion-based adjuvants, mineral salts, and innovative delivery systems such as liposomes or polymers (Sivakumar *et al.*, 2011, Moni *et al.*, 2023). Historically, mineral salts such as aluminum hydroxide, potassium aluminum sulfate (often referred to as alum), and aluminum phosphate have been effectively utilized for vaccine formulation for more the 90 years (Hogenesch *et al.*, 2018).

Aluminium salts were initiated for vaccine formulation since 1930s to 1950s with diphtheria and tetanus vaccines (Maschmann *et al.*, 1931, Zhao *et al.*, 2023). These adjuvants are the most preferred for use in vaccine formulations, due to their good safety profile and inducing efficacious immune response (Wu and Liu, 2021). Aluminum hydroxide gel has the capacity to adsorb protein antigens from an aqueous solution using the procedure named alum-adsorbed vaccine (Maschmann *et al.*, 1931). One of the significant advantages of aluminum-adsorbed vaccines is that they can elicit a high antibody titres detected from primary immunization (Volk and Bunney, 1939, Baylor *et al.*, 2002). This occurs due their capacity to establish a transitory depot on site of injection, which slowly releases the antigens. This activates antigen-presenting cells (APCs) to the injection site, facilitating their engulfment and transportation to the lymph nodes for a potent immune response, as illustrated in Figure 2.5. Vaccines formulated with aluminum hydroxide adjuvants have been shown to be safe following administration by subcutaneous and intramuscular injection (Volk and Bunney, 1942, Butler *et al.*, 1969, Smith, 2009, Hotez and Bottazzi, 2022). Various inactivated antigens including the recombinant epsilon toxoid, RVFV, diphtheria, Botulinum toxoid, and Covid-19 were formulated with aluminium hydroxide adjuvant and have shown to elicit high neutralising antibody immune response

(Maschmann *et al.*, 1931, Barnard and Botha, 1977, Smith, 2009, Chandran *et al.*, 2010, Hotez and Bottazzi, 2022). Currently, numerous research is being conducted to investigate new technologies, including innovative chemical compounds and nanoparticles for their compatibility for use as next-generation adjuvants (Mitchell *et al.*, 2021, Mbhele *et al.*, 2023).

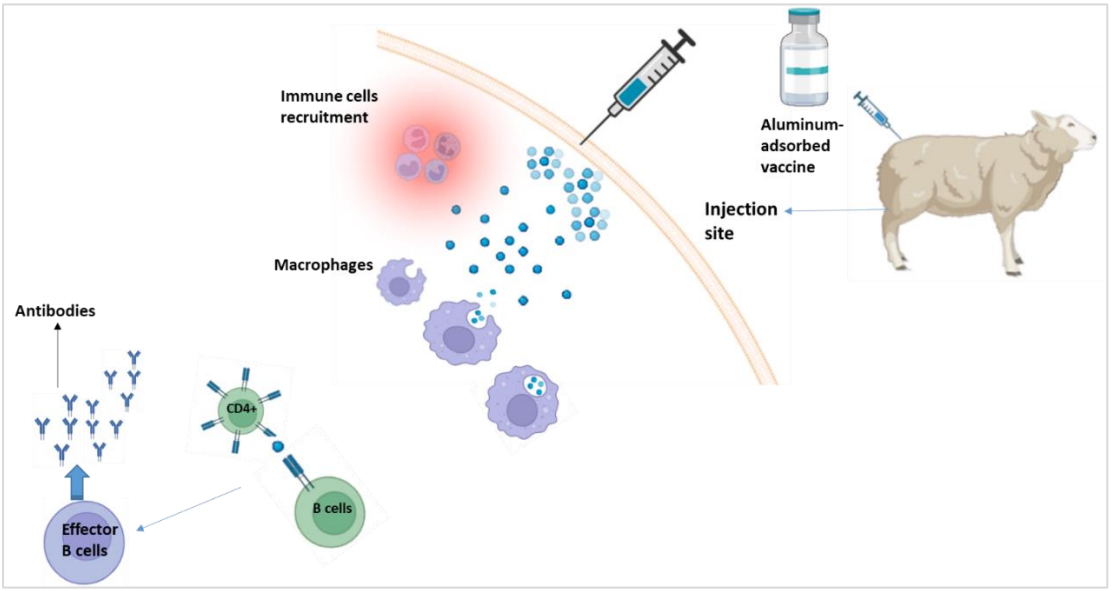


Figure 2. 5: A schematic diagram illustrating the action mechanism of the aluminum hydroxide adjuvant utilized in vaccine formulation. The aluminum hydroxide adjuvant facilitates the development of a depot at the site of injection, which establishes the slow release of the antigens. This process activates macrophages at the injection site, leading to their engulfment and transportation of the antigen to the lymph nodes, where they mediate an efficacious immune response. *Created with Biorender.com, and adapted from (Moni et al., 2023).*

2.11 Vaccination schedules of RVF

Vaccination of livestock against the RVF disease is conducted following warnings of possible outbreaks in affected regions. These warnings are reported to enable vaccination campaigns which are supported by the WOAH, WHO and the Food and Agriculture Organization of the United Nations (Fao and Africa—El Niño, 2015). This vaccination strategy is conducted due to the inter-epizootic nature of RVF, which makes it difficult to carry-out regular vaccinations of the disease in endemic regions (Dungu *et al.*, 2018). The WHO recommends vaccinating livestock before the onset of an outbreak. Vaccinating animals with live attenuated RVF vaccines during or after an outbreak is deemed insufficient, as it may lead to the emergence of genetic reassortants with virulent strains of the virus (Turell *et al.*, 1990, Grobbelaar *et al.*, 2011, Monath *et al.*, 2020). Additionally, vaccination during RVF outbreaks can

exacerbate the spread of the virus, as multi-dose syringes used in mass animal vaccination campaigns can easily transmit the virus from viraemic RVFV-positive animals to other susceptible animals within the herd (Faburay *et al.*, 2017). Furthermore, it has been reported that vaccination against RVF is inconsistent across Africa and the Arabian Peninsula, with most countries failing to implement vaccination programs due to the epizootic nature of RVF or lack of reported cases (Dungu *et al.*, 2018). In some cases reported, the vaccines administered during disease outbreaks failed to induce protective immune response on the anticipated time and the animals get infected by the pathogen before protective immunity against the disease could develop (Dungu *et al.*, 2013). This might raise concerns to the quality of the vaccine used for immunisation of animals, rather than looking into the vaccination schedule. Therefore, to simplify vaccination regimes, vaccines must be administered prior exposure to a disease so that it is given enough time to build immune response for protection against future infections. For instance, a low seropositivity of 10% was detected in cattle vaccinated with the inactivated RVF vaccine (ZH501 strain) and it was difficult to conclude if this has occurred due to the inefficacy of the vaccine or issues related with implementing vaccination regimes (Byomi *et al.*, 2014, Mroz *et al.*, 2017). Similarly, Elfadil et al. (2006) had also reported a low vaccination coverage of sheep and goats in 2004, ranging from 22.2% in the Jizan district to 39.3% in the Alarda district following immunisation campaigns with the live attenuated RVF Smithburn (Elfadil *et al.*, 2006). A new vaccination strategy is therefore required for implementation in RVF endemic regions. Given the limited understanding of RVF disease and the current vaccination strategies, it may be advantageous to explore the possibility of using the vaccine as a combination product with another commonly utilised product in the field, such as the pulpy kidney vaccine for immunisation of ruminants. This approach could enable farmers to administer a single dose for prevention of both RVF and pulpy kidney diseases.

2.12 The pulpy kidney disease

Pulpy kidney is a fatal form of enterotoxaemia that affects sheep and goats, and is caused by an overgrowth of the bacterium *Clostridium perfringens* type D in the gut (Lobato *et al.*, 2007). The disease typically affects lambs that are over the age of two weeks. This condition arises due to several factors including change in diet, particularly when ruminants are fed high amounts of grain or given access to lush green pasture, poor nutritional status, pregnancy toxaemia, the use of tranquiliser (phenothiazine), parasite infestation, overdose of a broad spectrum anthelmintic (netobimin) (Uzal and Songer, 2008, Jemal *et al.*, 2016, Farooq *et al.*, 2024). The rapid growth of the organism results in the production of the epsilon toxin, which accumulates in the gut as an inactive prototoxin. The toxin

is activated by peptide cleavage through the action of digestive enzymes, such as trypsin, chymotrypsin, and lambda toxin, which are produced by the bacteria (Worthington and Mülders, 1977, Uzal *et al.*, 2010). This activation result in the release of the 22-29 carboxyl and the 10-13 amino-terminal residues, consequently creating a reduced molecular mass of the epsilon toxin from 32.9 kDa to 28 kDa, and changes its isoelectric point from 8.02 to 5.36 (Alves *et al.*, 2014). This allows the toxin to escape the gut membrane and be absorbed into the systemic circulation, increasing capillary permeability in many organs and tissues, which leads to severe pathologies in the intestinal mucosa, kidneys, brain, and liver (Buxton, 1978). The epsilon toxin is the third most potent bacterial toxin known after the botulinum and tetanus toxins and was classified as a category B biological agent by the Centers for Disease Control and Prevention (CDC) of the United States until 2012 (Gill, 1982, Harkness *et al.*, 2012, Burnett, 2024). Pulpy kidney disease is characterised by devastating symptoms including fever, bloody diarrhoea, pale mucous membranes, teeth grinding, reduced milk production, blurred vision, and often leading to sudden death in per-acute cases (Renu *et al.*, 2021, Farooq *et al.*, 2024). The disease has a worldwide distribution and poses a substantial threat to animal health leading to economic instability within the agricultural sector (Hussain *et al.*, 2022). Pulpy kidney infections can be diagnosed by antigen detection using ELISA, genetic detection using PCR technique, or neutralisation assay in mice using antisera for *C. perfringens* type D epsilon toxin (Oakley and Warrack, 1953, Layana *et al.*, 2006, Albini *et al.*, 2008, Goldstein *et al.*, 2012). There is no effective therapeutic treatment against pulpy kidney infections in animals (Stiles *et al.*, 2013). However, upon early detection of the disease, animals may be given *C. perfringens* type D anti-epsilon toxin antibodies which provides immediate neutralisation of the toxin within infected animals. Prevention and control of the pulpy kidney disease in ruminants is achieved by limiting the food intake for animals, avoiding sudden changes in diet and environment, and vaccination of livestock (Farooq *et al.*, 2024). Livestock vaccination is regarded as the most effective way of safeguarding the livestock population against pulpy kidney infection (Uzal and Songer, 2008, Khiav and Zahmatkesh, 2021).

There are various clostridial vaccines that are commercially available for the immunization of ruminants. These vaccines exist in both monovalent or multivalent forms, and are generally affordable (Whaley, 2024). The multivalent clostridial vaccines consist of a combination of formaldehyde toxoids and bacterins from a variety of species (Tizard, 2021). Some of the commonly used vaccines are listed in Table 2.2). These vaccines are effective in protecting ruminants against a range of Clostridial diseases, such as tetanus, swelled head, gas gangrene, black disease, black leg, black quarter, and other diseases including pulpy kidney disease. Though there are a variety of multivalent pulpy kidney vaccines available, the monovalent form of the vaccine is manufactured by few pharmaceutical

companies including OBP (enterotoxaemia vaccines), and MSD Animal Health (Pulpyvax® 1-Shot and Pulpyvax®) (Table 2.2). The vaccines are administered to sheep and goats in two doses, provided 3-6 weeks apart. Adult sheep are vaccinated annually following the two doses, and 3-4 weeks prior to lambing. This approach has been shown to increase the antibody levels in the blood, providing passive immunity to newborn lambs up to 4-6 weeks of age through colostrum (De La Rosa *et al.*, 1997). The pulpy kidney vaccines have been widely used for prophylactic immunisation of ruminants against pulpy kidney, in conjunction with limiting the animal's food intake.

Table 2. 2: Monovalent and multivalent vaccines commercially available for prophylactic immunization of ruminants against pulpy kidney disease.

Name of vaccine	Vaccine composition	Supplier	Host	Dose and route of administration	References
Monovalent vaccines					
Enterotoxaemia	*Clostridium perfringens* type D, alum or oil adjuvant	OBP, SA	Sheep and goats	1 mL, S/C	Product leaflet, file:///C:/Users/Matome/Downloads/ENTEROTOXAEMIA%20(ALUM-PRECIPITATED)%20VACCINE%20FOR%20SHEEP%20AND%20GOAT.pdf file:///C:/Users/Matome/Downloads/ENTEROTOAXAEMIA%20VACCINE.pdf
Pulpyvax® 1-Shot	Toxoid produced by *C. perfringens* type D	MSD Animal Health, USA	Sheep	1 mL, intramuscular	Product leaflet, https://www.msd-animal-health.co.za/products/pulpyvax-1-shot/
Pulpivax®	Toxoids produced by *C. perfringens* type D, alum adjuvant	MSD Animal Health, USA	Sheep and goats	1 mL, S/C	Product leaflet, https://www.msd-animal-health.co.za/products/pulpyvax/
Bivalent/multivalent vaccines					
Lamb vaccine	*C. perfringens* (type D) toxoid and tetanus antitoxin	MSD Animal Health, USA	Sheep	2mL, S/C	Product leaflet, https://www.msd-animal-health.co.nz/products/lamb-vaccine/
Enterovac vaccine	Toxoids of *C. perfringens* type B and D	Intervac Pvt Ltd, Canada	Sheep, goats and cattle	1 mL (sheep and goats); 4 mL (cattle), S/C	Product leaflet, https://intervacpvtltd.com/product/intervac-enterovac-vaccine/

Multivax P-plus	Toxoids of *C. perfringens* types B, C and D, *Clostridium septicum, Clostridium tetani, Clostridium novyi* type B, and formalin inactivated cells and toxoids of *Clostridium chauvoei* and *Mannheimia* (*Pasteurella) haemolytica* and *Pasteurella trehalosi,* and aluminium hydroxide adjuvant	MSD Animal Health, USA	Sheep	2 mL, S/C	Product leaflet, https://www.msd-animal-health.co.za/products/multivax-p-plus/
Bravoxin	Toxoids of *C. perfringens* types A (α), B & C (β), and D (ε), *C. chauvoei* whole culture, *toxoid of C. novyi* type B, *C. septicum* toxoid, *C. tetani* toxoid, *C. sordellii* toxoid, *C. haemolyticum* toxoid, and aluminum potassium sulphate (alum)	MSD Animal Health, UK	Cattle and sheep	1 mL/ S/C	Product leaflet, https://www.msd-animal-health-hub.co.uk/Products/Bravoxin
Lambivac	*C. perfringens* beta and epsilon toxoids, *C. tetani* toxoid	MSD Animal Health, UK	Sheep and pigs	2 mL, S/C	Product leaflet, https://www.msd-animal-health-hub.co.uk/Products/Lambivac
Multiclos	Toxoids from cultures of *C. Perfringens type C and D, C. septicum, C. novyi* type B, and *C. sordellii*, as well as the anacultures of *C. chauvoei* and *C. novyi* type D	MSD Animal Health, USA	Cattle and sheep	5 mL (cattle), 3 mL 9sheep, S/C	Product leaflet, https://www.msd-animal-health.co.za/products/multiclos/

Enterotect P	*C. perfringens type D (epsilon toxoid), M. haemolytica* leukotoxin with inactivated *C. perfringens* type A (alpha toxoid)	Design Biologix CC, SA	Sheep and goats	1 mL, S/C	Product leaflet, https://www.msd-animal-health.co.za/products/enterotect-p/
Clostrivax O	Toxoids from *C. perfringens* types A and D, *C. tetani*, *C. Novyi* types A and B, *C. septicum, M. haemolytica* type A1 leukotoxin, formalin inactivated, and anacultures of formalin inactivated *C. chauvoei*, and *B. trehalose*	Design Biologix CC, SA	Sheep	2 mL, S/C	Product leaflet, https://designbio.co.za/wp-content/uploads/2023/08/PI-CO-Package-Insert-Clostrivax-O.pdf

2.13 Combination vaccines

The technology of combining multiple vaccine products into a single dose began in 1943 at the United States with the diphtheria, pertussis, tetanus (DPT) combination vaccine for human (Decker *et al.*, 1992). This technology allowed for single immunization shot of safe and efficacious vaccine against multiple life-threatening diseases in vaccination programmes for children (Pichichero, 2000). Following successful children's combination vaccines developed, veterinary combination vaccines were also considered and the first licensed product was for the prevention against anthrax and blackleg in 1940s (Provost and Perreau, 1978). Combination vaccines have been found to reduce production costs, and increase convenience and efficacy in the logistics of prophylactic projects in the field (Provost and Perreau, 1978). Utilising combination vaccines in the field minimises the chances of being forced to decide what vaccine or vaccines to use in a herd, weighing possible disease losses against the cost of vaccine, the cost of its administration, and the time and expense involved in the restraint of animals (Bovine, 2014). In addition, combination vaccines offer a significant advantage to small-scale livestock farmers, as they require only a single administration to provide protection against multiple pathogens (Safini *et al.*, 2022). This approach may also improve vaccination coverage for multiple diseases, as demonstrated in a study which evaluated the safety and efficacy of the RVF/PK combination vaccine in merino sheep (Bovine, 2014). This combination vaccine demonstrated a good safety profile and elicited neutralising antibody titres against both the RVFV and PK antigens in vaccinated sheep.

2.14 References

Adams, M. J., Lefkowitz, E. J., King, A. M., Harrach, B., Harrison, R. L., Knowles, N. J., Kropinski, A. M., Krupovic, M., Kuhn, J. H. & Mushegian, A. R. (2017). Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2017). *Archives of Virology,* 162**,** 2505-2538.

Ahmed Kamal, S. (2011). Observations on rift valley fever virus and vaccines in Egypt. *Virology Journal,* 8**,** 1-9.

Albini, S., Brodard, I., Jaussi, A., Wollschlaeger, N., Frey, J., Miserez, R. & Abril, C. (2008). Real-time multiplex PCR assays for reliable detection of Clostridium perfringens toxin genes in animal isolates. *Veterinary Microbiology,* 127**,** 179-185.

Alhaj, M. (2016). Safety and efficacy profile of commercial veterinary vaccines against Rift Valley fever: a review study. *Journal of Immunology Research,* 2016.

Alkan, C., Jurado-Cobena, E. & Ikegami, T. (2023). Advancements in Rift Valley fever vaccines: a historical overview and prospects for next generation candidates. *npj Vaccines,* 8**,** 171.

Allen, E. R., Krumm, S. A., Raghwani, J., Halldorsson, S., Elliott, A., Graham, V. A., Koudriakova, E., Harlos, K., Wright, D. & Warimwe, G. M. (2018). A protective monoclonal antibody targets a site of vulnerability on the surface of Rift Valley fever virus. *Cell Reports,* 25**,** 3750-3758. e4.

Alomar, A. A., Campbell, L. P., Mathias, D. K. & Burkett-Cadena, N. D. (2023). Rift Valley Fever Virus: A Zoonotic Vector-Borne Pathogen Affecting Human and Livestock Health: ENY2099/IN1403, 5/2023. *EDIS,* 2023.

Alves, G. G., De Ávila, R. a. M., Chávez-Olórtegui, C. D. & Lobato, F. C. F. (2014). Clostridium perfringens epsilon toxin: the third most potent bacterial toxin known. *Anaerobe,* 30**,** 102-107.

Anyamba, A., Linthicum, K. J., Mahoney, R., Tucker, C. J. & Kelley, P. W. (2002). Mapping potential risk of rift valley fever outbreaks in African savannas using vegetation index time series data. *Photogrammetric Engineering & Remote Sensing,* 68.

Anyamba, A., Linthicum, K. J., Small, J., Britch, S. C., Pak, E., De La Rocque, S., Formenty, P., Hightower, A. W., Breiman, R. F. & Chretien, J.-P. (2010). Prediction, assessment of the Rift Valley fever activity in East and Southern Africa 2006–2008 and possible vector control strategies. *The American Journal of Tropical Medicine and Hygiene,* 83**,** 43.

Barnard, B. & Botha, M. (1977). An inactivated rift valley fever vaccine. *Journal of the South African Veterinary Association,* 48**,** 45-48.

Baylor, N. W., Egan, W. & Richman, P. (2002). Aluminum salts in vaccines—US perspective. *Vaccine,* 20**,** S18-S23.

Besselaar, T. & Blackburn, N. (1991). Topological mapping of antigenic sites on the Rift Valley fever virus envelope glycoproteins using monoclonal antibodies. *Archives of virology,* 121**,** 111-124.

Besselaar, T. & Blackburn, N. (1992). The synergistic neutralization of Rift Valley fever virus by monoclonal antibodies to the envelope glycoproteins. *Archives of Virology,* 125**,** 239-250.

Beyer, T., Herrmann, M., Reiser, C., Bertling, W. & Hess, J. (2001). Bacterial carriers and virus-like-particles as antigen delivery devices: role of dendritic cells in antigen presentation. *Current Drug Targets-Infectious Disorders,* 1**,** 287-302.

Bhardwaj, N., Heise, M. T. & Ross, T. M. (2010). Vaccination with DNA plasmids expressing Gn coupled to C3d or alphavirus replicons expressing gn protects mice against Rift Valley fever virus. *PLOS Neglected Tropical Diseases,* 4**,** e725.

Bird, B. H., Albarino, C. G., Hartman, A. L., Erickson, B. R., Ksiazek, T. G. & Nichol, S. T. (2008). Rift valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. *Journal of Virology,* 82**,** 2681-2691.

Bird, B. H., Bawiec, D. A., Ksiazek, T. G., Shoemaker, T. R. & Nichol, S. T. (2007). Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. *Journal of Clinical Microbiology,* 45**,** 3506-3513.

Bird, B. H., Ksiazek, T. G., Nichol, S. T. & Maclachlan, N. J. (2009). Rift Valley fever virus. *Journal of the American Veterinary Medical Association,* 234**,** 883-893.

Bob, N. S., Barry, M. A., Diagne, M. M., Faye, M., Ndione, M. H. D., Diallo, A., Diop, M., Diop, B., Faye, O. & Loucoubar, C. (2022). Detection of rift valley fever virus lineage H from South Africa through the syndromic sentinel surveillance network in Senegal. *Open Forum Infectious Diseases*, Oxford University Press US, ofab655.

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M. & Haller, O. (2001). Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs. *Journal of Virology,* 75**,** 1371-1377.

Bouloy, M. & Weber, F. (2010). Molecular biology of Rift Valley fever virus. *The Open Virology Journal,* 4**,** 8.

Bovine, C. (2014). Contagious bovine pleuropneumonia. *Transboundry Animal Diseases Bulletin*, 24. Fao.org

Brinkmann, A., Ergünay, K. Radonić, A., Kocak Tufan, Z., Domingo, C. & Nitsche, A. (2017). Development and preliminary evaluation of a multiplexed amplification and next generation sequencing method for viral hemorrhagic fever diagnostics. *PLOS Neglected Tropical Diseases,* 11**,** e0006075.

Burnett, L. B. (2024). Clostridium perfringens toxin (epsilon toxin) attack. *Ciottone's Disaster Medicine (Third Edition),* 2024, 826-829.

Butler, N., Voyce, M., Burland, W. & Hilton, M. L. (1969). Advantages of aluminium hydroxide adsorbed combined diphtheria, tetanus, and pertussis vaccines for the immunization of infants. *Br Med J,* 1**,** 663-666.

Byomi, A., Samaha, H., Zidan, S. & Hadad, G. (2014). Some associated risk factors with the occurrence of Rift Valley Fever in animals and man in certain localities of Nile Delta, Egypt. *Assiut Veterinary Medical Journal,* 61**,** 10-17.

Caplen, H., Peters, C. & Bishop, D. H. (1985). Mutagen-directed attenuation of Rift Valley Fever virus as a method for vaccine development. *Journal of General Virology,* 66**,** 2271-2277.

Cêtre-Sossah, C., Pédarrieu, A., Juremalm, M., Jansen Van Vuren, P., Brun, A., Ould El Mamy, A. B., Héraud, J.-M., Filippone, C., Ravalohery, J.-P. & Chaabihi, H. (2019). Development and validation of a pen side test for Rift Valley Fever. *PLOS Neglected Tropical Diseases,* 13**,** e0007700.

Chandran, D., Naidu, S. S., Sugumar, P., Rani, G. S., Vijayan, S. P., Mathur, D., Garg, L. C. & Srinivasan, V. A. (2010). Development of a recombinant epsilon toxoid vaccine against enterotoxemia and its use as a combination vaccine with live attenuated Sheep Pox Virus against enterotoxemia and sheep pox. *Clinical and Vaccine Immunology.,* 17**,** 1013-1016.

Charlton, F. W., Hover, S., Fuller, J., Hewson, R., Fontana, J., Barr, J. N. & Mankouri, J. (2019). Cellular cholesterol abundance regulates potassium accumulation within endosomes and is an important determinant in bunyavirus entry. *Journal of Biological Chemistry,* 294**,** 7335-7347.

Chrun, T., Lacôte, S., Urien, C., Jouneau, L., Barc, C., Bouguyon, E., Contreras, V., Ferrier-Rembert, A., Peyrefitte, C. N. & Busquets, N. (2018). A Rift Valley fever virus Gn ectodomain-based DNA vaccine induces a partial protection not improved by APC targeting. *npj Vaccines,* 3**,** 14.

Collett, M. S. (1986). Messenger RNA of the M segment RNA of Rift Valley fever virus. *Virology,* 151**,** 151-156.

Daouam, S., Boumart, Z., Elarkam, A., Hamdi, J., Tadlaoui, K. & Ennaji, M. (2020). Comparative thermo-stability of two Rift Valley fever virus vaccine candidate CL13T with a recombinant arMP-12ΔNSm21/384. *Bioinformation,* 16**,** 547.

Daouam, S., Fakri, F. Z., Ennaji, M. M., Tadlaoui, K. O., Oura, C. & Elharrak, M. (2014). Heat stability of the Rift Valley fever virus clone 13 live vaccines. *Trials in Vaccinology,* 3**,** 61-64.

Daouam, S., Ghzal, F., Arkam, A., Naouli, Y., Jazouli, M., Ennaji, M., Tadlaoui, K., Oura, C. & El Harrak, M. (2015). Evaluation of the safety and efficacy of a live attenuated thermostable Rift Valley fever vaccine in sheep, goats and cattle. *Journal of Vaccines and Vaccination,* 6**,** 295.

Daubney, R., Hudson, J. & Garnham, P. (1931). Enzootic hepatitis or Rift Valley fever. An undescribed virus disease of sheep cattle and man from East Africa. *The Journal of Pathology and Bacteriology,* 34**,** 545-579.

De Boer, S., Kortekaas, J., De Haan, C., Rottier, P., Moormann, R. & Bosch, B. (2012a). Heparan sulfate facilitates Rift Valley fever virus entry into the cell. *Journal of Virology,* 86**,** 13767-13771.

De Boer, S., Kortekaas, J., Spel, L., Rottier, P., Moormann, R. & Bosch, B. (2012b). Acid-activated structural reorganization of the Rift Valley fever virus Gc fusion protein. *Journal of Virology,* 86**,** 13642-13652.

De Glanville, W. A., Nyarobi, J. M., Kibona, T., Halliday, J. E., Thomas, K. M., Allan, K. J., Johnson, P. C., Davis, A., Lankester, F. & Claxton, J. R. (2022). Inter-epidemic Rift Valley fever virus infection incidence and risks for zoonotic spillover in northern Tanzania. *PLoS Neglected Tropical Diseases,* 16**,** e0010871.

De La Rosa, C., Hogue, D. E. & Thonney, M. L. (1997). Vaccination schedules to raise antibody concentrations against∈-toxin of Clostridium perfringens in ewes and their triplet lambs. *Journal of Animal Science,* 75**,** 2328-2334.

Decker, M. D., Edwards, K. M., Bradley, R. & Palmer, P. (1992). Comparative trial in infants of four conjugate Haemophilus influenzae type b vaccines. *The Journal of Pediatrics,* 120**,** 184-189.

Didierlaurent, A.M., Morel, S., Lockman, L., Giannini, S.L., Bisteau, M., Carlsen, H., Kielland, A., Vosters, O., Vanderheyde, N., Schiavetti, F. and Larocque, D. (2009). AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *The Journal of immunology*, *183*, 6186-6197.

Dodd, K. A., Mcelroy, A. K., Jones, M. E., Nichol, S. T. & Spiropoulou, C. F. (2013). Rift Valley fever virus clearance and protection from neurologic disease are dependent on CD4+ T cell and virus-specific antibody responses. *Journal of Virology,* 87**,** 6161-6171.

Dondona, A. C., Aschenborn, O., Pinoni, C., Di Gialleonardo, L., Maseke, A., Bortone, G., Polci, A., Scacchia, M., Molini, U. & Monaco, F. (2016). Rift valley fever virus among wild ruminants, Etosha National Park, Namibia, 2011. *Emerging Infectious Diseases,* 22**,** 128.

Doyle, J. D., Barbeau, D. J., Cartwright, H. N. & Mcelroy, A. K. (2022). Immune correlates of protection following Rift Valley fever virus vaccination. *npj Vaccines,* 7**,** 129.

Drosten, C., Gottig, S., Schilling, S., Asper, M., Panning, M., Schmitz, H. & GüNther, S. (2002). Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. *Journal of Clinical Microbiology,* 40**,** 2323-2330.

Dungu, B., Donadeu, M. & Bouloy, M. (2013). Vaccination for the control of Rift Valley fever in enzootic and epizootic situations. *Vaccines and Diagnostics for Transboundary Animal Diseases,* 135, 61-72.

Dungu, B., Louw, I., Lubisi, A., Hunter, P., Von Teichman, B. F. & Bouloy, M. (2010). Evaluation of the efficacy and safety of the Rift Valley Fever Clone 13 vaccine in sheep. *Vaccine,* 28**,** 4581-4587.

Dungu, B., Lubisi, B. A. & Ikegami, T. (2018). Rift Valley fever vaccines: current and future needs. *Current opinion in virology,* 29**,** 8-15.

Elbeaino, T., Digiaro, M., Mielke-Ehret, N., Muehlbach, H.-P., Martelli, G. P. & Consortium, I. R. (2018). ICTV virus taxonomy profile: Fimoviridae. *Journal of General Virology,* 99**,** 1478-1479.

Elfadil, A., Hasab-Allah, K., Dafa-Allah, O. & Elmanea, A. (2006). The persistence of Rift Valley fever in the Jazan region of Saudi Arabia. *Revue Scientifique et Technique-Office International des Epizooties,* 25**,** 1131-1136.

Ellis, C. E., Mareledwane, V. E., Williams, R., Wallace, D. B. & Majiwa, P. A. (2014). Validation of an ELISA for the concurrent detection of total antibodies (IgM and IgG) to Rift Valley fever virus. *Onderstepoort Journal of Veterinary Research,* 81**,** 1-6.

Ellis, D., Simpson, D., Stamford, S. & Wahab, K. S. A. (1979). Rift Valley fever virus: some ultrastructural observations on material from the outbreak in Egypt 1977. *Journal of General Virology,* 42**,** 329-337.

Euler, M., Wang, Y., Nentwich, O., Piepenburg, O., Hufert, F. T. & Weidmann, M. (2012). Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. *Journal of Clinical Virology,* 54**,** 308-312.

Evans, A., Gakuya, F., Paweska, J., Rostal, M., Akoolo, L., Van Vuren, P. J., Manyibe, T., Macharia, J., Ksiazek, T. & Feikin, D. (2008). Prevalence of antibodies against Rift Valley fever virus in Kenyan wildlife. *Epidemiology & Infection,* 136**,** 1261-1269.

Faburay, B., Labeaud, A. D., Mcvey, D. S., Wilson, W. C. & Richt, J. A. (2017). Current status of Rift Valley fever vaccine development. *Vaccines,* 5**,** 29.

Fafetine, J. M., Tijhaar, E., Paweska, J. T., Neves, L. C., Hendriks, J., Swanepoel, R., Coetzer, J. A., Egberink, H. F. & Rutten, V. P. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. *Veterinary Microbiology,* 121**,** 29-38.

Fao, O. & Africa—El Niño, W. (2015). increased risk of Rift Valley fever—Warning to countries. *EMPRES WATCH,* 34.

Farooq, U., Khan, Y. A. & Ullah, R. (2024). Pulpy Kidney Disease: A Looming Threat to Goats-A Case Report. *Journal of Animal and Plant Research,* 1**,** 5-6.

Fawzy, M. & Helmy, Y. A. (2019). The one health approach is necessary for the control of Rift Valley fever infections in Egypt: A comprehensive review. *Viruses,* 11**,** 139.

Filone, C. M., Hanna, S. L., Caino, M. C., Bambina, S., Doms, R. W. & Cherry, S. (2010). Rift valley fever virus infection of human cells and insect hosts is promoted by protein kinase C epsilon. *PloS One,* 5**,** e15483.

Freiberg, A. N., Sherman, M. B., Morais, M. C., Holbrook, M. R. & Watowich, S. J. (2008). Three-dimensional organization of Rift Valley fever virus revealed by cryoelectron tomography. *Journal of Virology,* 82**,** 10341-10348.

Gaff, H. D., Hartley, D. M. & Leahy, N. P. (2007). An epidemiological model of Rift Valley fever. *Electronic Journal of Differential Equations (EJDE)[electronic only],* 2007**,** Paper No. 115, 12 p., electronic only-Paper No. 115, 12 p., electronic only.

Garcia, S., Crance, J. M., Billecocq, A., Peinnequin, A., Jouan, A., Bouloy, M. & Garin, D. (2001). Quantitative real-time PCR detection of Rift Valley fever virus and its application to evaluation of antiviral compounds. *Journal of Clinical Microbiology,* 39**,** 4456-4461.

Garrison, A. R., Alkhovsky, S. V., Avšič-Županc, T., Bente, D. A., Bergeron, É., Burt, F., Di Paola, N., Ergünay, K., Hewson, R. & Kuhn, J. H. (2020). ICTV virus taxonomy profile: Nairoviridae. *Journal of General Virology,* 101**,** 798-799.

Gaudreault, N. N., Indran, S. V., Balaraman, V., Wilson, W. C. & Richt, J. A. (2019). Molecular aspects of Rift Valley fever virus and the emergence of reassortants. *Virus Genes,* 55**,** 1-11.

Gerdes, G. (2004). Rift valley fever. *Revue scientifique et technique (International Office of Epizootics),* 23**,** 613-623.

Gerrard, S. R. & Nichol, S. T. (2007). Synthesis, proteolytic processing and complex formation of N-terminally nested precursor proteins of the Rift Valley fever virus glycoproteins. *Virology,* 357**,** 124-133.

Gill, D. M. (1982). Bacterial toxins: a table of lethal amounts. *Microbiological Reviews,* 46**,** 86-94.

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M. C., Takehara, K., Hilditch, C., Morikawa, S. & Bishop, D. H. (1991). Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punta Toro, Sicilian Sandfly fever, and Uukuniemi viruses. *Virology,* 180**,** 738-753.

Godde, C. M., Mason-D'croz, D., Mayberry, D., Thornton, P. K. & Herrero, M. (2021). Impacts of climate change on the livestock food supply chain; a review of the evidence. *Global Food Security,* 28**,** 100488.

Goldstein, M. R., Kruth, S. A., Bersenas, A. M., Holowaychuk, M. K. & Weese, J. S. (2012). Detection and characterization of Clostridium perfringens in the feces of healthy and diarrheic dogs. *Canadian Journal of Veterinary Research,* 76**,** 161-165.

Grobbelaar, A. A., Weyer, J., Leman, P. A., Kemp, A., Paweska, J. T. & Swanepoel, R. (2011). Molecular epidemiology of Rift Valley fever virus. *Emerging Infectious Diseases,* 17**,** 2270.

Habjan, M., Penski, N., Spiegel, M. & Weber, F. (2008). T7 RNA polymerase-dependent and-independent systems for cDNA-based rescue of Rift Valley fever virus. *Journal of General Virology,* 89**,** 2157-2166.

Harkness, J. M., Li, J. & Mcclane, B. A. (2012). Identification of a lambda toxin-negative Clostridium perfringens strain that processes and activates epsilon prototoxin intracellularly. *Anaerobe,* 18**,** 546-552.

Harmon, B., Schudel, B. R., Maar, D., Kozina, C., Ikegami, T., Tseng, C.-T. K. & Negrete, O. A. (2012). Rift Valley fever virus strain MP-12 enters mammalian host cells via caveola-mediated endocytosis. *Journal of Virology,* 86**,** 12954-12970.

Harmon, J. R., Spengler, J. R., Coleman-Mccray, J. D., Nichol, S. T., Spiropoulou, C. F. & Mcelroy, A. K. (2018). CD4 T cells, CD8 T cells, and monocytes coordinate to prevent Rift Valley fever virus encephalitis. *Journal of Virology,* 92**,** 10.1128/jvi. 01270-18.

Hayashi, M., Schultz, E. P., Lanchy, J.-M. & Lodmell, J. S. (2021). Time-resolved analysis of n-RNA interactions during RVFV infection shows qualitative and quantitative shifts in RNA encapsidation and packaging. *Viruses,* 13**,** 2417.

Heise, M., Whitmore, A., Thompson, J., Parsons, M., Grobbelaar, A., Kemp, A., Paweska, J., Madric, K., White, L. & Swanepoel, R. (2009). An alphavirus replicon-derived candidate vaccine against Rift Valley fever virus. *Epidemiology & Infection,* 137**,** 1309-1318.

Hofmann, H., Li, X., Zhang, X., Liu, W., Kühl, A., Kaup, F., Soldan, S. S., González-Scarano, F., Weber, F. & He, Y. (2013). Severe fever with thrombocytopenia virus glycoproteins are targeted by neutralizing antibodies and can use DC-SIGN as a receptor for pH-dependent entry into human and animal cell lines. *Journal of Virology,* 87**,** 4384-4394.

Hogenesch, H., O'hagan, D. T. & Fox, C. B. (2018). Optimizing the utilization of aluminum adjuvants in vaccines: you might just get what you want. *npj Vaccines,* 3**,** 51.

Hotez, P. J. & Bottazzi, M. E. (2022). Whole inactivated virus and protein-based COVID-19 vaccines. *Annual Review of Medicine,* 73**,** 55-64.

Huang, Y.-M. (1985). A new african species of Aedes (Diptera: Culicidae). *Mosquito Systematics,* 17**,** 108-120.

Hughes, H. R., Adkins, S., Alkhovskiy, S., Beer, M., Blair, C., Calisher, C. H., Drebot, M., Lambert, A. J., De Souza, W. M. & Marklewitz, M. (2020). ICTV virus taxonomy profile: Peribunyaviridae. *Journal of General Virology,* 101**,** 1-2.

Hussain, R., Guangbin, Z., Abbas, R. Z., Siddique, A. B., Mohiuddin, M., Khan, I., Rehman, T. U. & Khan, A. (2022). Clostridium perfringens types A and D involved in peracute deaths in goats kept in Cholistan ecosystem during winter season. *Frontiers in Veterinary Science,* 9**,** 849856.

Ikegami, T. (2012). Molecular biology and genetic diversity of Rift Valley fever virus. *Antiviral Research,* 95**,** 293-310.

Ikegami, T. (2017). Rift Valley fever vaccines: an overview of the safety and efficacy of the live-attenuated MP-12 vaccine candidate. *Expert Review of Vaccines,* 16**,** 601-611.

Ikegami, T. & Makino, S. (2009). Rift valley fever vaccines. *Vaccine,* 27**,** D69-D72.

Ikegami, T. & Makino, S. (2011). The pathogenesis of Rift Valley fever. *Viruses,* 3**,** 493-519.

Ikegami, T., Peters, C. & Makino, S. (2005). Rift valley fever virus nonstructural protein NSs promotes viral RNA replication and transcription in a minigenome system. *Journal of Virology,* 79**,** 5606-5615.

Jemal, D., Shifa, M. & Kebede, B. (2016). Review on pulpy kidney disease. *Journal of Veterinary Science and Technology,* 7**,** 361.

Jupp, P., Kemp, A., Grobbelaar, A., Leman, P., Burt, F., Alahmed, A., Mujalli, D. A., Khamees, M. A. & Swanepoel, R. (2002). The 2000 epidemic of Rift Valley fever in Saudi Arabia: mosquito vector studies. *Medical and Veterinary Entomology,* 16**,** 245-252.

Kabami, Z. (2023). Notes from the Field: Rift Valley Fever Outbreak—Mbarara District, Western Uganda, January–March 2023. *MMWR. Morbidity and Mortality Weekly Report,* 72.

Käfer, S., Paraskevopoulou, S., Zirkel, F., Wieseke, N., Donath, A., Petersen, M., Jones, T. C., Liu, S., Zhou, X. & Middendorf, M. (2019). Re-assessing the diversity of negative strand RNA viruses in insects. *PLoS Pathogens,* 15**,** e1008224.

Kainga, H., Mponela, J., Basikolo, L., Phonera, M. C., Mpundu, P., Munyeme, M., Simulundu, E. & Saasa, N. (2022). Assessment of Knowledge, Attitudes, and Practices towards Rift Valley Fever among Livestock Farmers in Selected Districts of Malawi. *Tropical Medicine and Infectious Disease,* 7**,** 167.

Kakach, L. T., Suzich, J. A. & Collett, M. S. (1989). Rift Valley fever virus M segment: phlebovirus expression strategy and protein glycosylation. *Virology,* 170**,** 505-510.

Kakach, L. T., Wasmoen, T. L. & Collett, M. S. (1988). Rift Valley fever virus M segment: use of recombinant vaccinia viruses to study Phlebovirus gene expression. *Journal of Virology,* 62**,** 826-833.

Khiav, L. A. & Zahmatkesh, A. (2021). Vaccination against pathogenic clostridia in animals: a review. *Tropical Animal Health and Production,* 53.

Kitandwe, P. K., Mckay, P. F., Kaleebu, P. & Shattock, R. J. (2022). An overview of Rift Valley fever vaccine development strategies. *Vaccines,* 10**,** 1794.

Kolakofsky, D. & Hacker, D. (1991). Bunyavirus RNA synthesis: genome transcription and replication. *Bunyaviridae***,** 143-159.

Komugisha, M., Kibwika, B., Kwesiga, B., Migisha, R., Muwanguzi, D., Lunkuse, S., Kayiwa, J., Nyakarahuka, L. & Ario, A. R. (2023). Outbreak of Rift Valley Fever among herdsmen linked to contact with body fluids of infected animals in Nakaseke District, Central Uganda, June–July, 2023. *Uganda National Institute of Public Health. https://uniph.go.ug/*.

Kortekaas, J., Dekker, A., De Boer, S., Weerdmeester, K., Vloet, R., De Wit, A., Peeters, B. & Moormann, R. (2010). Intramuscular inoculation of calves with an experimental Newcastle disease virus-based vector vaccine elicits neutralizing antibodies against Rift Valley fever virus. *Vaccine,* 28**,** 2271-2276.

Kortekaas, J., Oreshkova, N., Cobos-Jiménez, V., Vloet, R. P., Potgieter, C. A. & Moormann, R. J. (2011). Creation of a nonspreading Rift Valley fever virus. *Journal of Virology,* 85**,** 12622-12630.

Kroeker, A., Babiuk, S., Pickering, B., Richt, J. & Wilson, W. (2020). Livestock Challenge Models of Rift Valley Fever for Agricultural Vaccine Testing. *Frontiers in Veterinary Science*, 7, 238.

Kuhn, J. H., Adkins, S., Brown, K., De La Torre, J. C., Digiaro, M., Hughes, H. R., Junglen, S., Lambert, A. J., Maes, P. & Marklewitz, M. (2023a). ICTV Virus Taxonomy Profile: Cruliviridae 2023. *Journal of General Virology,* 104**,** 001930.

Kuhn, J. H., Adkins, S., Brown, K., De La Torre, J. C., Digiaro, M., Hughes, H. R., Junglen, S., Lambert, A. J., Maes, P. & Marklewitz, M. (2023b). ICTV Virus Taxonomy Profile: Mypoviridae 2023. *Journal of General Virology,* 104**,** 001931.

Kuhn, J. H., Adkins, S., Brown, K., De La Torre, J. C., Digiaro, M., Hughes, H. R., Junglen, S., Lambert, A. J., Maes, P. & Marklewitz, M. (2023c). ICTV Virus Taxonomy Profile: Wupedeviridae 2023. *Journal of General Virology,* 104**,** 001932.

Kwaśnik, M., Rożek, W. & Rola, J. (2021). Rift Valley fever–a growing threat to humans and animals. *Journal of Veterinary Research,* 65**,** 7-14.

Lagerqvist, N., Näslund, J., Lundkvist, Å., Bouloy, M., Ahlm, C. & Bucht, G. (2009). Characterisation of immune responses and protective efficacy in mice after immunisation with Rift Valley Fever virus cDNA constructs. *Virology Journal,* 6**,** 1-10.

Laughlin, L. W., Meegan, J. M., Strausbaugh, L. J., Morens, D. M. & Watten, R. H. (1979). Epidemic Rift Valley fever in Egypt: observations of the spectrum of human illness. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 73**,** 630-633.

Layana, J. E., Miyakawa, M. E. F. & Uzal, F. A. (2006). Evaluation of different fluids for detection of Clostridium perfringens type D epsilon toxin in sheep with experimental enterotoxemia. *Anaerobe,* 12**,** 204-206.

Le Roux, C. A., Kubo, T., Grobbelaar, A. A., Van Vuren, P. J., Weyer, J., Nel, L. H., Swanepoel, R., Morita, K. & Paweska, J. T. (2009). Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus in clinical specimens. *Journal of Clinical Microbiology,* 47**,** 645-651.

Léger, P., Tetard, M., Youness, B., Cordes, N., Rouxel, R. N., Flamand, M. & Lozach, P. Y. (2016). Differential use of the C-type lectins L-SIGN and DC-SIGN for phlebovirus endocytosis. *Traffic,* 17**,** 639-656.

Lihoradova, O. & Ikegami, T. (2014). Countermeasure development for Rift Valley fever: deletion, modification or targeting of major virulence factor NSs. *Future Virology,* 9**,** 27-39.

Linthicum, K., Davies, F., Kairo, A. & Bailey, C. (1985). Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya. *Epidemiology & Infection,* 95**,** 197-209.

Liu, J., Ochieng, C., Wiersma, S., Ströher, U., Towner, J. S., Whitmer, S., Nichol, S. T., Moore, C. C., Kersh, G. J. & Kato, C. (2016). Development of a TaqMan array card for acute-febrile-illness outbreak investigation and surveillance of emerging pathogens, including Ebola virus. *Journal of Clinical Microbiology,* 54**,** 49-58.

Liu, L., Celma, C. C. & Roy, P. (2008). Rift Valley fever virus structural proteins: expression, characterization and assembly of recombinant proteins. *Virology Journal,* 5**,** 1-13.

Lobato, F. C., Salvarani, F. M. & De Assis, R. A. (2007). Clostridioses dos pequenos ruminantes Clostridiosis of small ruminants. *Revista Portuguesa de Ciências Veterinárias RPCV,* 102**,** 23-34.

López-Gil, E., Lorenzo, G., Hevia, E., Borrego, B., Eiden, M., Groschup, M., Gilbert, S. C. & Brun, A. (2013). A single immunization with MVA expressing GnGc glycoproteins promotes epitope-specific CD8+-T cell activation and protects immune-competent mice against a lethal RVFV infection. *PLoS Neglected Tropical Diseases,* 7**,** e2309.

Lopez, N., Muller, R., Prehaud, C. & Bouloy, M. (1995). The L protein of Rift Valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules. *Journal of Virology,* 69**,** 3972-3979.

Lorenzo, G., Martín-Folgar, R., Hevia, E., Boshra, H. & Brun, A. (2010). Protection against lethal Rift Valley fever virus (RVFV) infection in transgenic IFNAR−/− mice induced by different DNA vaccination regimens. *Vaccine,* 28**,** 2937-2944.

Lozach, P.-Y., Kühbacher, A., Meier, R., Mancini, R., Bitto, D., Bouloy, M. & Helenius, A. (2011). DC-SIGN as a receptor for phleboviruses. *Cell Host & Microbe,* 10**,** 75-88.

Lumley, S., Horton, D. L., Hernandez-Triana, L. L. M., Johnson, N., Fooks, A. R. & Hewson, R. (2017). Rift Valley fever virus: strategies for maintenance, survival and vertical transmission in mosquitoes. *Journal of General Virology,* 98**,** 875-887.

Maes, P., Adkins, S., Alkhovsky, S. V., Avšič-Županc, T., Ballinger, M. J., Bente, D. A., Beer, M., Bergeron, É., Blair, C. D. & Briese, T. (2019). Taxonomy of the order Bunyavirales: second update 2018. *Archives of Virology,* 164**,** 927-941.

Makoschey, B., Van Kilsdonk, E., Hubers, W. R., Vrijenhoek, M. P., Smit, M., Wichgers Schreur, P. J., Kortekaas, J. & Moulin, V. (2016). Rift Valley fever vaccine virus clone 13 is able to cross the ovine placental barrier associated with foetal infections, malformations, and stillbirths. *PLOS Neglected Tropical Diseases,* 10**,** e0004550.

Maluleke, M. R., Phosiwa, M., Van Schalkwyk, A., Michuki, G., Lubisi, B. A., Kegakilwe, P. S., Kemp, S. J. & Majiwa, P. A. (2019). A comparative genome analysis of Rift Valley Fever virus isolates from foci of the disease outbreak in South Africa in 2008-2010. *PLoS Neglected Tropical Diseases,* 13**,** e0006576.

Maschmann, E., Küster, E. & Fischer, W. (1931). Über die Fähigkeit des Tonerde-Präparates B, Diphtherie-Toxin zu adsorbieren. *Berichte der deutschen chemischen Gesellschaft (A and B Series),* 64**,** 2174-2178.

Mbhele, Z., Thwala, L., Khoza, T. & Ramagoma, F. (2023). Evaluation of Aluminium Hydroxide Nanoparticles as an Efficient Adjuvant to Potentiate the Immune Response against Clostridium botulinum Serotypes C and D Toxoid Vaccines. *Vaccines,* 11**,** 1473.

Mcdonel, J. L. (1980). Clostridium perfringens toxins (type a, b, c, d, e). *Pharmacology & Therapeutics,* 10**,** 617-655.

Mcelroy, A. K., Albariño, C. G. & Nichol, S. T. (2009). Development of a RVFV ELISA that can distinguish infected from vaccinated animals. *Virology Journal,* 6**,** 1-11.

Mcmillen, C. M. & Hartman, A. L. (2018). Rift Valley fever in animals and humans: Current perspectives. *Antiviral Research,* 156**,** 29-37.

Michaely, L. M., Rissmann, M., Keller, M., König, R., Von Arnim, F., Eiden, M., Rohn, K., Baumgärtner, W., Groschup, M. & Ulrich, R. (2022). NSG-mice reveal the importance of a functional innate and adaptive immune response to overcome RVFV infection. *Viruses,* 14**,** 350.

Minke, J., Audonnet, J.-C. & Fischer, L. (2004). Equine viral vaccines: the past, present and future. *Veterinary Research,* 35**,** 425-443.

Mitchell, M. J., Billingsley, M. M., Haley, R. M., Wechsler, M. E., Peppas, N. A. & Langer, R. (2021). Engineering precision nanoparticles for drug delivery. *Nature Reviews Drug Discovery,* 20**,** 101-124.

Mohapatra, R. K., Kutikuppala, L. V., Kandi, V., Mishra, S., Rabaan, A. A., Costa, S., Al-Qaim, Z. H., Padhi, B. K. & Sah, R. (2023). Rift valley fever (RVF) viral zoonotic disease steadily circulates in the Mauritanian animals and humans: A narrative review. *Health Science Reports,* 6**,** e1384.

Monath, T. P., Kortekaas, J., Watts, D. M., Christofferson, R. C., Labeaud, A. D., Gowen, B. B., Peters, C. J., Smith, D. R., Swanepoel, R. & Morrill, J. C. (2020). Theoretical risk of genetic reassortment should not impede development of live, attenuated Rift Valley fever (RVF) vaccines commentary on the draft WHO RVF Target Product Profile. *Vaccine: X,* 5**,** 100060.

Moni, S. S., Abdelwahab, S. I., Jabeen, A., Elmobark, M. E., Aqaili, D., Ghoal, G., Oraibi, B., Farasani, A. M., Jerah, A. A. & Alnajai, M. M. A. (2023). Advancements in Vaccine Adjuvants: The Journey from Alum to Nano Formulations. *Vaccines,* 11**,** 1704.

Morrill, J. C. & Peters, C. (2011). Protection of MP-12–vaccinated rhesus macaques against parenteral and aerosol challenge with virulent Rift Valley Fever virus. *Journal of Infectious Diseases,* 204**,** 229-236.

Mpeshe, S. C., Luboobi, L. S. & Nkansah-Gyekye, Y. (2014). Optimal control strategies for the dynamics of Rift Valley Fever. *Available online at http://scik.org Communications in Optimization Theory*, 2014, 2014:5.

Mroz, C., Gwida, M., El-Ashker, M., Ziegler, U., Homeier-Bachmann, T., Eiden, M. & Groschup, M. (2017). Rift Valley fever virus infections in Egyptian cattle and their prevention. *Transboundary and Emerging Diseases,* 64**,** 2049-2058.

Muller, R., Saluzzo, J.-F., Lopez, N., Dreier, T., Turell, M., Smith, J. & Bouloy, M. (1995). Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. *The American Journal of Tropical Medicine and Hygiene,* 53**,** 405-411.

Murithi, R., Munyua, P., Ithondeka, P., Macharia, J., Hightower, A., Luman, E., Breiman, R. & Njenga, M. K. (2011). Rift Valley fever in Kenya: history of epizootics and identification of vulnerable districts. *Epidemiology & Infection,* 139**,** 372-380.

Näslund, J., Lagerqvist, N., Habjan, M., Lundkvist, Å., Evander, M., Ahlm, C., Weber, F. & Bucht, G. (2009). Vaccination with virus-like particles protects mice from lethal infection of Rift Valley Fever Virus. *Virology,* 385**,** 409-415.

Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Depner, K., Drewe, J. A., Garin-Bastuji, B., Gonzales Rojas, J. L., Gortázar Schmidt, C., Animal-Health, E. P. O. & Welfare (2020a). Rift Valley Fever: risk of persistence, spread and impact in Mayotte (France). *EFSA Journal,* 18**,** e06093.

Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Depner, K., Drewe, J. A., Garin-Bastuji, B., Rojas, J. L. G., Schmidt, C. G. & Michel, V. (2020b). Rift Valley Fever–epidemiological update and risk of introduction into Europe. *EFSA Journal,* 18**,** e06041.

Niklasson, B., Peters, C., Bengtsson, E. & Norrby, E. (1985). Rift Valley fever virus vaccine trial: study of neutralizing antibody response in humans. *Vaccine,* 3**,** 123-127.

Niklasson, B. S., Meadors, G. F. & Peters, C. J. (1984). Active and passive immunization against Rift Valley fever virus infection in Syrian hamsters. *Acta Pathologica Microbiologica Scandinavica Series C: Immunology,* 92**,** 197-200.

Oakley, C. & Warrack, G. H. (1953). Routine typing of Clostridium welchii. *Epidemiology & Infection,* 51**,** 102-107.

Oscherwitz, J. (2016). The promise and challenge of epitope-focused vaccines. *Human Vaccines & Immunotherapeutics,* 12**,** 2113-2116.

Papin, J. F., Verardi, P. H., Jones, L. A., Monge-Navarro, F., Brault, A. C., Holbrook, M. R., Worthy, M. N., Freiberg, A. N. & Yilma, T. D. (2011). Recombinant Rift Valley fever vaccines induce

protective levels of antibody in baboons and resistance to lethal challenge in mice. *Proceedings of the National Academy of Sciences,* 108**,** 14926-14931.

Pardigon, N., Vialat, P., Girard, M. & Bouloy, M. (1982). Panhandles and hairpin structures at the termini of germiston virus RNAs (bunyavirus). *Virology,* 122**,** 191-197.

Pendell, D., Lusk, J., Marsh, T., Coble, K. & Szmania, S. (2016). Economic assessment of zoonotic diseases: An illustrative study of rift valley fever in the United States. *Transboundary and Emerging Diseases,* 63**,** 203-214.

Pepin, M., Bouloy, M., Bird, B. H., Kemp, A. & Paweska, J. (2010). Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Veterinary Research,* 41**,** 61.

Peters, C., Jones, D., Trotter, R., Donaldson, J., White, J., Stephen, E. & Slone, T. (1988). Experimental Rift Valley fever in rhesus macaques. *Archives of Virology,* 99**,** 31-44.

Petrova, V., Kristiansen, P., Norheim, G. & Yimer, S. A. (2020). Rift valley fever: diagnostic challenges and investment needs for vaccine development. *BMJ Global Health,* 5**,** e002694.

Pichichero, M. E. (2000). New combination vaccines. *Pediatric Clinics of North America,* 47**,** 407-426.

Pichlmair, A., Habjan, M., Unger, H. & Weber, F. (2010). Virus-like particles expressing the nucleocapsid gene as an efficient vaccine against Rift Valley fever virus. *Vector-Borne and Zoonotic Diseases,* 10**,** 701-703.

Pittman, P. R., Liu, C., Cannon, T. L., Makuch, R. S., Mangiafico, J. A., Gibbs, P. H. & Peters, C. J. (1999). Immunogenicity of an inactivated Rift Valley fever vaccine in humans: a 12-year experience. *Vaccine,* 18**,** 181-189.

Pittman, P. R., Mcclain, D., Quinn, X., Coonan, K. M., Mangiafico, J., Makuch, R. S., Morrill, J. & Peters, C. J. (2016). Safety and immunogenicity of a mutagenized, live attenuated Rift Valley fever vaccine, MP-12, in a Phase 1 dose escalation and route comparison study in humans. *Vaccine,* 34**,** 424-429.

Provost, A. & Perreau, P. (1978). Combined vaccines in veterinary medicine in the developing countries. *Developments in Biological Standardization,* 41**,** 349-360.

Rahman, M. M., Islam, M. R. & Dhar, P. S. (2023). Recent re-emergence of Rift Valley fever: epidemiology, clinical characteristics, transmission, symptoms, diagnosis, prevention, and treatment. *International Journal of Surgery,* 109**,** 117-119.

Randall, R., Binn, L. & Harrison, V. (1964). Immunization against rift valley fever virus: Studies on the immunogenicity of lyophilized formalin-inactivated vaccine. *The Journal of Immunology,* 93**,** 293-299.

Renu, H. D., Mathur, M., Rani, S., Boyal, P., Mehra, M. & Asopa, S. (2021). Pathological study of kidneys in Clostridium perfringens type D enterotoxemia in sheep. *Journal of Entomology and Zoology Studies,* 9**,** 1395-7.

Roldão, A., Mellado, M. C. M., Castilho, L. R., Carrondo, M. J. & Alves, P. M. (2010). Virus-like particles in vaccine development. *Expert Review of Vaccines,* 9**,** 1149-1176.

Rostal, M. K., Liang, J. E., Zimmermann, D., Bengis, R., Paweska, J. & Karesh, W. B. (2017). Rift Valley fever: does wildlife play a role? *Ilar Journal,* 58**,** 359-370.

Safini, N., Elmejdoub, S., Bamouh, Z., Jazouli, M., Hamdi, J., Boumart, Z., Rhazi, H., Tadlaoui, K. O. & El Harrak, M. (2022). Development and evaluation of a combined contagious bovine Pleuropneumonia (CBPP) and lumpy skin disease (LSD) live vaccine. *Viruses,* 14**,** 372.

Said, A., Elmanzalawy, M., Ma, G., Damiani, A. M & Osterrieder, N. (2017). An equine herpesvirus type 1 (EHV-1) vector expressing Rift Valley fever virus (RVFV) Gn and Gc induces neutralizing antibodies in sheep. *Virology Journal,* 14**,** 1-8.

Salama, L. (2006). Studies on Rift Valley fever vaccine adjuvanted with aluminium phosphate. *Assiut Veterinary Medical Journal,* 52, 158-171.

Samy, A. M., Peterson, A. T. & Hall, M. (2017). Phylogeography of Rift Valley fever virus in Africa and the Arabian Peninsula. *PLOS Neglected Tropical Diseases,* 11**,** e0005226.

Sanders, B., Koldijk, M. & Schuitemaker, H. (2015). Inactivated viral vaccines. *Vaccine Analysis: Strategies, Principles, and Control***,** 45-80.

Selina, O., Imatdinov, I., Balysheva, V., Akasov, R., Kryukov, A., Balyshev, V. & Markvicheva, E. (2020). Microencapsulated plasmids expressing Gn and Gc glycoproteins of Rift Valley Fever virus enhance humoral immune response in mice. *Biotechnology Letters,* 42**,** 529-536.

Shoemaker, T., Boulianne, C., Vincent, M. J., Pezzanite, L., Al-Qahtani, M. M., Al-Mazrou, Y., Khan, A. S., Rollin, P. E., Swanepoel, R. & Ksiazek, T. G. (2002). Genetic analysis of viruses associated with emergence of Rift Valley fever in Saudi Arabia and Yemen, 2000-01. *Emerging Infectious Diseases,* 8**,** 1415.

Sindato, C., Karimuribo, E. & Mboera, E. (2012). The epidemiology and socio-economic impact of Rift Valley fever epidemics in Tanzania: A review. *The Onderstepoort Journal of Veterinary Research,* 79**,** 1.

Sivakumar, S., Safhi, M. M., Kannadasan, M. & Sukumaran, N. (2011). Vaccine adjuvants–current status and prospects on controlled release adjuvancity. *Saudi Pharmaceutical Journal,* 19**,** 197-206.

Smith, L. A. (2009). Botulism and vaccines for its prevention. *Vaccine,* 27**,** D33-D39.

Smithburn, K. (1949). Rift Valley fever: the neurotropic adaptation of the virus and the experimental use of this modified virus as a vaccine. *British Journal of Experimental Pathology,* 30**,** 1.

Smithburn, K., Haddow, A. & Gillett, J. (1948). Rift Valley fever. Isolation of the virus from wild mosquitoes. *British Journal of Experimental Pathology,* 29**,** 107.

Spik, K., Shurtleff, A., Mcelroy, A. K., Guttieri, M. C., Hooper, J. W. & Schmaljohn, C. (2006). Immunogenicity of combination DNA vaccines for Rift Valley fever virus, tick-borne encephalitis virus, Hantaan virus, and Crimean Congo hemorrhagic fever virus. *Vaccine,* 24**,** 4657-4666.

Stiles, B. G., Barth, G., Barth, H. & Popoff, M. R. (2013). Clostridium perfringens epsilon toxin: a malevolent molecule for animals and man? *Toxins,* 5**,** 2138-2160.

Strauss, J. H. & Strauss, E. G. (2008). Overview of viruses and virus infection. *Viruses and Human Disease*, 1.

Sun, M. H., Ji, Y. F., Li, G. H., Shao, J. W., Chen, R. X., Gong, H. Y., Chen, S. Y. & Chen, J. M. (2022). Highly adaptive Phenuiviridae with biomedical importance in multiple fields. *Journal of Medical Virology,* 94**,** 2388-2401.

Swanepoel, R. (2004). Rift Valley fever, In. Infectious diseases of livestock, Edited by JAW Coetzer & RC Tustin. Oxford University Press, Cape Town, South Africa, 2, 730-1605.

Teixeira, T., Kweder, S. L. & Saint-Raymond, A. (2020). Are the European Medicines Agency, US Food and Drug Administration, and other international regulators talking to each other? *Clinical Pharmacology & Therapeutics,* 107**,** 507-513.

Terasaki, K. & Makino, S. (2015). Interplay between the virus and host in Rift Valley fever pathogenesis. *Journal of Innate Immunity,* 7**,** 450-458.

Tizard, I. R. (2021). Sheep and goat vaccines. *Vaccines for Veterinarians***,** 215.

Tomley, F. M. & Shirley, M. W. (2009). Livestock infectious diseases and zoonoses. The Royal Society.

Turell, M. J., Saluzzo, J.-F., Tammariello, R. F. & Smith, J. F. (1990). Generation and transmission of Rift Valley fever viral reassortants by the mosquito Culex pipiens. *Journal of General Virology,* 71**,** 2307-2312.

Uzal, F., Vidal, J., Mcclane, B. & Gurjar, A. (2010). Clostridium perfringens toxins involved in mammalian veterinary diseases. *The Open Toxinology Journal,* 2**,** 24.

Uzal, F. A. & Songer, J. G. (2008). Diagnosis of Clostridium perfringens intestinal infections in sheep and goats. *Journal of Veterinary Diagnostic Investigation,* 20**,** 253-265.

Vaheri, A., Strandin, T., Hepojoki, J., Sironen, T., Henttonen, H., Mäkelä, S. & Mustonen, J. (2013). Uncovering the mysteries of hantavirus infections. *Nature Reviews Microbiology,* 11**,** 539-550.

Venter, M., Zaayman, D., Van Niekerk, S., Stivaktas, V., Goolab, S., Weyer, J., Paweska, J. T. & Swanepoel, R. (2014). Macroarray assay for differential diagnosis of meningoencephalitis in southern Africa. *Journal of Clinical Virology,* 60**,** 50-56.

Verma, S. K., Mahajan, P., Singh, N. K., Gupta, A., Aggarwal, R., Rappuoli, R. & Johri, A. K. (2023). New-age vaccine adjuvants, their development, and future perspective. *Frontiers in Immunology,* 14**,** 1043109.

Volk, V. K. & Bunney, W. E. (1939). Diphtheria Immunization with Fluid Toxoid and Alum Precipitated Toxoid—Preliminary Report. *American Journal of Public Health and the Nations Health,* 29**,** 197-204.

Volk, V. K. & Bunney, W. E. (1942). Diphtheria immunization with fluid toxoid and alum-precipitated toxoid. *American Journal of Public Health and the Nations Health,* 32**,** 690-699.

Walker, P. J., Siddell, S. G., Lefkowitz, E. J., Mushegian, A. R., Adriaenssens, E. M., Alfenas-Zerbini, P., Davison, A. J., Dempsey, D. M., Dutilh, B. E. & García, M. L. (2021). Changes to virus taxonomy and to the International Code of Virus Classification and Nomenclature ratified by the

International Committee on Taxonomy of Viruses (2021). *Archives of Virology,* 166**,** 2633-2648.

Wallace, D., Ellis, C., Espach, A., Smith, S., Greyling, R. & Viljoen, G. (2006). Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine,* 24**,** 7181-7189.

Wallace, D. B., Mather, A., Kara, P., Naicker, L., Mokoena, N. B., Pretorius, A., Nefefe, T., Thema, N. & Babiuk, S. (2020). Protection of cattle elicited using a bivalent lumpy skin disease virus-vectored recombinant Rift Valley fever vaccine. *Frontiers in Veterinary Science,* 7.

Warimwe, G. M., Gesharisha, J., Carr, B. V., Otieno, S., Otingah, K., Wright, D., Charleston, B., Okoth, E., Elena, L.-G. & Lorenzo, G. (2016). Chimpanzee adenovirus vaccine provides multispecies protection against Rift Valley fever. *Scientific Reports,* 6**,** 20617.

Warimwe, G. M., Lorenzo, G., Lopez-Gil, E., Reyes-Sandoval, A., Cottingham, M. G., Spencer, A. J., Collins, K. A., Dicks, M. D., Milicic, A. & Lall, A. (2013). Immunogenicity and efficacy of a chimpanzee adenovirus-vectored Rift Valley fever vaccine in mice. *Virology Journal,* 10**,** 1-9.

Whaley, J. (2024). Clostridial Disease Management and Vaccines for Sheep and Goats.

Who, W. H. O. (2019). Rift Valley fever Vaccines Target Product Profile. *R&D Blueprint***,** https://www.who.int/news-room/articles-detail/rift-valley-fever-vaccines-target-product-profile.

Willensky, S., Bar-Rogovsky, H., Bignon, E. A., Tischler, N. D., Modis, Y. & Dessau, M. (2016). Crystal structure of glycoprotein C from a hantavirus in the post-fusion conformation. *PLoS Pathogens,* 12**,** e1005948.

Worthington, R. & Mülders, M. (1977). Physical changes in the epsilon prototoxin molecule of Clostridium perfringens during enzymatic activation. *Infection and Immunity,* 18**,** 549-551.

Wright, D., Allen, E. R., Clark, M. H., Gitonga, J. N., Karanja, H. K., Hulswit, R. J., Taylor, I., Biswas, S., Marshall, J. & Mwololo, D. (2020). Naturally acquired Rift Valley fever virus neutralizing antibodies predominantly target the Gn glycoprotein. *Iscience,* 23.

Wright, D., Kortekaas, J., Bowden, T. A. & Warimwe, G. M. (2019). Rift Valley fever: biology and epidemiology. *The Journal of General virology,* 100**,** 1187.

Wu, Z. & Liu, K. (2021). Overview of vaccine adjuvants. *Medicine in Drug Discovery,* 11**,** 100103.

Zaher, M. R., Ahmed, H. A., Hamada, K. E. & Tammam, R. H. (2018). Colorimetric detection of unamplified Rift Valley Fever virus genetic material using unmodified gold nanoparticles. *Applied Biochemistry and Biotechnology,* 184**,** 898-908.

Zhao, T., Cai, Y., Jiang, Y., He, X., Wei, Y., Yu, Y. & Tian, X. (2023). Vaccine adjuvants: Mechanisms and platforms. *Signal Transduction and Targeted Therapy,* 8**,** 283.

Zouaghi, K., Bouattour, A., Aounallah, H., Surtees, R., Krause, E., Michel, J., Mamlouk, A., Nitsche, A. & M'ghirbi, Y. (2021). First serological evidence of Crimean-Congo hemorrhagic fever virus and Rift Valley fever virus in ruminants in Tunisia. *Pathogens,* 10**,** 769.

3 CHAPTER THREE: SAFETY AND IMMUNOGENICITY OF INACTIVATED RIFT VALLEY FEVER SMITHBURN VIRAL VACCINE IN SHEEP

Journal: Virology Journal (volume 20), Article number: 221. Published (2023)

Matome Selina Matsiela [1,2], **Leeann Naicker** [1], **Thandeka Khoza** [2*] **and Nobalanda Mokoena**[1*]

1. Onderstepoort Biological Products (Pty. Ltd), 100 Old Soutpan Road, Onderstepoort 0110, Pretoria South Africa.

2. Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg campus), Scottsville, 3209, KwaZulu-Natal, South Africa.

3.1 Abstract

The live-attenuated Rift Valley Fever Smithburn (SB) vaccine is one of the oldest products widely used in ruminants for control of RVF infections. Vaccinations with RVF Smithburn result in residual pathogenic effect and is limited for use in non-pregnant animals. Commercially available RVFV inactivated vaccines are considered safer options to control the disease. These products are prepared from virulent RVFV isolates and present occupational safety concerns. This research study evaluates the ability of an inactivated SB vaccine strain to elicit neutralising antibody response in sheep. The RVF Smithburn vaccine was inactivated with binary ethylenimine at 37 °C. Inactivated RVFV cultures were adjuvanted with Montande™ Gel-01 and aluminium hydroxide ($Al(OH)_3$) gel for immunogenicity and safety determination in sheep. The commercial RVF inactivated vaccine and a placebo were included as positive and negative control groups, respectively. Inactivated RVFV vaccine formulations were safe with all animals showing no clinical signs of RVFV infection and temperature reactions following prime-boost injections. The aluminium hydroxide formulated vaccine induced an immune response as early as 14 days post primary vaccination with neutralising antibody titre of 1:20 and a peak antibody titre of 1:83 was reached on day 56. A similar trend was observed in the animal group vaccinated with the commercial inactivated RVF vaccine obtaining the highest antibody titre of 1:128 on day 56. The neutralizing antibody levels remained within a threshold for the duration of the study. Merino sheep vaccinated with Montanide™ Gel-01-Smithburn were characterised with overall lower immune response when compared to aluminium hydroxide vaccine emulsions. These finding suggests that the inactivated RVF Smithburn vaccine strain adjuvanted with aluminium-hydroxide can be used an alternative to the products prepared from virulent RVFV isolates for protection of ruminants against the disease. The vaccine can further be evaluated for safety in pregnant ewes.

Keywords: Aluminium hydroxide adjuvant; Montanide™ Gel-01 adjuvant; Rift valley fever virus; Smithburn vaccine; Virus inactivation.

3.2 Introduction

The Rift Valley Fever virus (RVFV) is a zoonotic arbovirus classified under the genus *Phlebovirus*, family *Phenuiviridae* and order Bunyavirales. It is a causative agent of an acute mosquito-borne viral disease that result in abortions in pregnant animals and neonatal mortalities (Swanepoel, 2004, Flick and Bouloy, 2005, Bird *et al.*, 2009, Ikegami, 2017). The RVFV is considered a priority pathogen by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) due to its pathological effect in domestic ruminants and humans in African countries and neighbouring regions (Daubney *et al.*, 1931, Pepin *et al.*, 2010). Also, the RVFV is classified as Category A Priority Pathogen by the National Institute of Allergy and Infectious Diseases in the United States of America. If not effectively controlled, RVF poses a threat and has great potential to spread into non-endemic regions. Control and prevention of RVF outbreaks depends upon adequate management of sustainable animal vaccination programs (Grossi-Soyster and Labeaud, 2020).

Veterinary vaccines for protection of susceptible animals are commercially available (Table 3.1) and have been successfully used as effective means of controlling the spread of RVFV in endemic and high risk countries, such as, South Africa, the Arabian Peninsula, Kenya, Tanzania, Egypt and Sudan (Faburay *et al.*, 2017). Vaccines against RVF are supplied as live-attenuated or inactivated products (Table 3.1). Live-attenuated vaccines are developed from virulent RVFV isolates following repeated passage in cultured cells to alter their virulence and ensure they do not cause disease in vaccinated animals (Lauring *et al.*, 2010). These vaccines replicate in host cells and are able to induce long term protective immunity with a single dose (Faburay *et al.*, 2017). This is consistent with efficacy reports of RVF Smithburn, Clone-13, MP-12, and thermostable Clone-13 vaccines which have been demonstrated to induce high immunity in ruminants (Morrill *et al.*, 1991, Faburay *et al.*, 2017, Ikegami, 2017). However, safety concerns widely limit the application of live-attenuated vaccines, due to their potential of reversion to virulence.

Live-attenuated vaccines have been reported to cause abortions and/or foetal malformations in pregnant ewes (Smithburn, 1949, Botros *et al.*, 2006, Faburay *et al.*, 2017, Boumart *et al.*, 2020). The evidence of vertical transmission of the attenuated virus is in alignment with abortogenic and teratogenic properties of pathogenic strains (Oymans *et al.*, 2020). Consequently, most live-attenuated RVF vaccines are limited for use in non-pregnant animals (Botros *et al.*, 2006, Grobbelaar *et al.*, 2011, Monath *et al.*, 2020). The inactivated vaccines are considered safer alternatives and may be used during pregnancy. These vaccines are produced by propagating live virus in cultured cells, followed by chemical, heat or radiation treatment to disable their ability to infect and replicate inside

the host (Alhaj, 2016, Matsiela *et al.*, 2022). The resultant product eliminates the aspect of possible reversion to virulence. Though inactivated RVF vaccines require booster dosing to induce protective immune responses, followed by repeated application on a yearly basis, they offer improved safety. Commercially available inactivated vaccines are manufactured from virulent field isolates. This poses occupational risk as the virus can be transmitted to humans through handling virulent RVFV material (Petrova *et al.*, 2020). This study aims to improve the safety profile of the Smithburn live-attenuated RVFV vaccine strain while retaining its immunogenicity. The Smithburn virus vaccine was inactivated with BEI and formulated with Montanide™ Gel-01 and aluminium hydroxide adjuvants for evaluation of safety and immunogenicity in sheep.

Table 3. 1: Commercially available RVF vaccines that are licensed for use in livestock

Vaccine	Type	Company	Route of administration/dose	Use in pregnant animals	References
Live Rift Valley Fever (Smithburn strain)	Live-attenuated	OBP, South Africa;	1 mL S/C in sheep, goat and cattle.	No	(Alhaj, 2016, Ikegami, 2017, Dungu *et al.*, 2018)
RIFTVAX TM (Smithburn strain) vaccine	Live-attenuated	KEVEVAPI, Kenya)	1 mL S/C in sheep and goats, 2 mL in cattle	No	(Alhaj, 2016, Ikegami, 2017, Dungu *et al.*, 2018)
RVFV (Clone-13) vaccine	Live-attenuated	OBP, South Africa	1 mL S/C in sheep, goats and cattle.	Yes	(Alhaj, 2016, Ikegami, 2017, Dungu *et al.*, 2018)
Formalin-inactivated RVFV (South African field strain)	Inactivated	OBP, South Africa	1 mL S/C in sheep and goats, 2 mL in cattle.	Yes	(Barnard and Botha, 1977, Alhaj, 2016, Ikegami, 2017)
Riftovax live-attenuated thermostable Clone 13 vaccine (CL-13T)	Live-attenuated	MCI Sante Animale, Morocco	1 mL S/C in sheep, goats and cattle	Yes	(Dungu *et al.*, 2018, Nielsen *et al.*, 2020b)
BEI-inactivated RVFV (ZH501 strain) vaccine	Inactivated	VSVRI, Egypt	1 mL S/C in sheep and goats, 2 mL in cattle	Yes	(Alhaj, 2016, Dungu *et al.*, 2018)

3.3 Materials and Methods

3.3.1 Cells and virus

Adherent Baby Hamster Kidney (BHK-21) and African Green Monkey Kidney (Vero) cell lines, supplied by European Collection of Authenticated Cell Cultures (ECACC, UK) were maintained at 37 °C in a humidified incubator with 5% CO_2. The cells were grown using standard cell culture techniques in Glasgow Minimum Essential Media (GMEM) (Gibco, USA) supplemented with 10% (v/v) bovine serum (Cell Sera, Australia) and antibiotics (100,000 U/L penicillin (Sigma Aldrich, USA), 100 mg/L streptomycin (Sigma Aldrich, USA) and 5,3 mg/mL amphotericin B (Sigma Aldrich, USA).

The RVF Smithburn virus strain used in the production of vaccine manufactured by Onderstepoort Biological Products SOC Ltd was obtained following internal procedures. The RVF vaccine strain was propagated in the BHK-21 cells in 850 cm^2 roller bottles. The virus was quantified by viral plaque assays on Vero cell monolayers and results expressed as log_{10} PFU/mL.

3.3.2 Production and inactivation of RVFV Smithburn

The live-attenuated RVFV Smithburn was produced in BHK-21 at 37 °C on a rolling apparatus and monitored daily until (2-3 days) 90% cytopathic effect has been reached. Infectious RVFV Smithburn was quantified by plaque forming unit (PFU) assay on Vero cell culture (Anderson *et al.*, 2011).

The binary ethyleneimine (BEI) was selected as an inactivating agent and prepared following the method described by Bahnemann (Bahnemann, 1975). The RVFV culture was used at a minimum titre of 1,00E+06 PFU/mL and the following final concentrations of BEI were evaluated for inactivation of the Smithburn strain: 0.5 mM, 1 mM, 1.5 mM and 2 mM. Following the addition of each concentration of BEI to the RVFV Smithburn whole cell cultures, they were incubated at 37 °C with continuous stirring at 150 rpm for 24 hours. Each concentration of BEI was prepared in more than one flask and evaluated at 2-hour intervals. The untreated virus was included as the control. The BEI virus inactivation reaction was immediately neutralized by adding 1 M sodium thiosulphate pentahydrate ($H_{10}Na_2O_8S_2$) to the sampled aliquots at final concentration of 10% volume of BEI added. Neutralized inactivated samples were stored at 4 °C until tested for RVFV infectivity on cell culture. Inactivation was conducted in three independent experiments.

Virus inactivation was validated using the Real-Time Cell Analysis (RTCA) system (xCELLigence™, DP ACEA Biosciences Inc, US) as part of quality assurance (Moetlhoa *et al.*, 2021). The Vero cell suspension

at 1-5 x 10^4 cells/mL was seeded on the RTCA 16-well plates and a 100 µL of the culture media supplemented with 10% serum was added to make up a volume of 200 µL. Cell growth was monitored by recording the cell index (CI) hourly for up to 48 hours to obtain exponential cell growth before infection with the inactivated-RVFV Smithburn aliquots. The 16-wellplates were incubated for 1 hour at 37 °C with 5% CO_2 following infection with 100 µL inactivated-RVFV aliquots. Cell culture media was added and the plates were further incubated at 37 °C with 5% CO_2 and monitored on the RTCA system for up to 100 hours. The Vero cell culture, untreated with the virus were included as the control in the analysis.

3.3.3 Production of inactivated RVFV Smithburn vaccines

The RVFV Smithburn vaccine was prepared on BHK-21 cells and harvested virus culture was inactivated at the predetermined BEI concentration. Complete inactivation of the virus was confirmed and validated by infecting a monolayer of Vero cell culture with the killed Smithburn virus on 96-well microplates and 16-well RTCA E-plates. Two vaccine emulsions were formulated from the inactivated RVFV Smithburn cultures, one with aluminium hydroxide gel and the other with Montanide™ Gel 01 PR adjuvants (SEPPIC, France). The vaccine formulations were prepared under sterile conditions at room temperature using a high-speed homogenizer, D500 (Wiggens, China) at 13,000 rpm. Both vaccine formulations were subjected to standard quality control tests prior use.

3.3.4 Animals, housing and care

Twenty merino sheep of mixed gender and between the ages of 6 to 12 months, were sourced from Langfontein farm in Mpumalanga, South Africa. The animals were pre-screened for presence of antibodies against RVFV using serological assays prior to the commencement of the trial. The serum neutralisation test (SNT) was conducted in-house at OBP and the samples were sent to Agricultural Research Council at the Onderstepoort Veterinary Institute for enzyme-linked immunosorbent assay (ELISA) (Frey and Liess, 1971, Ellis *et al.*, 2014). Animals that were free from RVFV antibodies were delivered to OBP for vaccine safety and immunogenicity studies. The sheep were housed in stables provided controlled environmental conditions at ambient temperature (20-25 °C), and lights switched off at night to mimic the natural environment. Animals were then allowed to acclimatize for 7 days prior the trial. Rectal temperatures of animals were recorded daily starting from the 4 days before beginning of trial. The animals were fed Epol Ram, Lamb and Ewe pellets, Eragrostis and allowed access to water *ad lib*. The stables were cleaned daily and wood shavings covering the floor replaced once a week. Animals enrolled in the study were identified by ear tags on the right-hand. Animal

handling was done in accordance with the standard operating procedures at the experimental animal department at OBP.

3.3.5 Safety and immunogenicity of inactivated RVFV Smithburn vaccines in sheep

Animals were assigned four groups (A, B, C and D), with each group containing 5 sheep for safety and immunogenicity studies. Groups A and B were allocated for the aluminium hydroxide and Montanide™ Gel-01 adjuvanted RVFV vaccinations, respectively. Individual animals were vaccinated via the subcutaneous (SC) route on the inner left thigh with 1 mL of the adjuvanted inactivated RVFV vaccines on day 0 and day 28 (Table 3.2). The animals in group C and D were for vaccination with the commercial inactivated RVFV vaccine supplied by OBP and sterile vaccine diluent, respectively, thus serving as positive and negative controls. The general clinical health of the individual animals, including the RVFV symptoms (such as fever, nasal discharge, loss of appetite, weakness, and bloody diarrhoea) were monitored and recorded once daily for the duration of the study. Rectal body temperatures of the animals were recorded at least once daily for 21 days post each vaccination.

Table 3. 2 Vaccination of sheep with inactivated RVFV Smithburn formulation

Group	No. of animals	Vaccine	Dose of administration	Days of vaccination	Route of administration
A	5	Montanide™ Gel-01-RVFV (Smithburn)	1 mL	D0, D28	S/C
B	5	Aluminium hydroxide-RVFV (Smithburn)	1 mL	D0, D28	S/C
C	5	Commercial inactivated RVFV vaccine	1 mL	D0, D28	S/C
D	5	Sterile vaccine diluent	1 mL	D0, D28	S/C

3.3.6 Blood collection

Each sheep participating in this study was bled in 4–5 mL clot activator and gel separating yellow top tubes on days 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 for serological analysis of humoral immune response. The blood collected was centrifuged at 2500× *g* at 4 °C for an hour. The serum samples were harvested into sterile cryovials and heated at 56 °C in a water bath for 30 min to inactivate non-specific viral inhibitory substances. The heat inactivated serum samples were stored at − 20 °C until used for serological analysis.

3.3.7 Evaluation of the antibody immune response through Enzyme linked immunosorbent assay (ELISA)

The levels of IgM and IgG antibodies induced in sheep against the RVFV were estimated using ELISA assay, based on recombinant nucleoprotein (N protein). The ELISA was conducted at the ARC-OVI for concurrent detection of IgM and IgG antibodies against RVFV. The procedure was conducted according to the method described by (Ellis *et al.*, 2014). The net absorbance read at $OD_{(450\ nm)}$ was expressed as S/P% of the positive control, and all serum samples with S/P% of ≥7 were considered positive (Ellis *et al.*, 2014).

3.3.8 Evaluation of the antibody immune response through serum neutralisation test (SNT)

The SNT assay was used to detect the presence of specific neutralising antibodies in serum of vaccinated sheep. The antibody levels measured by this test are mostly induced against the Gn/Gc glycoproteins of RVFV and afford protection in infected and vaccinated animals. The SNT method was performed as described by Frey and Liess (Frey and Liess, 1971). Briefly, the heat inactivated serum samples were serially diluted two-fold using GMEM cell culture media supplemented with streptomycin and amphotericin B antibiotics (Sigma Aldrich, USA). Positive and negative anti-RVFV serums, were included as controls. The live RVFV Smithburn was added in each well (50 µL) to achieve a final concentration of 100 $TCID_{50}$/mL. After 1-hour incubation of the sera with the virus at 37°C, 100 µL of Vero cells were added in 96-well microtitre plates. The cell monolayer was examined for presence of CPE under light microscope after four days. Antibody titres were expressed as the reciprocal of the serum dilution that inhibited 50% of virus-induced CPE (Muench, 1938).

3.3.9 Ethical approval

The veterinary clinical trial experiment was performed according to the approved protocol by the OBP Animal Ethics Committee (South African Veterinary Council Facility Registration Number: FR1514054) under approval number OBP2019/002 and Department of Agriculture, Land Reform and Rural Development under section 20 of Animal Diseases Act (Act 35 of 1984); approval number 12/11/1/1/(b) MG.

3.3.10 Statistical analysis

Statistical analysis was conducted to compare the significant difference of the antibody immune response induced in sheep by the inactivated RVFV Smithburn formulations. A two-way analysis of variance (ANOVA) followed by a post hoc Tukey's test was utilized to determine difference in antibody immune response over time. Graphpad prism version 5.0 software for Windows was utilized for statistical analysis at a significance level of 5% ($p < 0.05$).

3.4 Results

3.4.1 Inactivation profile of RVFV using binary ethylenimine

The inactivation of RVFV Smithburn was evaluated at different concentrations of BEI (0.5 mM, 1 mM, 1.5 mM and 2 mM). The absence of virus infectivity was determined as a function of $TCID_{50}$ titres, for selection of a suitable BEI concentration that completely inactivates RVFV. In addition, the virus inactivation was validated by measuring the inability of inactivated RVFV culture to induce cell death following incubation at 37 °C. Inactivation kinetics of RVFV after treatment with different concentrations of BEI are demonstrated on Figure 3.1. The data showed that higher concentrations of BEI required a short time period to achieve complete inactivation when compared to lower concentration. The lowest BEI concentration (0.5 mM) completely inactivated RVFV Smithburn following 18-hour incubation period as indicated by no CPE on Vero cells after incubation. All other BEI concentrations higher than 0.5 mM (1 mM, 1.5 mM and 2 mM) achieved complete inactivation after 8-hour of treatment, with higher concentration 2 mM achieving inactivation after 6-hour of treatment (Figure 3.1). This data was supported by absence of CPE after infecting monolayers of Vero cells with the lowest log dilution of the inactivated virus culture (cell morphology presented on Figure 3.2). The untreated RVFV control induced continuous CPE on infected cells from 0 up to 24-hour post incubation, and reduced virus titre with time as a result of incubation at 37 °C. The overall results indicated that the rate of RVFV inactivation is directly proportional to the concentration of BEI used in the inactivation process.

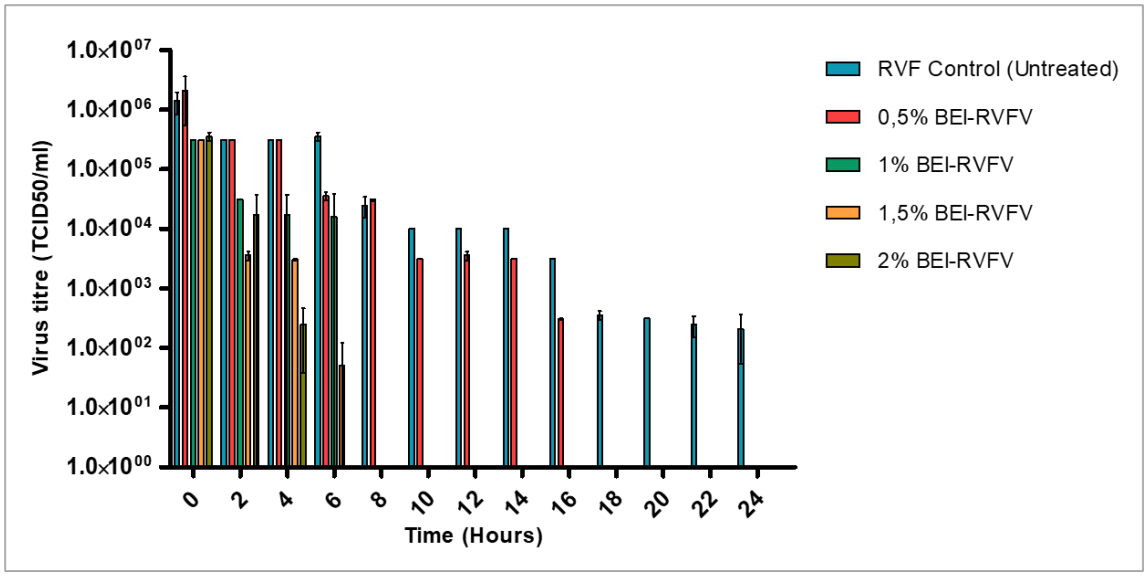


Figure 3. 1: Inactivation profile of rift valley fever virus (RVFV) with binary ethylenimine (BEI). Average titres of the 2-hour interval RVFV (Smithburn) inactivated with 4 different concentrations of BEI (0.5 mM, 1 mM, 1.5 mM and 2 mM) were determined. Titres were measured using the tissue culture infectious dose (TCID50) assay. RVFV culture was included and used as a negative control. Complete inactivation of RVFV was first observed at BEI concentration 2 mM after 6-hour of treatment, followed by 1.5 mM and 1 mM BEI after 8 hour of treatment. Complete inactivation of RVFV by 0.5% BEI was observed after 18-hour of treatment. Error bars represent standard error of the mean (SEM)

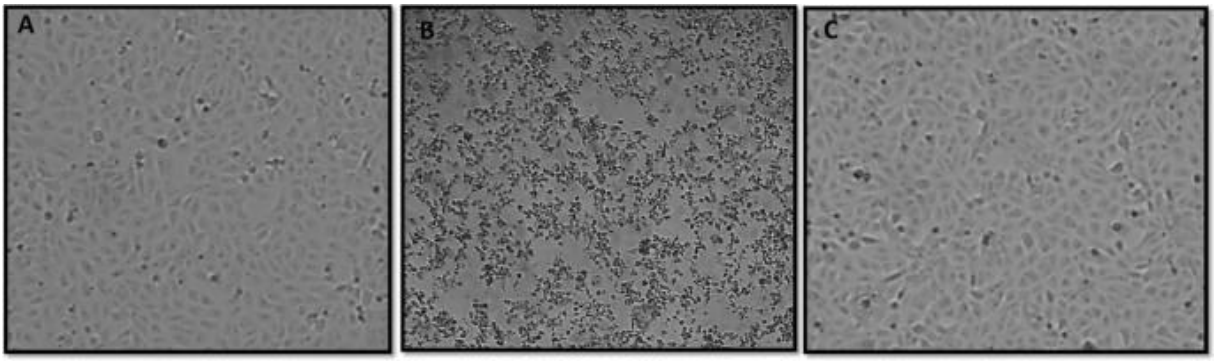


Figure 3. 2: Monolayer of Vero cell culture on the fifth day of incubation after treatment with rift valley fever virus (RVFV) Smithburn. **a** Non-treated cell control, **b** cells treated with the lowest dilution of the live RVFV Smithburn, **c** cells treated with the lowest dilution of the inactivated RVFV. Cytopathic effect was not observed in Vero cells infected with the lowest dilution of the inactivated RVFV. The non-treated cell culture and live RVFV were respectively used as positive and negative controls

3.4.2 Preparation of the inactivated RVFV Smithburn vaccine pilot batch

A pilot batch of RVFV Smithburn was prepared and inactivated with 0.5 mM BEI at 37 °C for 24-hour. Inactivated vaccine used to infect Vero monolayer resulted in no CPE following 5 days of incubation

at 37 °C (Figure 3.3). A monolayer of cells non-treated with the virus and cells treated with live RVFV were included as controls. The live RVFV induced CPE in infected Vero cell culture and no CPE was observed with cells only control. The cells infected with the inactivated RVFV Smithburn vaccine were comparable with the cells only control (Figure 3.3). Complete inactivation of the virus was further validated using the RTCA by monitoring the cell index (CI) of Vero cell culture to indicate various growth phases before and after treatment with inactivated RVFV (Matsiela *et al.*, 2022). The CI profile of Vero cells seeded on E-plate wells followed a typical growth curve with CI value of 0–10 in the first 48-hour of the experiment. The CI reached as high as 10 indicating absence of virus infectivity in cells following 48-hour incubation on the RTCA system (Figure 3.3). Following infection of cells with live RVFV, a gradual decrease in the CI value was observed from 50 to 130-hour of incubation due to CPE formation of infected cells (Figure 3.3). The CI of cells treated with inactivated RVFV followed a typical growth phase as the cell only control indicating complete inactivation of the RVFV Smithburn (Figure 3.3). The slight difference in CI values between cells infected with inactivated virus and cells only control was attributed to the media components added. The inactivated virus contained neutralized BEI reaction mix whereas the cells only controls were topped up with fresh GMEM media which provided more nutrients.

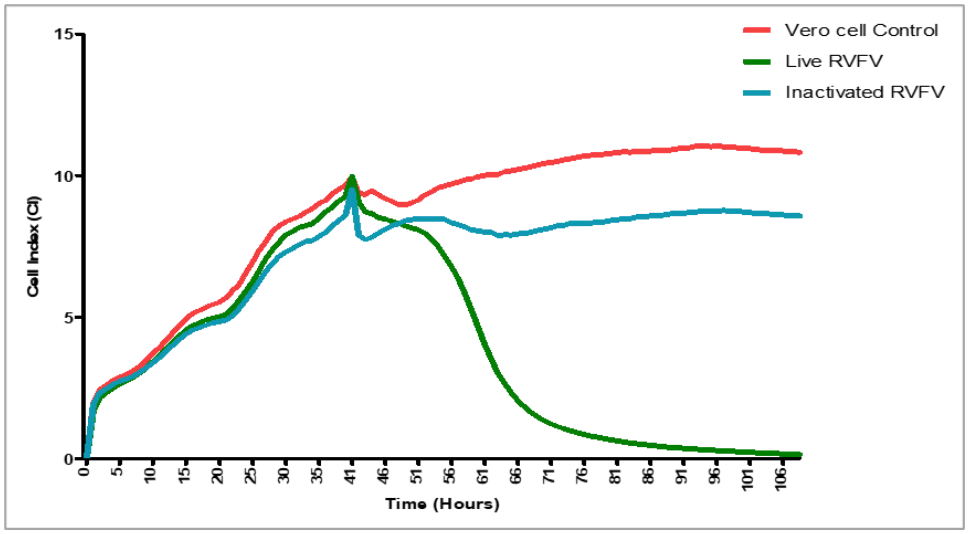


Figure 3. 3: Validation of the inactivation of rift valley fever virus (RVFV) Smithburn using real time cell analysis (RTCA) assay. Growth profile of Vero cells infected with the inactivated and live RVFV Smithburn was recorded. No cell death was recorded in Vero cell culture infected with the inactivated RVFV Smithburn as indicated by the stationary cell index following treatment. The live RVFV induced a decline in cell index showing cell death following treatment with the virus. Vero cell culture was included as positive control.

The RVFV culture was split into two aliquots and formulated with aluminium hydroxide gel and Montanide™ Gel-01 adjuvants to achieve two vaccine emulsions. The vaccines were formulated at the same dose as the commercial inactivated RVF vaccine. Inactivated vaccine was tested for sterility before and after formulation using the standard test methods. Vaccine emulsions were tested on blood tryptose agar with bovine blood, and inoculated on thioglycolate and soy media. Sterility test revealed that the vaccines were free from bacterial and fungal contamination as confirmed during the period of observation after incubation on designated media for 14days.

3.4.3 Safety evaluation of the inactivated RVFV Smithburn vaccine in sheep

Merino sheep were vaccinated with inactivated RVFV Smithburn vaccines. The commercial inactivated RVFV vaccine and sterile vaccine diluent served as positive and negative controls, respectively. Merino sheep were monitored daily for clinical signs of infection, reduced mobility and possible mortalities. Rectal temperatures were recorded 4 days prior the start of the trial and 14-days post each vaccination. Animals in all groups showed no signs of distress nor temperature increments following vaccination. The mean temperature records of the four groups of animals are indicated in Figure 3.4. All the vaccinated and placebo animals maintained a normal range of the physiological temperature of sheep (38–40 °C) following primary and secondary vaccination with the inactivated RVFV Smithburn vaccine (Figure 3.4). No detectable clinical signs of RVFV infection, or other signs of localized inflammations on vaccination sites, nor mortalities recorded in vaccinated animals.

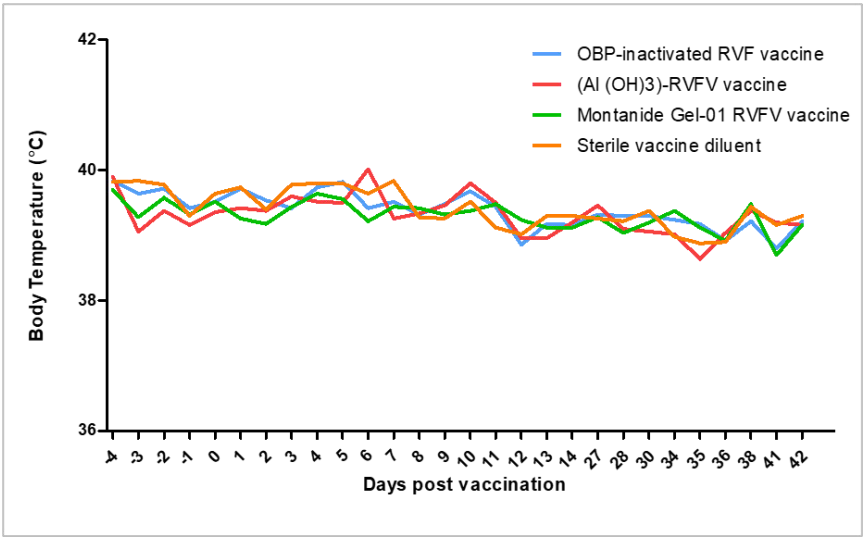


Figure 3. 4: Average body temperatures of sheep vaccinated with the inactivated Rift Valley Fever virus (RVFV) vaccines. Sheep retained the average normal temperature range of 38.3–39.9 °C, suggesting all inactivated vaccine formulations are safe for use in target species

3.4.4 Immunogenicity of the inactivated RVFV vaccine in sheep

To evaluate the immunogenicity of the inactivated RVFV vaccines in sheep, antibodies against RVFV were measured in serum collected at 7-days interval from 0 to 63 days post vaccination. The ELISA method was primarily applied to estimate levels of IgM and IgG antibodies. The ≥7 S/P% is indicative of positive immune response for IgM and IgG antibodies (Ellis *et al.*, 2014). The neutralizing antibody titres of ≥1:4 when using SNT method are indicative of seroconversion and ≥1:16 is considered sufficient to confer protection against the RVF disease as per OBP internal standard operating procedures.

3.4.4.1 Determination of the IgM antibody level

Two groups of animals were used to determine and compare the immunogenicity of the inactivated RVFV Smithburn vaccine formulations, namely a Montanide™ Gel 01-SB and $Al(OH)_3$-SB. The commercial inactivated RVFV vaccine and the sterile vaccine diluent were included as positive and negative controls, respectively. The results presented in Figure 3.5 showed that the IgM antibodies against RVFV were not induced in the animal group vaccinated with placebo vaccine (sterile vaccine diluent). A low IgM antibody immune response, below the threshold was observed in animals vaccinated with the commercial inactivated RVFV vaccine on days 14–49 post vaccination (Figure 3.5). The $Al(OH)_3$-SB vaccine induced marginal IgM antibody immune response on day 7 and 14-days of primary vaccination. The highest neutralising IgM antibody levels of 27 S/P% was observed in animals vaccinated with Montanide™ Gel-01-SB vaccine emulsion after 7-days of primary vaccination (Figure 3.5). The antibody levels gradually decreased to 1 S/P% after 28 days of primary vaccination.

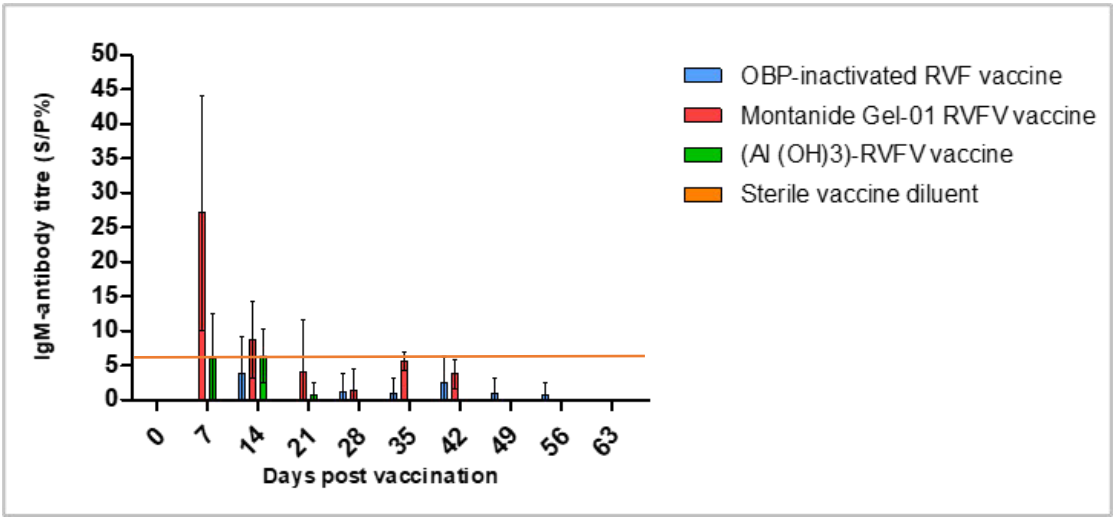


Figure 3. 5: Total IgM antibody levels conferred by the inactivated Rift Valley Fever virus (RVFV) vaccines in sheep. IgM antibody titres against the RVFV nucleoprotein were measured using ELISA. Serum samples were collected from sheep injected with inactivated RVFV vaccine candidates at 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post vaccination. The commercial OBP-inactivated RVF vaccine and sterile vaccine diluent were respectively included as positive and negative controls. Data sets are presented as the mean ± SEM in the same treatment.

3.4.4.2 Determination of the IgG antibody level

Figure 3.6 represents data obtained for IgG antibody immune response induced in sheep immunized with the inactivated RVFV Smithburn vaccine candidates. Sheep injected with $Al(OH)_3$-SB vaccine obtained higher IgG antibody immune response (120 S/P%) at day 14 post primary vaccination (Figure 3.6). The IgG antibodies in this group of animals were maintained throughout the trial with a peak antibody response (128 S/P%) observed at day 21 post primary vaccination (Figure 3.6). The animal group immunized with Montanide™ Gel-01-SB vaccine induced IgG-antibody immune response (101 S/P%) after 14-days of primary vaccination. A 32 S/P% IgG antibody response was detected in the positive control animal group immunized with the commercial inactivated vaccine after 14-days of primary vaccination, with a peak antibody response (75 S/P%) observed after 7-days of booster vaccination (Figure 3.6). No IgG antibodies were detected in the animal group vaccinated with the placebo vaccine throughout the trial (Figure 3.6). Both inactivated vaccine formulations induced IgG response that was comparable to the commercial vaccine and remained above the threshold throughout the duration of the trial.

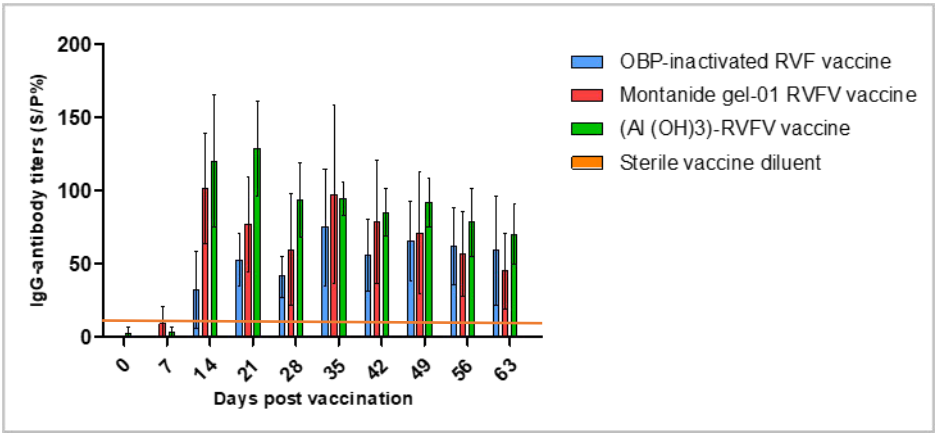


Figure 3. 6: Total IgG antibody levels conferred by the Rift Valley Fever virus (RVFV) formulations in sheep. IgG antibody titres against the RVFV nucleoprotein were measured using ELISA. Serum samples were collected from sheep injected with inactivated RVFV vaccine candidates at 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63-days post vaccination. The commercial OBP-inactivated RVF vaccine and sterile vaccine diluent were respectively included as positive and negative controls. Data sets are presented as the mean ± SEM in the same treatment

3.4.4.3 RVFV-neutralising antibody titre determined by SNT

SNT tests were conducted to determine neutralising antibody titres against-RVFV in sheep and results were expressed as reciprocal of the dilution which neutralized 50% of the RVFV. Sheep injected with the commercial inactivated RVFV vaccine (manufactured by OBP) developed neutralising antibody titres of 1:22 after 28-days of primary vaccinations. The vaccine reached the peak antibody titre (1:128) 7 days following booster vaccination, and the antibodies were maintained up to the end of the trial (day 63) (Figure 3.7). No detectable neutralising antibody response was observed for the negative animal control group vaccinated with sterile vaccine diluent. The Montanide™ Gel-01-SB vaccine emulsion on the other hand induced low neutralising antibody titres in sheep with a peak antibody response (1:23) on day 56 post primary vaccination (Figure 3.7). The $Al(OH)_3$-SB vaccine emulsion seroconverted with 1:21 from day 14 post primary vaccination with peak neutralising antibody response (1:83) observed on day 56 post vaccination (Figure 3.7). The antibody titres induced by this vaccine in sheep were maintained throughout the duration of the trial (Figure 3.7). Furthermore, statistical analysis also showed no significance difference ($P>0.05$) between the neutralising antibody immune response induced by the $Al(OH)_3$-SB vaccine emulsion and the commercial OBP inactivated RVF vaccine.

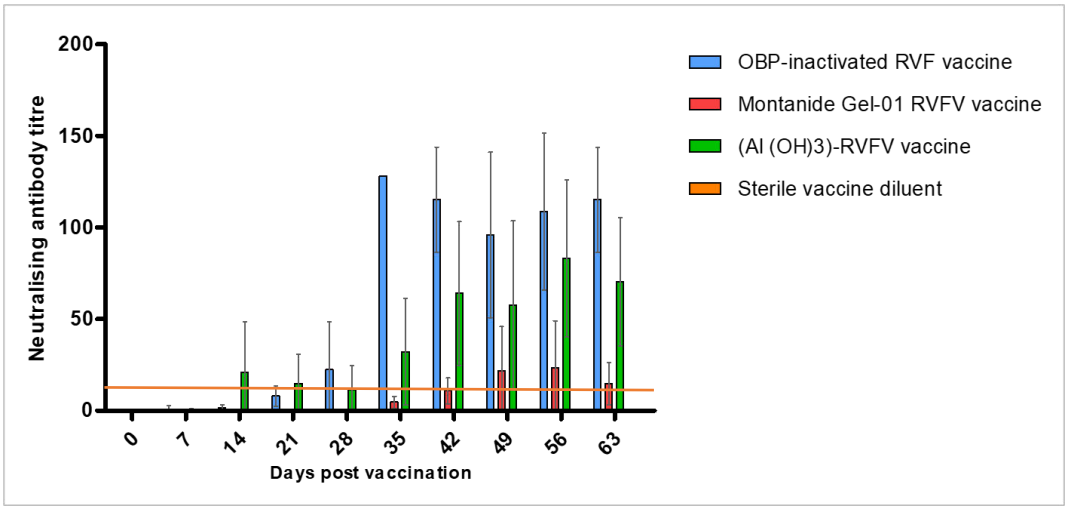


Figure 3. 7: Neutralizing antibody immune response conferred by the inactivated RVFV vaccines in sheep. Neutralizing antibody titres were measured using serum neutralization test for day 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 post vaccination. The commercial OBP-inactivated RVF vaccine and sterile vaccine diluent were respectively included as positive and negative controls. Error bars represent standard error of the mean (SEM).

3.5 Discussion

RVF is an important zoonotic viral disease that affects livestock and humans. Effective prevention and control of the spread of RVFV in endemic countries is achieved through vaccination of susceptible animals (Smith *et al.*, 2018). Livestock vaccination programs result in reduced outbreaks and transmission to humans. Live-attenuated RVFV Smithburn vaccine is one of the commercially available vaccines recommended for use to prevent RVF outbreaks. However, this vaccine strain is associated with several limitations such as possible reversion to virulent state of the virus, teratogenicities as well as abortions in pregnant animals (Botros *et al.*, 2006). Inactivated vaccines have been demonstrated to be safe for use in pregnant animals but are manufactured from virulent field isolates presenting the risk of infection for individuals processing the vaccine strains prior to inactivation. The vaccine drawbacks present a need for research towards the development of suitable RVFV vaccine candidates with improved safety and immunogenicity to protect against the spread of the virus in livestock, and safe for handling by laboratory personnel.

Inactivated vaccine improvement is dependent on the type of inactivating agent and the adjuvant used for vaccine formulation (Al-Olayan *et al.*, 2016). This study evaluated the immune response of sheep vaccinated with the BEI-inactivated RVFV Smithburn vaccine. BEI is an inactivating agent previously introduced for inactivation of viruses in vaccine production, and it was shown to be more advantageous for use as an alternative to formalin due to its protein preservative effect (Blackburn and Besselaar, 1991). Inactivation of RVFV Smithburn using BEI was evaluated at 0.5 mM, 1 mM, 1.5 mM and 2 mM, and complete inactivation was observed in all BEI concentrations in test within 24-hours of treatment. BEI was therefore used at the lowest concentration of 0.5 mM for 24-hours of treatment to ensure complete inactivation of RVFV while preserving viral proteins. Two vaccine formulations of the RVFV were prepared using Montanide™ Gel 01 and aluminium hydroxide gel adjuvants, to select a suitable adjuvant for formulation of the inactivated RVF-SB vaccine. The vaccines were prepared at the same dosage as the commercial inactivated vaccine derived from RVF HB74 virulent strain manufactured by OBP. Both inactivated RVF-SB formulations were subjected to the standard vaccine quality control process to ensure that they met specifications for freedom from extraneous agents, safety and potency, prior to use in clinical trials. Safety and immunogenicity of the inactivated RVF–SB emulsions were evaluated in target Merino sheep, following primary and secondary vaccination. No local reactions and clinical side effects were observed after each inoculation of vaccine in animals. Safety of the commercial inactivated RVF has been established extensively (Alhaj, 2016), and in this study as anticipated the vaccine induced no reaction with all 5

sheep injected. No febrile reactions were observed nor any form of discomfort in animals. Body temperatures of the animals were within a normal range of the physiological temperature after each inoculation. A study conducted by Magd et al., 2014 also demonstrated safety of the BEI inactivated RVFV vaccine formulated with 20% Montanide Gel-01 and aluminium hydroxide, which resulted in no mortalities nor clinical signs of RVFV infections in mice (Magd *et al.*, 2014). Safety test results suggests that the inactivated RVFV formulated with aluminium hydroxide gel and Montanide™ Gel-01 are safe for use in sheep following primary and secondary vaccinations.

The humoral immunogenicity of the vaccine formulations, in the form of IgM and IgG were monitored and analysed at 7-day intervals from day 0 up to day 63 post vaccination. The neutralising IgM antibodies were detected from 7 to 14 days post primary vaccination in animal groups vaccinated with Montanide™ Gel-01-SB vaccine and $Al(OH)_3$-SB vaccine emulsions. The animal group vaccinated with Montanide™ Gel-01-SB vaccine obtained high positive IgM antibody levels, which were shown to decrease to negative response from day 21 after primary vaccination until the end of the trial. This was expected as the Montanide gel adjuvant is known to induce strong short term inflammatory immune response to trigger an efficacious response, and this was evident in our study as highest levels of IgM were obtained with this adjuvant. Several studies have also described IgM antibodies as a form of early onset of immunity which appear briefly as early as 3-days in response to infection and decreases few days following accumulation of IgG antibodies in circulation (Fafetine *et al.*, 2007, Wallace *et al.*, 2020). Wallace et al., 2020 has reported detection of IgM antibodies following 3 days post challenge with virulent RVFV M35/74 strain, which were shown to rapidly decrease from day 9 to 14 due to an increase in IgG antibody levels (14 to 21-days post challenge) (Wallace *et al.*, 2020). Similar observations occurred in our study where the IgM antibodies were detected 7 to 14-days post primary vaccination of sheep with Montanide™ Gel-01-SB vaccine and $Al(OH)_3$-SB vaccine emulsions. These antibodies decreased to negative levels following accumulation of IgG antibodies depicted from 14-days post primary vaccination (Figure 3.5 and Figure 3.6) which is a typical profile of adaptive immune response. The animal group vaccinated with Montanide™ Gel-01-SB vaccine induced IgG antibody levels after 7-days post primary vaccination as determined with an ELISA method. The antibodies were maintained at high levels from day 14 until end of the trial (Figure 3.6). Literature has reported that IgG antibodies begin to accumulate in circulation after 7-days of infection or vaccination, and this was in accord with data obtained after vaccination of sheep with Montanide™ Gel-01-SB vaccine. The animal group vaccinated with $Al(OH)_3$-SB vaccine emulsion induced highest IgG antibody immune response when compared to both Montanide™ Gel-01-SB vaccine emulsion and the commercial inactivated RVFV (Figure 3.6). The antibody immune response induced by $Al(OH)_3$-SB

vaccine remained at high levels from day 14 until end of the trial. The commercial inactivated RVFV vaccine had induced low IgM antibody titres in sheep 14-days post primary vaccination, which were below the threshold of 7 S/P%. However, the vaccine induced positive IgG antibodies which were detected 14 days post primary vaccination, with peaks observed following 7-days post booster vaccination and remaining at high levels until the end of the study. Similar results were obtained in a study reported by Ronchi *et al.* (2022) who had detected negative IgM antibodies and high IgG antibodies against RVFV 7 days after vaccination of sheep with an inactivated RVFV vaccine formulated with 10% Montanide Pet Gel A (Ronchi *et al.*, 2022). The vaccine reported by Ronchi *et al.* (2022) and OBP commercial inactivated product are both formulated from virulent RVFV strains.

The high levels of IgG antibodies are an indication of a long-term immune response induced in sheep following vaccination. These IgG antibodies are traditionally considered to correlate with protective humoral immune response to viruses, when characterized with neutralising capabilities (Corrales-Aguilar *et al.*, 2016). The ELISA data generated in this study measured antibodies induced against the N protein of RVFV which is known to be the most immunogenic protein within the *Phenuiviridae* family. Several studies have also reported that the epitopes exposed on the N protein are not involved in mediating virus attachment and entry, and thus may not induce neutralising antibody immune response (Jegerlehner *et al.*, 2004, Carragher *et al.*, 2008, El Bakkouri *et al.*, 2011). Additional assays were employed to make an informed decision on potential efficacy of inactivated RVF Smithburn vaccine candidates. Therefore, serum neutralising test against RVFV was included as confirmatory assay to detect neutralising antibodies induced by the RVF SB vaccine candidates. The commercial inactivated RVFV vaccine conferred the highest neutralising antibody immune response observed 28 days after primary vaccination (Figure 3.7). The $Al(OH)_3$-SB vaccine emulsion conferred neutralising antibody immune response as early as 14 days after primary vaccination and the response increased following booster dose. The antibody titres were maintained at protective levels until the end of the trial. There was no significant difference in antibody titres induced by commercial inactivated RVFV vaccine and $Al(OH)_3$-SB vaccine with P=0,0004 and P=0,0003 for ELISA and SNT data, respectively. The data suggest the inactivated $Al(OH)_3$-SB formulation might result in similar protection levels to the current OBP inactivated RVF commercial vaccine product since they were evaluated at the same dosage and formulated with same adjuvant. The slight difference in antibody levels obtained with SNT and IgG ELISA for both $Al(OH)_3$-SB emulsion and the commercial inactivated RVFV vaccines can be associated with the use of different RVFV strains used in the formulation. The commercial inactivated RVFV vaccine induced highest neutralising antibodies through SNT which may be explained by fact that the vaccine is prepared from formulation with virulent RVFV when compared to the attenuated

SB vaccine strain. The IgG levels induced by Montanide™ Gel-01-SB vaccine comprised of low levels of neutralising antibodies as depicted in Figure 3.7 when compared to aluminium hydroxide gel vaccine products. The Montanide™ Gel-01-SB vaccine emulsion induced high level of non-neutralising antibodies specific to RVFV and requires further evaluation in virulent virus challenge studies. The results generated in this study proved the concept that inactivated RVF vaccine prepared from attenuated Smithburn strain adjuvanted with aluminium hydroxide gel can potentially serve as an alternative candidate vaccine for protection of livestock against RVF.

3.6 Conclusions

Immunogenicity and safety of an inactivated RVFV Smithburn was evaluated in sheep after primary and secondary inoculations. The results obtained indicated that the inactivated RVFV Smithburn formulated with aluminium hydroxide gel conferred strong neutralising antibody immune response in sheep. Though challenge with virulent RVFV strain was not conducted, there was no significant statistical difference in antibody titres between commercial inactivated RVFV vaccine and $Al(OH)_3$-SB formulated product, suggesting the potential of this formulation to confer protection similar to the current OBP inactivated RVF vaccine. This vaccine formulation may be further developed into a full vaccine product to confer protection against RVFV challenge, also including data for duration of immunity. The safety of the vaccine may further be evaluated in pregnant animals.

3.7 References

Al-Olayan, E. M., Mohamed, A. F., El-Khadraqy, M. F., Shebl, R. I. & Yehia, H. (2016). An Alternative Inactivant for Rift Valley Fever Virus using Cobra Venom-derived L-Amino Oxidase, which is Related to its Immune Potential. *Brazilian Archives of Biology and Technology,* 59.

Alhaj, M. (2016). Safety and efficacy profile of commercial veterinary vaccines against Rift Valley fever: a review study. *Journal of Immunology Research,* 2016.

Anderson, B., Rashid, M. H., Carter, C., Pasternack, G., Rajanna, C., Revazishvili, T., Dean, T., Senecal, A. & Sulakvelidze, A. (2011). Enumeration of bacteriophage particles: Comparative analysis of the traditional plaque assay and real-time QPCR-and nanosight-based assays. *Bacteriophage,* 1, 86-93.

Bahnemann, H. (1975). Binary ethylenimine as an inactivant for foot-and-mouth disease virus and its application for vaccine production. *Archives of Virology,* 47, 47-56.

Barnard, B. & Botha, M. (1977). An inactivated rift valley fever vaccine. *Journal of the South African Veterinary Association,* 48, 45-48.

Bird, B. H., Ksiazek, T. G., Nichol, S. T. & Maclachlan, N. J. (2009). Rift Valley fever virus. *Journal of the American Veterinary Medical Association,* 234, 883-893.

Blackburn, N. & Besselaar, T. (1991). A study of the effect of chemical inactivants on the epitopes of Rift Valley fever virus glycoproteins using monoclonal antibodies. *Journal of Virological Methods,* 33, 367-374.

Botros, B., Omar, A., Elian, K., Mohamed, G., Soliman, A., Salib, A., Salman, D., Saad, M. & Earhart, K. (2006). Adverse response of non-indigenous cattle of European breeds to live attenuated Smithburn Rift Valley fever vaccine. *Journal of Medical Virology,* 78, 787-791.

Boumart, Z., Bamouh, Z., Hamdi, J., Safini, N., Tadlaoui, K., Bettinger, G., Watts, D. & Elharrak, M. (2020). Safety and immunogenicity of the Rift Valley fever arMP-12 ΔNSm21/384 candidate vaccine in pregnant ewes. *Vaccine: X,* 6, 100070.

Carragher, D. M., Kaminski, D. A., Moquin, A., Hartson, L. & Randall, T. D. (2008). A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *The Journal of Immunology,* 181, 4168-4176.

Corrales-Aguilar, E., Trilling, M., Reinhard, H., Falcone, V., Zimmermann, A., Adams, O., Santibanez, S. & Hengel, H. (2016). Highly individual patterns of virus-immune IgG effector responses in humans. *Medical Microbiology and Immunology,* 205, 409-424.

Daubney, R., Hudson, J. & Garnham, P. (1931). Enzootic hepatitis or Rift Valley fever. An undescribed virus disease of sheep cattle and man from East Africa. *The Journal of Pathology and Bacteriology,* 34, 545-579.

Dungu, B., Lubisi, B. A. & Ikegami, T. (2018). Rift Valley fever vaccines: current and future needs. *Current Opinion in Virology,* 29, 8-15.

El Bakkouri, K., Descamps, F., De Filette, M., Smet, A., Festjens, E., Birkett, A., Van Rooijen, N., Verbeek, S., Fiers, W. & Saelens, X. (2011). Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. *The Journal of Immunology,* 186, 1022-1031.

Ellis, C. E., Mareledwane, V. E., Williams, R., Wallace, D. B. & Majiwa, P. A. (2014). Validation of an ELISA for the concurrent detection of total antibodies (IgM and IgG) to Rift Valley fever virus. *Onderstepoort Journal of Veterinary Research,* 81, 1-6.

Faburay, B., Labeaud, A. D., Mcvey, D. S., Wilson, W. C. & Richt, J. A. (2017). Current status of Rift Valley fever vaccine development. *Vaccines,* 5, 29.

Fafetine, J. M., Tijhaar, E., Paweska, J. T., Neves, L. C., Hendriks, J., Swanepoel, R., Coetzer, J. A., Egberink, H. F. & Rutten, V. P. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. *Veterinary Microbiology,* 121, 29-38.

Flick, R. & Bouloy, M. (2005). Rift Valley fever virus. *Current Molecular Medicine,* 5, 827-834.

Frey, H. R. & Liess, B. (1971). Vermehrungskinetik und Verwendbarkeit eines stark zytopathogenen VD-MD-Virusstammes für diagnostische Untersuchungen mit der Mikrotiter-Methode. *Zentralblatt für Veterinärmedizin Reihe B,* 18, 61-71.

Grobbelaar, A. A., Weyer, J., Leman, P. A., Kemp, A., Paweska, J. T. & Swanepoel, R. (2011). Molecular epidemiology of Rift Valley fever virus. *Emerging Infectious Diseases,* 17, 2270.

Grossi-Soyster, E. N. & Labeaud, A. D. (2020). Rift valley fever: Important considerations for risk mitigation and future outbreaks. *Tropical Medicine and Infectious Disease,* 5, 89.

Ikegami, T. (2017). Rift Valley fever vaccines: an overview of the safety and efficacy of the live-attenuated MP-12 vaccine candidate. *Expert Review of Vaccines,* 16, 601-611.

Jegerlehner, A., Schmitz, N., Storni, T. & Bachmann, M. F. (2004). Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *The Journal of Immunology,* 172, 5598-5605.

Lauring, A. S., Jones, J. O. & Andino, R. (2010). Rationalizing the development of live attenuated virus vaccines. *Nature Biotechnology,* 28, 573-579.

Magd, D. M. A., Ebeid, M., Moustafa, A.-M. M., Shalakamy, E. M. S. & Wahab, M. G. A. (2014). Effecacy of Montanide gel inactivated RVF vaccine in comparison with aluminum hydroxide gel inactivated one. *BENHA Veterinary Medical Journal*, 27, 239-247

Matsiela, M. S., Naicker, L., Dibakwane, V. S., Ntombela, N., Khoza, T. & Mokoena, N. (2022). Improved safety profile of inactivated Neethling strain of the lumpy skin disease vaccine. *Vaccine: X,* 12, 100209.

Moetlhoa, B., Naicker, L., Hayeshi, R., Grobler, A., Mokoena, N. B. & Mawadza, C. (2021). Application of a real-time cell analysis system in the process development and quantification of Rift Valley fever virus clone 13. *Access Microbiology,* 3.

Monath, T. P., Kortekaas, J., Watts, D. M., Christofferson, R. C., Labeaud, A. D., Gowen, B. B., Peters, C. J., Smith, D. R., Swanepoel, R. & Morrill, J. C. (2020). Theoretical risk of genetic reassortment should not impede development of live, attenuated Rift Valley fever (RVF) vaccines commentary on the draft WHO RVF Target Product Profile. *Vaccine: X,* 5, 100060.

Morrill, J., Carpenter, L., Taylor, D., Ramsburg, H., Quance, J. & Peters, C. (1991). Further evaluation of a mutagen-attenuated Rift Valley fever vaccine in sheep. *Vaccine,* 9, 35-41.

Muench, H. R. (1938). A simple method of estimating 50 per cent end points. *American Journal of Tropical Medicine and Hygiene,* 27, 493-497.

Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Depner, K., Drewe, J. A., Garin-Bastuji, B., Rojas, J. L. G., Schmidt, C. G. & Michel, V. (2020). Rift Valley Fever–epidemiological update and risk of introduction into Europe. *EFSA Journal,* 18, e06041.

Oymans, J., Wichgers Schreur, P. J., Van Keulen, L., Kant, J. & Kortekaas, J. (2020). Rift Valley fever virus targets the maternal-foetal interface in ovine and human placentas. *PLoS Neglected Tropical Diseases,* 14, e0007898.

Pepin, M., Bouloy, M., Bird, B. H., Kemp, A. & Paweska, J. (2010). Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Veterinary Research,* 41, 61.

Petrova, V., Kristiansen, P., Norheim, G. & Yimer, S. A. (2020). Rift valley fever: diagnostic challenges and investment needs for vaccine development. *BMJ Global Health,* 5, e002694.

Ronchi, G. F., Testa, L., Iorio, M., Pinoni, C., Bortone, G., Dondona, A. C., Rossi, E., Capista, S., Mercante, M. T. & Morelli, D. (2022). Immunogenicity and safety studies of an inactivated vaccine against Rift Valley fever. *Acta Tropica,* 232, 106498.

Smith, D. R., Johnston, S. C., Piper, A., Botto, M., Donnelly, G., Shamblin, J., Albariño, C. G., Hensley, L. E., Schmaljohn, C. & Nichol, S. T. (2018). Attenuation and efficacy of live-attenuated Rift Valley fever virus vaccine candidates in non-human primates. *PLoS Neglected Tropical Diseases,* 12, e0006474.

Smithburn, K. (1949). Rift Valley fever: the neurotropic adaptation of the virus and the experimental use of this modified virus as a vaccine. *British Journal of Experimental Pathology,* 30, 1.

Swanepoel, R. (2004). Rift Valley fever, In. Infectious diseases of livestock, Edited by JAW Coetzer & RC Tustin. *Oxford University Press,* Cape Town, South Africa, 2, 730-1605.

Wallace, D. B., Mather, A., Kara, P., Naicker, L., Mokoena, N. B., Pretorius, A., Nefefe, T., Thema, N. & Babiuk, S. (2020). Protection of cattle elicited using a bivalent lumpy skin disease virus-vectored recombinant Rift Valley fever vaccine. *Frontiers in Veterinary Science,* 7.

4 CHAPTER FOUR: GROWTH OPTIMISATION OF *CLOSTRIDIUM PERFRINGENS* TYPE D FOR INCREASED PRODUCTION OF THE EPSILON TOXIN.

4.1 Abstract

Epsilon toxin produced by *Clostridium perfringens* type D is the widely used antigen in vaccine formulation for prevention against pulpy kidney (PK) disease. The vaccine production process mainly involves cultivation of the bacteria for expression of the epsilon toxin used in the formulation of monovalent product or in combination with other antigens. The use of the epsilon toxin in combination vaccine products therefore require increased yields to ensure formulation of efficacious product. Subsequently, it is of great importance to optimise *C. perfringens* type D fermentation process to achieve optimal growth for vaccine manufacturing of PK. This study identified suitable anaerobic fermentation conditions for *C. perfringens* type D to achieve maximum production yields of the epsilon toxin. The bacterium was cultured in yeast and potassium hydrogen phosphate (PYP) media at pH of 7.2 and a temperature of 37 ± 2 °C, with a stirring speed of 100 rpm. Three anaerobic conditions were evaluated for improved yield of the toxin, namely: (a) a closed system with no air flow, (b) a system supplied with nitrogen gas for the first two hours of the fermentation process, and (c) a continuous nitrogen gas supply throughout the fermentation process. The bacterium displayed different growth kinetics under the studied culturing conditions. The growth of *C. perfringens* type D in the closed fermentation system was characterised by a delayed lag phase of up to 6-hours, and reached maximum growth after 15 hours of cultivation. In constrast, the bacterial growth in the restricted (2 hours) and continuous nitrogen supplied systems had a shorter lag phase (up to 3-hours) and reached maximum growth within 9-hours of cultivation. These results demonstrated that *C. perfringens* type D grew optimally in the PYP media under anaerobic conditions supplied with nitrogen gas. Although conditions B and C resulted in similar growth kinetics, higher epsilon toxin yields were obtained with nitrogen supplied for the first 2 hours of fermentation as determined with radial immunodiffusion (RID) assay. The yields were 17.9 TCP/mL and 17.35 TCP/mL for B and C, respectively. Similar results were obtained when the toxin was quantified with Enzyme linked immunosorbent assay (ELISA) with the epsilon toxin concentrations of 4.9 mg/mL and 4.5 mg/mL for anaerobic conditions B and C, respectively. In contrast, condition A resulted in low yield of the epsilon toxin detected at 15.86

TCP/mL (RID) and 2.1 mg/mL (ELISA).These results suggest that fermentation of *C. perfringens* type D with nitrogen gas at first 2 hours of the fermentation process improved epsilon toxin yields.

Keywords: *Clostridium perfringens* type D, epsilon toxin, pulpy kidney disease, ELISA, radial immunodiffusion assays

4.2 Introduction

The *Clostridium* genus comprises various anaerobic, gram-positive, spore-forming, and rod-shaped bacilli (Guo *et al.*, 2020). These organisms are widely distributed in soil, water and the faeces of healthy animals, existing in both vegetative and spore forms (Javed *et al.*, 2012). Over 100 species of Clostridial organisms are reported as part of the normal gut flora of healthy animals and humans (Phukan *et al.*, 1997, Bokori-Brown *et al.*, 2011, Guo *et al.*, 2020). Several species of these organisms are pathogenic in nature and have the potential to induce enterotoxaemia in ruminants. Enterotoxaemia is mainly caused by *Clostridium perfringens* and occurs when the organisms replicate uncontrollably in their normal habitat in the gut, resulting in the production of lethal toxins which are absorbed into the bloodstream, causing both enteric and systemic diseases in ruminants (Rood *et al.*, 1997, Uzal *et al.*, 2004, Pawaiya *et al.*, 2020). The *C. perfringens* produce up to twenty distinct toxins that result in various diseases, including yellow lamb disease, lamb dysentery, haemorrhagic enteritis, struck and PK (Revitt-Mills *et al.*, 2015, Hussain *et al.*, 2018). These diseases are associated with different strains of the *C. perfringens*, which are characterised into seven toxin types (A, B, C, D, E, F and G) dependent on the genes encoding for typing toxins, namely alpha, beta, epsilon and iota toxins, as well as the recently added enterotoxin (CPE) and necrotic enteritis B-like toxin (NetB) (Table 4.1) (Rood *et al.*, 2018, Hussain *et al.*, 2022). Additionally, the individual *C. perfringens* strains can produce minor or non-typing toxins, such as beta-2, perfringolysin O, necrotic enteritis F toxin, binary enterotoxin and others (Kiu *et al.*, 2017).

Table 4. 1: The seven toxinotypes of *Clostridium perfringens* species and typing toxins produced in host intestinal gut, modified from (Forti *et al.*, 2020).

Clostridium perfringens	Name of toxin produced					
	Alpha	Beta	Epsilon	Iota	Enterotoxin	NetB
A	√					
B	√	√	√			
C	√	√				
D	√		√			
E	√			√		
F	√				√	
G	√					√

C. perfringens type D produces the epsilon toxin which is the causative agent of the common PK enteric disease in domestic ruminants (Uzal and Songer, 2008). The toxin is produced as an inactive prototoxin with an estimated molecular weight of 32.9 kDa (Habeeb, 1975). The inactive prototoxin is activated by proteolytic cleavage using enzymes such as trypsin and chymotrypsin, which results in the release of the amino and carboxy terminals from the protein (Bhown and Habeeb, 1977, Worthington and Mülders, 1977). This toxin plays a major role in the fatal PK disease, associated with principal symptoms such as oedema and neurological disorders, leading to subsequent death of the affected animals (Finnie, 2003, Garcia *et al.*, 2013). The epsilon toxin has become an important tool in the field of veterinary medicine used for development of vaccines for effective control and prevention of PK disease (Morcrette *et al.*, 2019).

A number of commercial vaccines developed from epsilon toxoids, are available for the prevention of PK disease. These vaccines exist in both polyvalent combination and monovalent forms. The polyvalent combination vaccines were produced for prevention of PK and other Clostridial diseases, these vaccines include One Shot Ultra (Pfizer Inc, USA), Coglavax (Ceva Sante Animale, France), and Multivax P (MSD animal health, USA). The monovalent vaccines are produced by inactivating the *C. perfringens* type D whole cell or culture filtrates with formaldehyde, resulting in complete inactivation of the epsilon toxin, known as epsilon toxoid (Morcrette *et al.*, 2019). The epsilon toxoid vaccines are

formulated with adjuvants for enhanced efficacy (Jemal *et al.*, 2016). The monovalent PK vaccines currently available include Pulpyvax (MSD animal health, USA), and Enterotoxaemia vaccine (Onderstepoort Biological products (OBP), SA). Enterotoxaemia vaccine is formalin inactivated, and consists of whole cell culture of the *C. perfringens* type D strain that has demonstrated effective control and prevention of the disease through systematic vaccination of sheep and goats for more than 6 decades (Sutton, 1952, Jansen, 1960). In the past decade alone, over 78 million doses have been sold to the South African market (OBP internal communication). PK vaccines have been explored as part of combination vaccine for various diseases targeting ruminants (Ardehali, 1984, Pulotov *et al.*, 2021, Araghi *et al.*, 2022). This is primarily due to adherence of the vaccination regime of ruminants against multiple diseases by commercial and small scale famers (Hopker *et al.*, 2021, Pulotov *et al.*, 2021). Consequently, innovative procedures must be explored for optimising production of the epsilon toxin, which is required at increased yields for formulation of an efficacious vaccine product. This chapter aims to optimise growth of the *C. perfringens* type D by evaluating the effect of nitrogen supply and selection of suitable anaerobic fermentation conditions for increased production of the epsilon toxin.

4.3 Materials and Methods

4.3.1 Bacterial strains and culture media

The *C. perfringens* type D (ET663 strain) was obtained from OBP inventory as a 2 mL lyophilised pellets, and used for production of the epsilon toxin in synthetic media. All culture media used in this study are listed in Table 4.2, and were prepared in water for injection (WFI). The WFI utilised was obtained internally at OBP, and was purified using filtration, water softening and reverse osmosis. The media was adjusted to required pH using 1 M sodium hydroxide (Sigma Aldrich, USA) and 10% (v/v) ortho-phosphoric acid (88% for analysis EMPARTA® ACS, Merck Millipore, Germany) prior to sterilisation by autoclaving at 121 °C for 15 minutes. Unlike other culture media, the blood tryptone agarose (BTA) media was autoclaved at 121 °C for 30 minutes. The sterility of the media after autoclaving was confirmed by incubation at 37 ± 2 °C for 48 hours, and the absence of bacterial/fungal growth contaminations indicated that the media was sterile and suitable for use.

Table 4. 2: Preparation of culture media.

Culture media	Components
Brain Heart Infusion (BHI) (pH 7.2±0.2)	37 g/L BHI (Thermo Scientific™ Oxoid™, UK)
Tryptone yeast and glucose (TYG) media (pH 7.2±0.2)	30 g/L tryptone (Sigma Aldrich, USA), 4 g/L glucose (Roquette, UK), 20 g/L yeast extract (Merck Millipore, Germany), 0.5 g/L L-cysteine (Marsing & Co-Africa (Pty) Ltd, South Africa), 1 g/L potassium thioglycolate (Sigma Aldrich, USA) and 50 g/L cooked meat medium (Oxoid). Hemin solution was used as a supplement to the media, and was prepared by dissolving 100 mg hemin (Merck Millipore, Germany) in 1 L WFI, and added 20 mL of 1N sodium hydroxide (Merck Millipore, Germany). An additional supplement (Vitamin K1, Merck Millipore, Germany) was added to the media before use.
Peptone Yeast and potassium hydrogen phosphate (PYP) *C. perfringens* type D synthetic media (pH 7.2±0.2).	15 g/L neutralised bacteriological peptone (Thermo Scientific™ Oxoid™, USA), 2 g/L yeast extract (Merck Millipore, Germany), 4 g/L tryptone (Sigma Aldrich, USA), 2 g/L beef extract (Thermo Scientific™ Oxoid™, USA), 1 g/L L-cysteine (Marsing & C0-Africa (Pty) Ltd, SA), 1.3 g/L potassium dihydrogen phosphate (Minema Chemicals, SA), and 2.1 g/L disodium hydrogen phosphate (Sigma Aldrich, USA).

Blood tryptose agar (BTA) (pH 7.1±0.2)	35.2 g/L Tryptose Blood Agar Base (Thermo Scientific™ Oxoid™, USA). Following autoclave the media was left to cool to ±60 °C and 6.25 % (v/v) sterile citrated bovine blood (OBP, SA) was added and mixed thoroughly, before the media was dispensed in petri dishes at ±20 mL volume.
Soy (pH 7.1±0.2)	2.5 g/L glucose (Roquette, UK), 5 g/L sodium chloride (Merck Millipore, Germany), 3 g/L soya peptone (Thermo Scientific™ Oxoid™, USA), 17 g/L casein hydrolysate acid (Thermo Scientific™ Oxoid™, UK), and 2.5 g/L dipotassium hydrogen phosphate (Merck Millipore, Germany)

4.3.2 Preparation of the *C. perfringens* type D inoculum

The pre-inoculum culture of the *C. perfringens* type D was prepared by first reconstituting the lyophilised pellets of the organism in 2 mL BHI media and used to inoculate the 1 L of TYG medium, supplemented with sterile 1% (v/v) hemin solution and 0.2% (v/v) Vitamin K1 in a 2 L round bottom flask. The culture was anaerobically incubated at 37 ± 2 °C overnight without stirring. The anaerobic conditions were maintained using AnaeroGen™ 2.5 L Sachet (Thermo Scientific™ Oxoid™, UK), composed of ascorbic acid and activated carbon which rapidly absorbs oxygen and releases carbon dioxide in a sealed incubation chamber. Following overnight incubation, the pre-inoculum culture (5% (v/v) was seeded into 800 mL sterile PYP synthetic medium (without dextrin) in a 1 L Q-plus bioreactor, and further incubated overnight at 37 ± 2 °C with gentle stirring at 100 rpm, and continuous nitrogen purging through the 0.2 µm membrane filters (Sartorius, Germany).

4.3.3 Cultivation of the *C. perfringens* type D for expression of the epsilon toxin

The *C. perfringens* type D was cultured in PYP media (pH 7.2 ± 0.2) enhanced with 12,8 g/L dextrin (Minema Chemicals, SA) in three 1 L bioreactor vessels. The overnight inoculum culture (5% v/v) was seeded in the three bioreactor vessels consisting of different anaerobic conditions (as illustrated on Figure 4.1). The systems were maintained at a controlled temperature of 37 ± 2 °C, non-controlled pH 7.2 ± 0.2 and continuous stirring at 100 rpm; condition A (closed system, with no airflow), condition B (nitrogen gas purged for the first 2-hours of the fermentation process), condition C (nitrogen gas purged continuously until harvest time). The cultures were incubated overnight at 37 ± 2 °C, with gentle stirring at 100 rpm. These conditions were used to study and characterise the growth kinetics of the *C. perfringens* type D and expression of the epsilon toxin. Samples were collected at a 3-hour interval up to 21 hours (T0, T3, T6, T9, T12, T15, T18, and T21) and used to monitor bacterial cell

growth and epsilon toxin production yield. The optical density (OD) of the bacterial culture at 3-hour interval was measured using the Genesys 20 spectrophotometer model 4001/4 (Thermofisher Scientific, USA) at a wavelength of 600 nm in triplicates. The *C. perfringens* type D sample cultures were transferred into polystyrene microphotometer cuvettes (Lasec® SA (Pty) Ltd, SA) to a 2 mL capacity, and subsequently analysed. The mean absorbance of the crude culture at 600 nm wavelength was measured and recorded for the various time points of the *C. perfringens* type D culture.

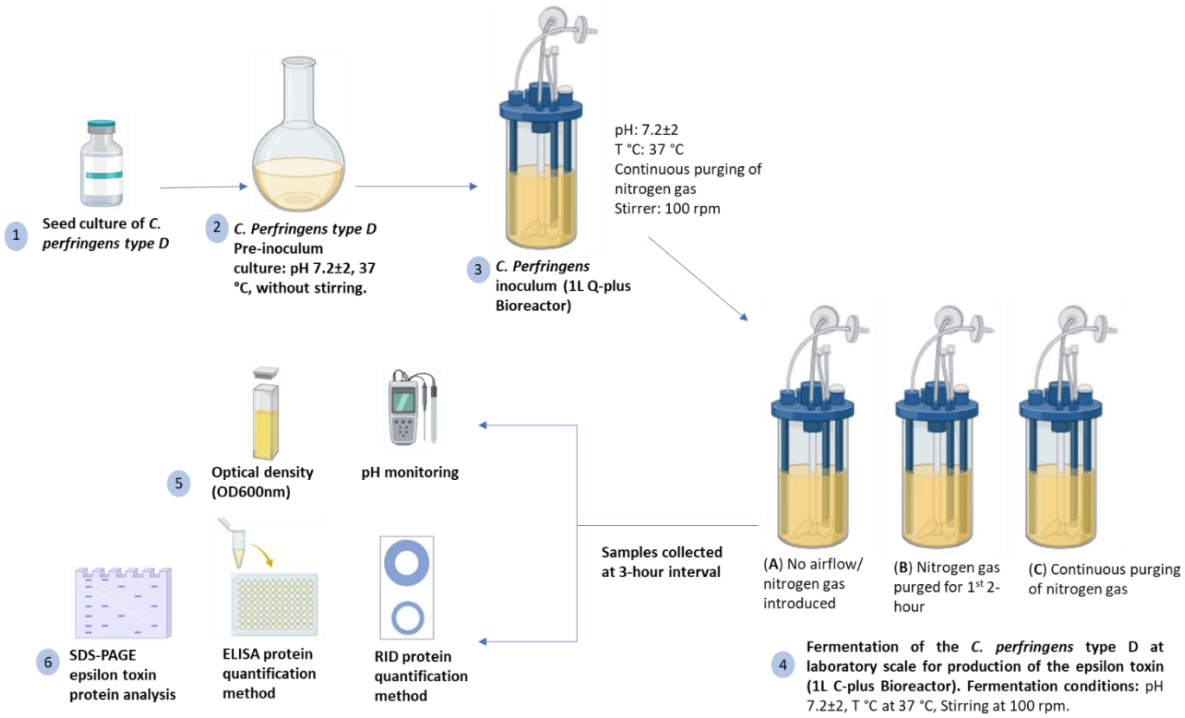


Figure 4. 1: Schematic illustration of the fermentation of *C. perfringens* type D for production of the epsilon toxin. Samples were taken at 3-hour interval and used to determine the growth kinetics of the bacteria and production of the epsilon toxin.

4.3.4 Purity test of the harvested *C. perfringens* type D culture

Purity of the harvested culture was confirmed using two techniques; selective growth culture conditions incubated under aerobic and anaerobic environment, which also allows determining the morphology of colonies formed on BTA solid media after incubation. The test was performed as previously described by Uzal *et al.* (2004). To test the purity of the *C. perfringens* type D harvested culture, its sample was aseptically streaked with a glass rod on BTA solid media and incubated

anaerobically and aerobically in duplicate at 37 ± 2 °C for 24 hours. The harvested culture was also assayed using gram staining and viewed under light microscope at 10x magnification to determine if it contained the pure purple rod-shaped bacilli (Gram, 1884).

4.3.5 Analysis of the epsilon toxin expression using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis was used to evaluate the expression of epsilon toxin in the *C. perfringens* type D culture supernatant. This assay was conducted in accordance with the method described by Laemmli (1970). Briefly, a 50 µL of the harvested culture supernatant was combined with 10 µL of non-reducing treatment buffer comprising [4% (w/v) SDS, 25% (v/v) 0.5 M Tris-HCl, 20% (v/v) glycerol and 0.002 (w/v) bromophenol blue], followed by incubation at 95 °C for 10 minutes. The protein samples (20 µL) were subsequently loaded into 12.5% (w/v) SDS-PAGE gels. A PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermofisher Scientific, USA) was added as a protein size standard at a volume of 2 µL per gel. The gels were then subjected to electrophoresis using a Tris glycine SDS-PAGE running buffer [consisting of 0.3% (w/v) Tris base, 1.4% (w/v) glycine, 1% (w/v) SDS and pH 8.3] at 90-100V, 20A for 90 minutes. This procedure was conducted using the BioRad® Mini protein electrophoresis apparatus (BioRad, Brazil). After protein separation, the gels were stained with Coomassie Blue R250 (Sigma Aldrich, USA) for protein analysis.

4.3.6 Epsilon toxin identification using western blot

Western blot was used to confirm the identity of the epsilon toxin in the harvested *C. perfringens* type D culture after fermentation (Burnette, 1981). The method was conducted as previously described by Gallagher *et al.* (2011) using the iBlot® Gel Transfer device (Invitrogen) for dry transfer of proteins. Briefly, harvested culture supernatant was resolved in the SDS-PAGE gel and transferred onto a nitrocellulose membrane, using the iBlot® Dry Blotting system (Thermofisher Scientific Invitrogen, USA) with default parameters set at 7 minutes. Following protein transfer, the membrane was rinsed with a saline buffered solution for 5 minutes and incubated in a blocking solution (described in Annexture A) at room temperature with gentle agitation for 1-hour to prevent non-specific binding. The membrane was subsequently washed with a saline buffered solution for 5 minutes, followed by incubation in blocking solution containing primary anti-epsilon toxin antibodies (National Institute for Biological Standard and Control (NIBSC), France) at a ratio of 1:1000 overnight with gentle agitation. The membrane was then washed 3x with a saline buffered solution to remove any unbound primary antibodies for 5 minutes with each wash. Blocking solution containing secondary anti-horse IgG conjugate tagged with horseradish peroxidase (Abcam Ltd, UK) at a final ratio of 1:2000, was added

to the membrane and incubated for 1-hour with gentle agitation. The membrane was washed 3x with a saline buffered solution to remove any unbound secondary antibodies for 5 minutes with each wash. Following the 3-washes of the membrane, a 1-Step™ TMB-Blotting Substrate Solution (Thermofisher Scientific, USA) was added to the membrane and incubated at room temperature with gentle agitation until the protein bands are visible on membrane. Colour detection reaction was stopped by discarding the substrate solution and thoroughly rinsed the membrane with WFI.

4.3.7 Confirmation of the epsilon toxin identity by liquid chromatography-mass spectrometry (LC-MS)

The LC-MS system was used for peptide mapping of the protein bands obtained following gel electrophoresis of the *C. perfringens* type D culture supernatant as per method described by Shevchenko *et al.* (2006) with modification. Briefly, the protein bands obtained following SDS-PAGE gel electrophoresis were cut-out from the gel and diced into small pieces and placed in a 2 mL microcentrifuge tube and mixed with 25 mM ammonium bicarbonate (NH_4HCO_3) in 50% Acetonitrile (ACN) using vortex. The supernatant was decanted and the gels were dried for 20 minutes using speed vac. After complete drying of the gels, the proteins were reduced in gel by adding 25 µL of 10 mM Dithiothreitol in 25 mM NH_4HCO_3 and mixed by vortex followed by centrifugation at 2500 x *g* for 5 minutes at 25 °C, and incubation at 60 °C for 1-hour. Samples were cooled to room temperature, then 100 % ACN was added and incubated for ten minutes before the supernatant was decanted. A 25 µL of 55 mM iodoacetamide was added to the gel pieces and mixed by vortex and centrifuged at 2500 x *g* for 45 minutes at 25 °C. The supernatant was removed and the gel pieces were washed with 100 µL of NH_4HCO_3 followed by vortex for 10 minutes and centrifuged at 2500 x *g* for 5 minutes at 25 °C. The supernatant was decanted and the gel pieces were dehydrated with 100 µL of 25 mM NH_4HCO_3 in 50 % ACN, the mixture was vortexed for 5 minutes followed by centrifugation at 2500 x g for 10 minutes at 25 °C before removing the supernatant. The gel pieces were completely dried using speed vac and treated with trypsin buffer (13 ng/mL trypsin in 10 mM NH_4HCO_3 containing 10% (v/v) ACN to a final volume of 50 µL or more to completely cover the gel pieces. The mixture was placed on ice for 30 min and added 20 µL of 25 mM NH_4HCO_3 to the gel pieces and the mixture was incubated at 37 °C overnight. The digested solution (aqueous extraction) was transferred into a clean 0.5 mL microcentrifuge tube, followed by addition of 0.1% formic acid and the samples were dried under vacuum. Dried samples were re-suspended in 2% acetonitrile and 0.2% formic acid for LC-MS analysis using a Dionex Ultimate 3000 RSLC system (Dionex, Voisins-le-Bretonneux, France) coupled to an AB Sciex 5600 TripleTOF mass spectrometer (Thermo Fisher, Waltham, MA, USA) and equipped with a nanoelectrospray ion source. Injected peptides were inline de-salted using an Acclaim PepMap C18

trap column (75 µm × 2 cm; 2 min at 5 µL/min using 2% ACN and 0.2% formic acid. Trapped peptides were gradient eluted and separated on a Waters nanoEase CSH C18 column (75 µm × 25 cm, 1.7 µm particle size) at a flow-rate of 0.3 µL/min with a gradient of 6-40% B over 15 minutes (A: 0.1% formic acid; B: 80% ACN and 0.1% formic acid). The 5600 TripleTOF mass spectrometer was operated in positive ion mode. Data-dependent acquisition (DDA) was employed; precursor (MS) scans were acquired from *m/z* 400-1500 (2+-5+ charge states) using an accumulation time of 100 ms followed by 40 fragment ion (MS/MS) scans, acquired from *m/z* 100-1800 with 20 ms accumulation time each (Shilov *et al*., 2007). Raw data files were searched with Protein Pilot V5.0 software (SCIEX), using a database containing sequences from *C. perfringens* downloaded from UniProt. Trypsin was set as the digestion enzyme, cysteine alkylation (iodoacetamide) was allowed as a fixed modification and biological modifications allowed in the search parameters. A 1% false discovery rate filter was applied at the protein level for refinement of identifications.

4.3.8 Quantification of the epsilon toxin

The epsilon toxin yields obtained after fermentation of the *C. perfringens* type D were estimated using both radial immunodiffusion (RID) and enzyme linked immunosorbent assay (ELISA) assays. The RID assay was utilised for primary quantification of the toxin followed by confirmation of the concentrations using ELISA assay.

4.3.8.1 Quantification of the epsilon toxin using RID assay

RID assay was conducted according to the method described by Brandi *et al.* (2016), using a 1.4% (w/v) agarose gel, prepared in 100 mL PBS by boiling at 115 ±5 °C, and allowed to cool to ±75 °C. After cooling, sodium azide (Merck Millipore, Germany) at a final concentration of 1% (w/v) was added to the gel, to prevent bacterial and fungal growth contaminants. The gel was further allowed to cool to 56 °C, and specific anti-epsilon toxin antibody serum (NIBSC, France), was added into the gel at final concentration of 2.1 IU/mL and gently mixed while avoiding to create bubbles. The gel was transferred in between two glass plates, placed vertically on the casting equipment and left to solidify at 4 °C overnight, to form a 2 mm gel. Wells were carefully generated on the gel, using the telescopic punch with 3 mm external diameter and a plastic matrix with holes used as a frame. A 10 µL of culture supernatants were added into the wells in duplicate. The standard epsilon toxin (obtained from OBP, SA) and PBS were included as positive and negative controls, respectively. The samples were left to diffuse radially into the gel for 30 minutes before placed in a humidified chamber (prepared using a paper towel soaked in WFI and placed in a sealable incubation chamber) and further incubated at 37

± 2°C for 48 hours. This is to allow the antigen to further diffuse radially and form ring precipitin at the optimal antigen-antibody concentration. The gel was washed with 0.1 M sodium chloride overnight and then rinsed with WFI for 30 minutes. The gel was heat-dried with a hair drier until completely attached onto the glass-plate surface, to allow for standard observation of a ring precipitin. Following drying, the gel was stained with Coomassie blue solution for 30 minutes followed by a destaining solution to allow visualisation of the ring precipitin. The diameter of the ring was assessed using a caliper and subsequently documented. The epsilon toxin concentration was interpolated on the linear graph generated and used as a standard for quantification of the epsilon toxin at the Research and Development Bacterial department at OBP (Appendix A).

4.3.8.2 Quantification of the epsilon toxin using ELISA assay

Quantification of the epsilon toxin in the *C. perfringens* type D culture supernatant was conducted using the direct antigen ELISA (Bio K 268/2,BioX-Diagnostics, Belgium), manufactured for specific detection of the epsilon toxin. Prior to use of the kit in the detection of the epsilon toxin, it was modified to allow quantification of the toxin by developing the standard curve of the protein (obtained from Bio-X Diagnostics, Belgium). The standard curve was developed using the Bicinchoninic acid (BCA) protein quantification kit (Thermofisher Scientific, USA) following manufactrurers instructions. The standard curve was used to correlate protein concentrations in relation to the absorbance measured at 450 nm wavelength. The ELISA method was carried out to determine protein concentrations at various dilutions of the *C. perfringens* type D culture supernatant. The following dilutions (200x, 400x, 600x, 800x, 1000x and 1200x) of the epsilon culture supernatant were prepared in PBS and ELISA carried out following manufacturer's instructions. The average absorbance at 450 nm was evaluated using the Power Wave HT spectrophotometer (Agilent Technologies, USA). The mean absorbance was obtained and interpolated on the epsilon toxin ELISA standard curve to determine the concentrations of the epsilon toxin in culture supernatant. The culture dilutions with the concentrations that falls within the working range were utilised for estimation of the epsilon toxin in the harvested *C. perfringens* type D.

4.3.9 Activation of the epsilon prototoxin with trypsin

Complete activation of the epsilon prototoxin was evaluated using trypsin (from porcine pancreas, Sigma Aldrich, Germany) at five different final concentrations (0.01 mg/mL, 0.02 mg/mL, 0.032 mg/mL, 0.05 mg/mL and 0.1 mg/mL prepared in PBS. For the digestion, the harvested culture of *C. perfringens* type D was centrifuged at 4000 x *g* to obtain the culture supernatant and divided into 6 aliquots, of which 5 were treated with trypsin at various concentrations. Complete activation was evaluated at 30 minutes, 60 minutes, and 150 minutes following incubation at 37 ± 2 °C with stirring at 100 rpm. The untreated culture supernatant was included as a control. Complete activation of the epsilon prototoxin was evaluated on SDS-PAGE gel.

4.3.10 Experimental animal model, housing and care

The CD-1 mice were randomly selected and used for assessing toxicity of the epsilon toxin both before and after activation with trypsin. Mice were bred at OBP Experimental unit and used at eight to twelve weeks of age, with an estimated weight of 20 ±2 g. All protocols were conducted in accordance with the standard operating procedures at OBP. The animals were placed in groups of three in clean cages containing Corncob bedding, and in a controlled environment with temperature and relative humidity of 23 ± 2 °C and ±50%, respectively. The cages were labelled according to the treatment received, and mice were provided with clean water ad libitum and fed JCW Petfood mice chow. At the end of the experiment, the animals were euthanised using carbon dioxide narcosis and asphyxiation.

4.3.11 Mouse median lethal dose MLD_{50}/mL

The mouse median lethal dose (MLD_{50}/mL) was performed to measure toxicity of the epsilon toxin before and after activation with trypsin. Ten-fold serial dilutions (10^{-1} to 10^{-3}) of the bacterial culture supernatant was conducted using saline buffered solution. A group of mice (n=4, per dilution of culture) were injected with 0.2 mL intravenously of the diluted culture and mortalities were recorded after 48 hours of injection. The neat (undiluted) *C. perfringens* culture supernatant was included. The uninjected group of mice were included as negative controls.

4.3.12 Inactivation of the trypsinised epsilon toxin

Inactivation of the epsilon toxin using formalin (37% (v/v) formaldehyde, stabilized with 10% (v/v) methanol) (Merck Millipore, Germany) was evaluated at final concentrations of (0.3% v/v, 0.5% v/v and 0.7% v/v) to achieve complete inactivation. The trypsinised culture supernatant was subdivided into multiple aliquots, and the various concentrations of formalin were added in duplicates into the

culture supernatants and incubated at 37 ± 2 °C for 48-hours with stirring at 100 rpm. Samples were collected at 24 and 48-hours to evaluate complete inactivation of the epsilon toxin in culture supernatants. The untreated culture supernatant was included as the negative control. Complete inactivation of the epsilon toxin was confirmed using toxicity testing in mice as described previously in section 4.3.13. The untreated epsilon toxin and saline buffered solution were included as negative and positive controls, respectively. Mice that record no mortalities after injection indicates complete inactivation of the toxin.

4.3.13 Ethical considerations

Procedures conducted in CD-1 mice were carried out in accordance with the standards as set out by the OBP Experimental unit, based on Animal Protection Act (Act 71 of 1962) and Animal Disease Act (Act 5 of 1985). The protocol utilised were approved by the OBP Animal Ethics Committee (South African Veterinary Council Facility Registration Number: FR1514054). The research study was also supported by the national Department of Agriculture, Land Reform and Rural Development under Section 20 of the Animal Diseases Act (Act 35 of 1984) South Africa, approval number 12/11/1/1/(b) MG (2302).

4.3.14 Statistical analysis

Statistical analysis was conducted to compare the significant difference of the *C. perfringens* type D growth rate, PH and the epsilon toxin concentrations as estimated by both RID and ELISA assays in all the three conditions A, B and C. A two-way analysis of variance (ANOVA) followed by a Bonferroni's post-test was utilised to compare the three fermentation conditions over time. GraphPad prism version 5.0 software for Windows was utilised for the statistical analysis at a signifcance level of 5% ($p < 0.05$).

4.4 Results

4.4.1 Growth characterisation of the *C. perfringens* type D

The growth kinetics of the *C. perfringens* type D was studied under three anaerobic conditions. Condition A was completely sealed with no airflow, whereas conditions B and C were supplied with nitrogen gas in the first 2-hours and continuously during fermentation process, respectively. All other fermentation parameters were kept the same across the three experimental conditions. The results indicated that *C. perfringens* type D grew rapidly under controlled anaerobic conditions with the cell growth rate in condition C being faster than that in condition B. Furthermore, the bacterium cultured in condition B and C reached exponential growth phase between 6 and 9-hours of cultivation, both reaching the maximum OD_{600nm} of 1.8 after 9-hours (Figure 4.2 A). In contrast, *C. perfringens* type D cultured in condition A showed a lag phase of 6 hours and only reached stationary phase after 15-hours of cultivation, indicating slow growth condition. The much slower growth rate in condition A is attributed to the traces of oxygen contents diffused into media during inoculation. Constant growth was observed for all three conditions and have reached stationary phase, which was attributed to the accumulation of metabolites excreted by the bacteria during exponential growth phase and depleted culture nutrients (Esmaeilnejad-Ahranjani *et al.*, 2023). According to the statistical analysis, there was no significant difference ($P>0.05$) between bacterial growth rates in all the three fermentation conditions. The pH analysis of *C. perfringens* type D fermentation under all three conditions showed a decrease in pH once the bacterium has entered exponential phase (Figure 4.2 B). Similarly, there was no significant difference ($P>0.05$) observed between the rates of pH decrease across all the fermentation conditions, in exception to pH detected after 9 hours of fermentation between conditions A and C ($P<0.05$).

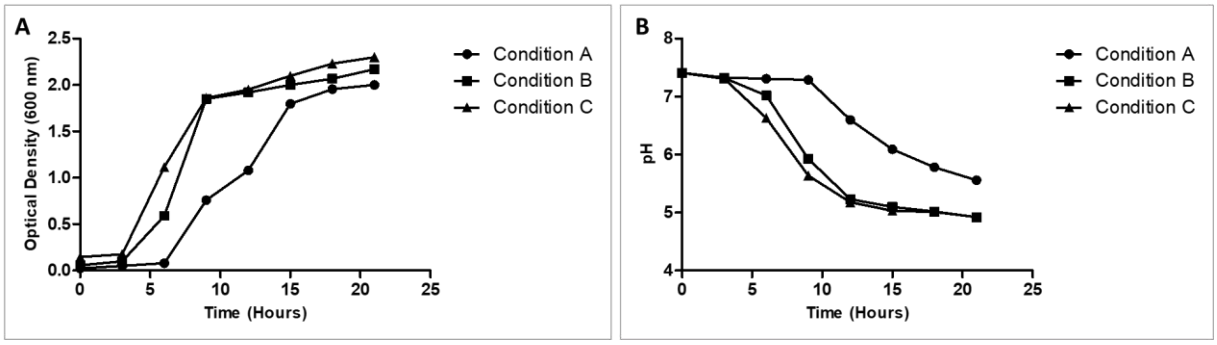


Figure 4. 2: Growth characterisation of the *C. perfringens* type D under different anaerobic conditions. (A) Growth and (B) pH profile of the *C. perfringens* type D. The *C. perfringens* type D culture was fermented in 1 L Q-plus bioreactors, where A represent a closed system with no air flow. The conditions where nitrogen gas was supplied for the first 2-hours and continuously are indicated by B and C, respectively. Each data point represents an average mean of the three replicates.

4.4.1.1 Purity of the C. perfringens type D *culture*

Purity of the bacterium was determined from the harvested culture and the organism was shown to be free from contaminants as confirmed by absence of growth on BTA solid media following incubation under aerobic conditions for 24 hours at 37 ± 2 °C (Figure 4.3 A). Pure culture colonies of the *C. perfringens* type D were obtained on BTA solid media, surrounded by zones of hemolysis, after incubation under anaerobic conditions as anticipated (Figure 4.3 B). The purity of the bacterial culture was also confirmed using gram staining technique, which confirmed the purple straight sided-rods, appearing in single forms, in clusters and also formed chains, viewed under light microscope at 10x magnification (Figure 4.3 C).

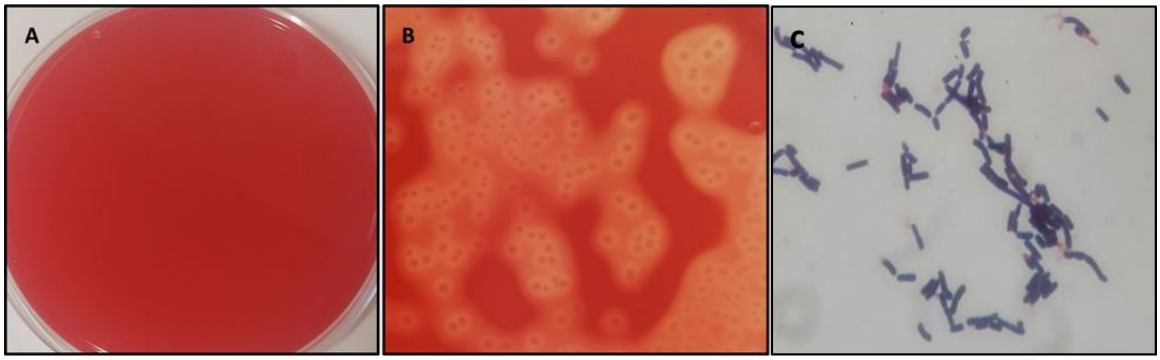


Figure 4. 3: Purity test of the *C.perfringens* type D culture after an overnight fermentation at anaerobic conditions, temperature 37 ± 2 °C, a starting pH of 7.2 and gentle stirring at 100 rpm. (**A**) no growth detected on BTA solid media inoculated with the harvested culture and incubated aerobically at 37 ± 2 °C overnight. (**B**) Pure *C. perfringens* type D colonies were obtained on BTA solid media following incubation at anaerobic condition at 37 ± 2 °C. (**C**) Pure gram-positive *C. perfringens* type D was confirmed under light microscopy at 10x magnification.

4.4.2 Expression of the epsilon toxin by the *C. perfringens* type D

Expression of the epsilon toxin secreted during the *C. perfringens* type D fermentation under three experimental conditions was evaluated using the SDS-PAGE. A protein band with molecular size of 35 kDa, corresponding to the predicted size of the epsilon toxin was observed on SDS-PAGE for all three fermentation conditions (Figure 4.4). Expression of the epsilon toxin was observed from 9 hours for conditions B and C. The SDS-PAGE results also showed that the epsilon toxin was secreted in high quantities during *C. perfringens* type D fermentation as indicative by intense protein bands in comparison to other proteins secreted by the bacteria. This is due to dextrin that was used as a carbohydrate supplement to the culture media of the *C. perfringens* type D, which was shown to favour expression of the epsilon toxin in high quantities when compared to other proteins the bacteria can express, such as alpha, kappa and theta toxins (Hauschild and Pivnick, 1965). The expression of the epsilon toxin in culture condition A was characterised by lower yields when compared to conditions B and C (Figure 4.4). The epsilon toxin expression was observed from 12-21 hours of the *C. perfringens* type D cultivation.

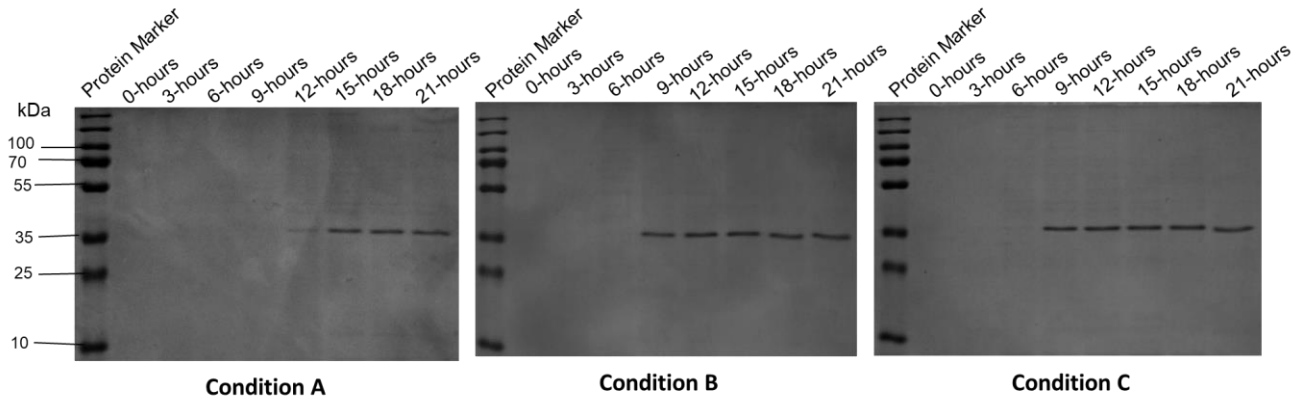


Figure 4. 4: SDS-PAGE analysis of the epsilon toxin expression by the *C. perfringens* type D cultured under different anaerobic conditions. Condition A (closed system, no airflow), and condition B and C were supplied with nitrogen gas for the first 2-hours and continuously during fermentation process, respectively.Epsilon toxin is represented by a 35 kDa protein band across all conditions, a PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermofisher Scientific, USA) was used to estimate sizes of the expressed proteins.

4.4.3 Validation of the epsilon toxin expression using western blot analysis and LC-MS

Western blot technique was used to confirm the identity of the 35 kDa protein expressed during fermentation of *C. perfringens* type D. Figure 4.5 shows positive identification of the 35 kDa protein as epsilon prototoxin by binding to the specific anti-epsilon antibodies. These results were consistent with the LC-MS analysis where digestion of this 35 kDa protein produced peptides with 96% identity to the reference protein produced by the *C. perfringens* type D, obtained using accession number E7D8R1 E7D8R1_CLOPF (Table 4. 3).

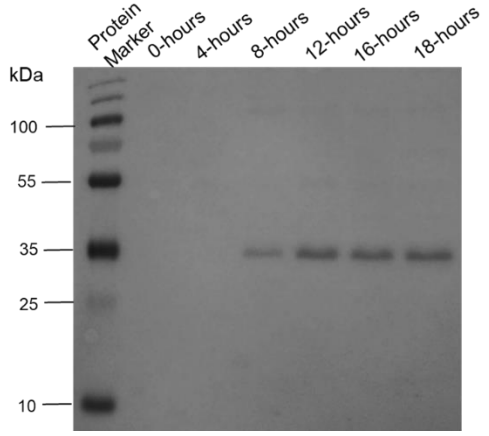


Table 4. 3: LCMS/MS of the epsilon prototoxin peptides

Peptide sequence of the epsilon toxin	Positions of peptides on a reference protein
ASYDNVDTLIEKGR	47 - 60
ALLTNDTQQEQK	120 - 131
ATTTHTVGTSIQATAK	147 - 162
FTVPFNETGVSLTTSYSFANTNTNTNSK	163 - 190
EITHNVPSQDILVPANTTVEVIAYLKK	191 - 117

Figure 4. 5: Western Blot analysis of the epsilon toxin expressed by *C. perfringens* type D. Protein extracts were separated on a 12.5% SDS-PAGE gel and transferred onto a nitrogen cellulose. The 35 kDa protein band observed on the SDS-PAGE gel analysis had adhered to the specific anti-epsilon toxin antibodies, confirming the identity of the epsilon toxin produced by the *C. perfringens* type D culture fermented under anaerobic conditions maintained with continuous nitrogen purging.

4.4.4 Quantification of the epsilon toxin in *C. perfringens* type D culture supernatants using RID

The RID assay was used to determine the concentrations of the epsilon toxin expressed by the *C. perfringens* type D during fermentation process. Figure 4.6 shows a ring formed for samples collected during the exponential growth phase of all the three tested conditions indicating that the test samples contained epsilon toxin. However, the diameter of the ring was varied between the conditions suggesting that the growth conditions used for culturing the *C. perfringens* type D results in different epsilon toxin yields. This is in agreement with Figure 4.7 which shows the epsilon toxin yields during fermentation. The maximum concentration yields (17.9 TCP/mL) was obtained in condition B, after 12-hours of cultivation and was maintained up to 21-hours of harvest. The statistical analysis

confirmed no significant difference (P>0.05) observed between the epsilon toxin yields expressed in all the three fermentation conditions. These results were consistent with the *C. perfringens* type D growth kinetics (Figure 4.2 A), and the toxin concentrations determined by ELISA, which demonstrated that cultivation of *C. perfringens* type D requires maintained anaerobic conditions to obtain maximum toxin yield, and there is no need for continuous nitrogen purging during fermentation.

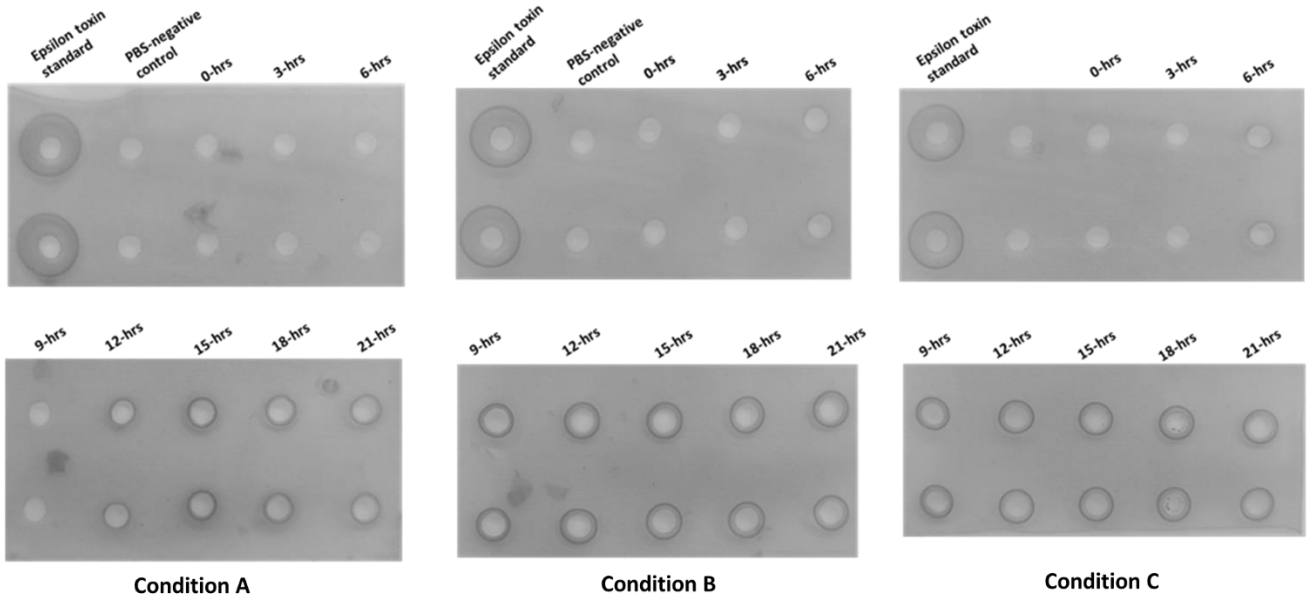


Figure 4. 6: Quantification of the epsilon toxin using RID assay. Condition A (closed system, with no airflow), conditions B and C (Nitrogen gas supplied for 1st 2-hours and continuously during the fermentation process, respectively). The epsilon toxin was expressed earlier after 6 and 9-hours of fermentation in anaerobic conditions C and B, respectively. A ring formed around the sample wells increased in diameter from 12-21 hours for both conditions B and C, indicating increasing production yield of the toxin. The expression of the epsilon toxin in condition A was delayed and only produced from 12-21 hours of cultivation at lower yields, indicated by smaller ring diameters.

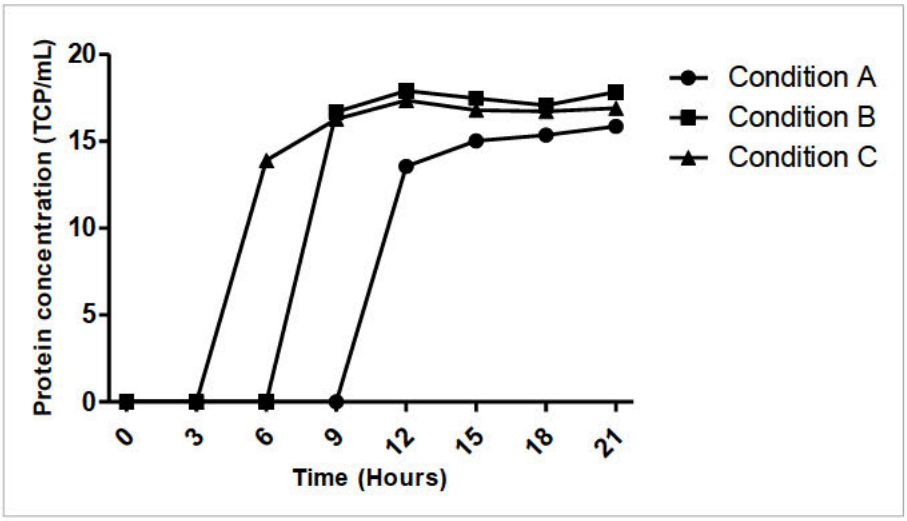


Figure 4. 7: Quantification of the epsilon toxin in the *C. perfringens* type D culture supernatant using RID assay. Condition A (closed system, no airflow), conditions B and C, were supplied with nitrogen gas for 1st 2-hours and continuously during the fermentation process. The epsilon toxin was expressed earlier after 6 and 9-hours of the *C. perfringens* type D cultivation under anaerobic conditions C and B, respectively. The epsilon toxin was produced at high yields in both conditions B and C. Expression of the epsilon toxin in condition A yielded lower concentrations of the epsilon toxin detected from 12-21 hours of cultivation. Each data points represents an average mean of the two replicates.

4.4.5 Quantification of the epsilon toxin in *C. perfringens* type D culture supernatants using ELISA

Quantification of the epsilon toxin using ELISA method was utilised as a confirmatory analysis to the RID assay. A standard curve of the epsilon toxin standard was developed prior to the utilisation of the commercial ELISA method. Figure 4.8 A displays an ELISA standard curve derived from the absorbance (OD_{450nm}) of the standard epsilon toxin dilutions consisting of different protein concentrations (µg/mL) (Figure 4.8 A). The correlation coefficient of both graphs was 0.99, showing the best data fit. In order to quantify expressed epsilon toxin, different dilutions of the *C. perfringens* type D culture supernatant were prepared. A consistent decrease in the concentration of the epsilon toxin was observed as the dilution factor increased (Table 4.4). The epsilon toxin was diluted to a 1:1200 ratio, which was determined to be within the standard working range, and yielded a protein concentration of 1601.5 µg/mL (prior to including the dilution factor) (Table 4.4). This 1:1200 ratio was found to be suitable for pre-diluting the culture supernatant in order to quantify the concentrations of epsilon toxin using the ELISA assay. It is important to note that the determined dilution factor may vary based on the concentration of the epsilon toxin in the culture supernatant, however, the mean concentration should fall within the working range of the standard curve to ensure accurate determination of the

toxin concentration. Figure 4.8 B shows that the *C. perfringens* type D cultured under maintained anaerobic conditions B and C had first expressed the epsilon toxin during the exponential growth phase, after 6-hours of cultivation (condition C), obtaining the concentration of 1.272 mg/mL. The maximum epsilon toxin yields obtained with culture condition C was 4.557 mg/mL after 12-hours of *C. perfringens* type D cultivation. In condition B, the epsilon toxin was first expressed after 9-hours of cultivation and obtained the maximum yields of 4.9 mg/mL after 12-hours of cultivation (Figure 4.8 B). Expression of the toxin in culture condition A was delayed, and had shown first protein expression detected after 12-hours of cultivation at a concentration of 1.198 mg/mL, and obtained an exponential increase, reaching concentration of 2.961 mg/mL after 21-hours of cultivation (Figure 4.8 B). The statistical analysis of the three conditions confirmed that conditions B and C exhibited significantly different ($P<0.001$) rates of epsilon toxin expression from 9 to 21 hours and 6 to 21 hours of bacterial cultivation, respectively, when compared to condition A. Furthermore, a highly significant difference ($P<0.001$) was observed between the epsilon toxin concentrations in conditions B and C from 6 to 21 hours of cultivation.

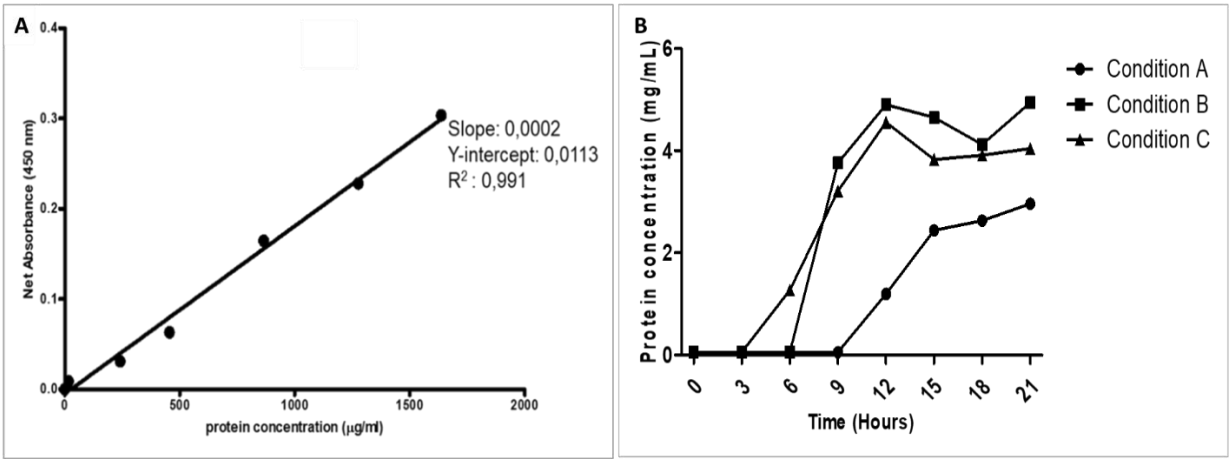


Figure 4. 8: Quantification of the epsilon toxin using ELISA assay. (A) Standard curve of the epsilon toxin developed using the ELISA method to determine net absorbance (450nm) of the protein concentrations. The correlation coefficient of the graph was 0,99, showing the best data fit. (B) The expression kinetics of the epsilon toxin under different anaerobic conditions of the *C. perfringens* type D fermentation. Condition A (Closed system, no airflow), conditions B and C were supplied with nitrogen gas at 1st 2-hours and continuously during the fermentation process, respectively. The epsilon toxin was expressed earlier after 6 and 9-hours of cultivation in conditions C and B, respectively. The toxin was expressed at high yileds under both conditions B and C. The expression of the epsilon toxin under condition A was delayed and produced from 12-21 hours of cultivation. Each data points presents an average mean of the two data points.

Table 4. 4 Validation of the ELISA assay for quantification of the epsilon toxin in *C. perfringens* type D culture supernatants.

Sample Dilution	Average absorbance at 450 nm	Protein concentration (y=0,0002x-0,0113) (µg/mL)
1:200	1.708	8596.5
1:400	1.262	6366.5
1:600	0.853	4321.5
1:800	0.730	3706.5
1:1000	0.555	2831.5
1:1200	0.309	1601.5

4.4.6 Activation of the epsilon prototoxin

The epsilon toxin is activated by trysin cleavage into approximately 28.6 kDa. Different concentrations of trypsin were evaluated to determine the optimal concentration required to achieve complete cleavage of the prototoxin. Figure 4.9 A shows that concentrations of trypsin at 0.05 mg/mL and 0.1 mg/mL achieved complete activation of the prototoxin after 1-hour of treatment, as evident with the absence of the prototoxin protein band on the SDS-PAGE gel. However, a high concentration of residual trypsin was detected in the activated culture supernatant (Figure 4.9 A). Subsequently,a lower concentration of the enzyme at 0.032 mg/mL was evaluated to achieve complete activation of the toxin at an increased incubation time (up to 150 minutes). Figure 4.9 B shows complete activation of the epsilon prototoxin after 150 minutes incubation with 0.032 mg/mL trypsin and no residual trypsin was present in the digested sample.

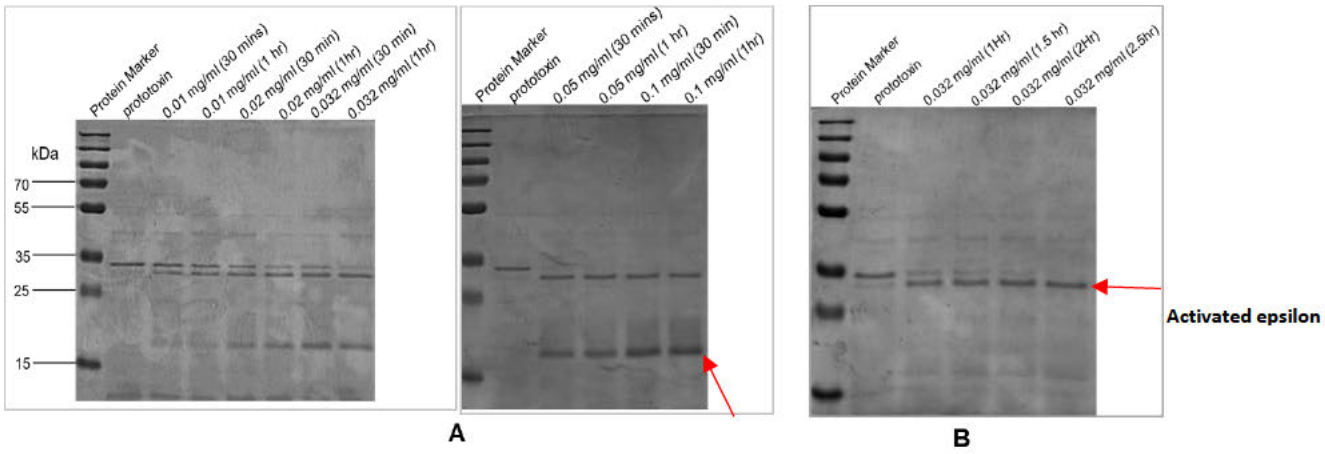


Figure 4. 9: Activation of the epsilon toxin with various concentrations of trypsin.(A) Activation of the epsilon prototoxin evaluated with different concentrations of trypsin (0.01 mg/mL, 0.02 mg/mL, 0.032 mg/mL, 0.05 mg/mL and 0.1 mg/mL) over a period of 30 min and 60 min at 37 ± 2 °C. The epsilon prototoxin was completely activated using 0.05 mg/mL and 0.1 mg/mL following 30 minutes of treatment at 37 ± 2 °C. **(B)** Activation of the epsilon prototoxin evaluated with trypsin at final concentration of 0.032 mg/mL over a period of 150 minutes at 37 ± 2 °C. The epsilon prototoxin was completely activated after 150 minutes with no residual trypsin observed in the activated culture.

4.4.7 Toxicity evaluation of the activated epsilon toxin in mice

Toxicity of the cleaved epsilon toxin was evaluated in mice. A ten-fold serial dilution of the activated sample cultures was prepared and each injected in a group of four mice. The prototoxin was included as a control to establish if increased toxicity was achieved after protein activation. Results presented on Table 4.5 indicated that the prototoxin expressed by the *C. perfringens* type D under maintained anaerobic conditions, obtained the toxicity level of 10^1 MLD_{50}/mL, which was increased to 10^2 MLD_{50}/mL after activation with 0.032 mg/mL trypsin. This was evident with the mortalities recorded in mice injected with a 10^{-1} dilution (prototoxin) and 10^{-2} dilution (activated toxin). The toxicity induced by the prototoxin may have resulted from proteases and trypsin excreted by *C. perfringens* type D as by-products of metabolism during fermentation (Kulshrestha, 1974, Freedman *et al.*, 2014).

Table 4. 5: Toxicity of the epsilon toxin before and after activation with trypsin, evaluated in mice.

	MLD$_{50}$/mL					
Sample culture	**Neat**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
Harvested Prototoxin	†††	†††	√√√	√√√	√√√	√√√
Activated epsilon toxin	†††	†††	†††	√√√	√√√	√√√

Key: † represents dead mouse, √ represents live mouse

4.4.8 Inactivation of the epsilon toxin with formalin

Formalin was evaluated at various concentrations to achieve complete inactivation of the epsilon toxin (with toxicity level of 10^2 MLD$_{50}$/mL). The untreated epsilon toxin and saline buffered solution were included as the negative and positive controls, respectively. Complete inactivation of the toxin was achieved using formalin at final concentration of 0.7% (v/v) after 48-hours of incubation at 37 ± 2 °C, since no mice mortality was recorded (Table 4.6). Lower concentrations of formalin (0.3% (v/v) and 0.5% (v/v)) did not induce complete inactivation of the epsilon toxin. This was evident with mortalities recorded in mice injected with the neat formalinised-culture. No mortalities were observed in mice injected with saline buffered solution.

Table 4. 6: Inactivation profile of the epsilon toxin at various concentrations of formalin at 37 ± 2 °C for 48 hours.

	MLD$_{50}$/mL			
Sample culture	**Neat**	**10^{-1}**	**10^{-2}**	**10^{-3}**
24-hours of inactivation with formalin				
Untreated epsilon toxin	†††	†††	√√√	√√√
Epsilon toxin (0.3% formalin)	†††	†††	√√√	√√√
Epsilon toxin (0.5% formalin)	†††	†††	√√√	√√√
Epsilon toxin (0.7% formalin)	†††	†††	√√√	√√√

Saline buffered solution	√√√	-	-	-
48-hours of inactivation with formalin				
Untreated epsilon toxin	†††	√√√	√√√	√√√
Epsilon toxin (0.3% (v/v) formalin)	†††	√√√	√√√	√√√
Epsilon toxin (0.5% (v/v) formalin)	†††	√√√	√√√	√√√
Epsilon toxin (0.7% (v/v) formalin)	√√√	√√√	√√√	√√√
Saline buffered solution	√√√	-	-	-

Key: † represents dead mouse, √ represents live mouse

4.5 Discussion

Protection of small ruminants against PK disease is largely reliant upon the administration of highly potent vaccines formulated with high concentration of epsilon toxoid. These vaccines are attained by utilising high concentrations of the epsilon toxin in their formulations, which are produced by *C. perfringens* type D fermentation. The efficiency of *C. perfringens* type D fermentation is influenced by factors such as the composition of the media, the degree of anaerobicity during fermentation, and aeration (Esmaeilnejad-Ahranjani *et al.*, 2023). For this study, growth kinetics of the *C. perfringens* type D at the end of fermentation showed that the culture was of uniform morphology and gram positive, appearing as purple, rod-shaped bacilli as anticipated. Furthermore, single and round colonies on agar plates were observed, surrounded by a zone of haemolysis, which is characteristic of *C. perfringens* organisms (Javed *et al.*, 2012, Singh *et al.*, 2018).

The *C. perfringens* type D growth profile studies indicated that the nitrogen purging enhances the anaerobicity of the fermentation system by eliminating traces of oxygen during fermentation, resulting in an increased rate of bacterial growth (Figure 4.2). In comparison to conditions without nitrogen purging, the bacteria cultured with nitrogen exhibited a reduced lag time of 3-hours, and the exponential phase was shortened from 15 to 9-hours. This demonstrated that the presence of oxygen in the fermentation system hinders the growth of *C. perfringens* type D, which in turn results in low toxin yields. Conversely, accelerated growth of the bacteria results in a progressive reduction in the pH during fermentation. This was observed during the exponential phase of bacterial growth, where a gradual decrease in pH occurred, and reached below pH 5.5 at stationary growth phase. These findings are consistent with the data reported by Esmaeilnejad-Ahranjani *et al*. (2023), where a reduction in pH was observed during the culturing of *C. perfringens* type D under anaerobic conditions and reached a pH of 5.5 during the stationary growth phase. The decrease in pH during the fermentation process indicates a rapid consumption of nutrients and excretion of metabolites, with the toxin included (Esmaeilnejad-Ahranjani *et al.*, 2023).

The community of *C. perfringens* is commonly referred to as strict anaerobes, although they can survive in aerobic conditions and low concentrations of superoxide or hydroxyl-radical-generating compounds (Briolat and Reysset, 2002, Kiu and Hall, 2018). This study sought to evaluate effects of nitrogen purging on epsilon toxin yields during cultivation of *C. perfringens* type D. The highest concentration of the epsilon toxin was obtained in the culture that was maintained under anaerobic conditions and provided with nitrogen gas for the first two hours of fermentation, resulting in a concentration of 4.9 mg/mL and 17.9 TCP/mL after 12 hours of cultivation. Similarly, the culture that

was continuously supplied with nitrogen yielded a concentration of 4.557 mg/mL and 17.35 TCP/mL after 12-hours of cultivation. The results indicate that there is little difference in the production of the epsilon toxin when nitrogen is supplied for the first two hours of cultivation or continuously. On the other hand, the culture that was not supplied with nitrogen gas produced lower yields of the epsilon toxin, with a concentration of 2.961 mg/mL and 15.86 TCP/mL after 21-hours of cultivation. This study has demonstrated that growth conditions with low oxygen concentrations are not optimal for cultivating the *C. perfringens* type D. The bacteria thrive in the absence of oxygen, and nitrogen purging in the fermentation system eliminates traces of oxygen, and provides suitable conditions for cultivating *C. perfringens* type D, resulting in high yields of the epsilon toxin.

The epsilon toxin is produced as an inactive prototoxin, which requires digestion for activation (Worthington *et al.*, 1973). An activated epsilon toxin is pathogenic to ruminants and had also been shown to be antigenic (Minami *et al.*, 1997, Uzal *et al.*, 2004, Harkness *et al.*, 2012). In this study, the prototoxin produced by the *C. perfringens* type D, induced low level of toxicity before enzymatic cleavage with trypsin. The low level of toxicity may have resulted from partial cleavage of the toxin by proteases such as trypsin, which are secreted by the organism during fermentation process (Kulshrestha, 1974, Freedman *et al.*, 2014). The improved toxicity of the epsilon toxin was further evaluated through activation using trypsin at various concentrations. The high concentrations of trypsin (0.05 and 0.1 mg/mL) produced a complete activated toxin at a short treatment time (30 minutes), as well as the residual enzyme detected in the activated culture. The presence of residual trypsin in the activated culture may not be suitable for formulation of the vaccine considering the Good Manufacturing Practices of vaccine products and animal safety and welfare. Therefore, it is also important to ensure that the final residual trypsin contained in the activated *C. perfringens* type D culture remains at lower concentrations. A study conducted by Morcrette *et al.* (2019) demonstrated complete activation of the epsilon prototoxin at final concentration of 1:100 (w/w) ratio and used a cocktail of EDTA free enzyme inhibitors to hinder protease activity after activation. However, residual protease inhibitor were detected in the final activated culture, which may also compromise vaccine safety (Morcrette *et al.*, 2019). The low concentration of trypsin (0.032 mg/mL) had induced complete activation of the prototoxin at an extended incubation time to 150 minutes, obtaining no residual enzyme following activation. This concentration of trypsin was shown to be suitable for digestion of the epsilon toxin used in vaccine formulation. The data obtained had also shown that the toxicity of the epsilon toxin increased following treatment with trypsin. These findings were in alignment with the results of the study conducted by Lyerly and Wilkins (1991), who further stated that epsilon prototoxin and epsilon toxoid had the same TCP but varied in antigenicity (Lyerly, 1991). The results

obtained suggests that treatment of the prototoxin with trypsin enzyme improves its antigenicity. The trypsinised epsilon toxin was further inactivated with formalin at different concentrations and complete inactivation of the toxin was observed with formalin concentration of 0.7% (v/v) after 48 hours of treatment at 37 ± 2 °C with constant stirring at 100 rpm, as indicated by the absence of mortalities recorded in mice injected with the neat formalin treated culture. These findings were in agreement with the results reported by Lobato *et al*. (2010), who used formaldehyde at final concentration of 0.5% (v/v) to inactivate the recombinant epsilon toxin for vaccine formulation (Lobato *et al.*, 2010). Formalin can be effectively utilised to inactivate epsilon toxin and complete inactivation should be demonstrated by injection of the neat inactivated culture in mice before vaccine formulation. Confirmation of complete inactivation of the toxin is necessary to avoid formulation of incomplete inactivated epsilon toxin, which may result in a risk of residual toxicity in vaccinated animals.

4.6 Conclusions

This chapter examined various anaerobic conditions for optimal production of *C. perfringens* type D epsilon toxin. The findings revealed that purging the fermentation system with nitrogen gas is necessary to maintain the anaerobic conditions required for optimal growth of the organism and high production yields of the epsilon toxin. Optimised production of the epsilon toxin allows for the antigen to be used in the formulation of the combination vaccine without compromising the antigenicity of the product.

4.7 References

Araghi, A., Taghizadeh, M., Doust, S. R. H., Paradise, A. & Dezfouli, S. A. (2022). Field Evaluation of Novel Combination Vaccines Against Foot and Mouth Disease Virus and Clostridium perfringens Toxoid Using Different Immunization Protocols. *Jundishapur Journal of Microbiology,* 15.

Ardehali, M. (1984). Preparation of one combined vaccine against anthrax, enterotoxaemia, infections necrotic hepatitis and braxy diseases in sheep and goats. *Razi Vaccine and Serum Research Institute - RVSRI,* agris@fao.org.

Bhown, A. S. & Habeeb, A. (1977). Structural studies on ε-prototoxin of Clostridium perfringens type D. Localization of the site of tryptic scission necessary for activation to ε-toxin. *Biochemical and Biophysical Research Communications,* 78, 889-896.

Bokori-Brown, M., Savva, C. G., Fernandes Da Costa, S. P., Naylor, C. E., Basak, A. K. & Titball, R. W. (2011). Molecular basis of toxicity of Clostridium perfringens epsilon toxin. *The FEBS Journal,* 278, 4589-4601.

Brandi, I. V., Santos, E. M. S., De Carvalho, B. M. A., Durães, C. a. F., Farias, P. K. S., Sari, R. S., Cangussu, A. S. R. & Junior, A. P. (2016). Total combining power: Technique for the evaluation of the quality control process of clostridiosis vaccines. *Journal of Microbiological Methods,* 130, 164-168.

Briolat, V. & Reysset, G. (2002). Identification of the Clostridium perfringens genes involved in the adaptive response to oxidative stress. *Journal of Bacteriology,* 184, 2333-2343.

Burnette, W. N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry,* 112, 195-203.

Esmaeilnejad-Ahranjani, P., Majidi, B., Paradise, A. & Hasanzadeh, M. (2023). Optimization and scale-up of Clostridium perfringens type D culture and epsilon-toxin production: Effects of stirring, glucose and pH adjustment. *Toxicon,* 234, 107302.

Finnie, J. (2003). Pathogenesis of brain damage produced in sheep by Clostridium perfringens type D epsilon toxin: a review. *Australian Veterinary Journal,* 81, 219-221.

Forti, K., Ferroni, L., Pellegrini, M., Cruciani, D., De Giuseppe, A., Crotti, S., Papa, P., Maresca, C., Severi, G. & Marenzoni, M. L. (2020). Molecular characterization of Clostridium perfringens strains isolated in Italy. *Toxins,* 12, 650.

Freedman, J. C., Li, J., Uzal, F. A. & Mcclane, B. A. (2014). Proteolytic processing and activation of Clostridium perfringens epsilon toxin by caprine small intestinal contents. *MBio,* 5, e01994-14.

Gallagher, S., Winston, S. E., Fuller, S. A. & Hurrell, J. G. (2011). Immunoblotting and immunodetection. *Current Protocols in Cell Biology,* 52, 6.2. 1-6.2. 28.

Garcia, J., Adams, V., Beingesser, J., Hughes, M. L., Poon, R., Lyras, D., Hill, A., Mcclane, B. A., Rood, J. I. & Uzal, F. A. (2013). Epsilon toxin is essential for the virulence of Clostridium perfringens type D infection in sheep, goats, and mice. *Infection and Immunity,* 81, 2405-2414.

Gram, C. (1884). Ueber die isolirte Farbung der Schizomyceten in Schnitt-und Trockenpraparaten. *Fortschritte der Medicin,* 2, 185-189.

Guo, P., Zhang, K., Ma, X. & He, P. (2020). Clostridium species as probiotics: potentials and challenges. *Journal of Animal Science and Biotechnology,* 11, 1-10.

Habeeb, A. (1975). Studies on ε-prototoxin of Clostridium perfringens type D physicochemical and chemical properties of ε-prototoxin. *Biochimica et Biophysica Acta (BBA)-Protein Structure,* 412, 62-69.

Harkness, J. M., Li, J. & Mcclane, B. A. (2012). Identification of a lambda toxin-negative Clostridium perfringens strain that processes and activates epsilon prototoxin intracellularly. *Anaerobe,* 18, 546-552.

Hauschild, A. & Pivnick, H. (1965). Effect of carbohydrates on toxinogenesis by clostridium perfringens type D. *Canadian Journal of Microbiology,* 11, 15-22.

Hopker, A., Pandey, N., Bartholomew, R., Blanton, A., Hopker, S., Dhamorikar, A., Goswami, J., Marsland, R., Metha, P. & Sargison, N. (2021). Livestock vaccination programme participation among smallholder farmers on the outskirts of National Parks and Tiger Reserves in the Indian states of Madhya Pradesh and Assam. *Plos One,* 16, e0256684.

Hussain, K., Ijaz, M., Durrani, A., Anjum, A., Nasir, A., Farooqi, S., Aqib, A. & Ahmad, A. (2018). Bacterial count and predisposing factors of Clostridium perfringens (targeting CPA gene) infection along with antimicrobial sensitivity in diarrheic sheep in Pakistan. *Tropical Biomedicine,* 35, 434–441.

Hussain, R., Guangbin, Z., Abbas, R. Z., Siddique, A. B., Mohiuddin, M., Khan, I., Rehman, T. U. & Khan, A. (2022). Clostridium perfringens types A and D involved in peracute deaths in goats kept in Cholistan ecosystem during winter season. *Frontiers in Veterinary Science,* 9, 849856.

Jansen, B. (1960). The experimental reproduction of pulpy kidney disease. *Journal of the South African Veterinary Association,* 31, 205-208.

Javed, S., Rafeeq, M., Tariq, M. M., Awan, M. A., Rashid, N. & Mumtaz, A. (2012). Study on in-vitro biochemical growth characterization and assessment of hemolytic toxin of Clostridium perfringens type B and D. *Pakistan Journal of Zoology,* 44.

Jemal, D., Shifa, M. & Kebede, B. (2016). Review on pulpy kidney disease. *Journal of Veterinary Science and Technology,* 7, 361.

Kiu, R., Caim, S., Alexander, S., Pachori, P. & Hall, L. J. (2017). Probing genomic aspects of the multi-host pathogen Clostridium perfringens reveals significant pangenome diversity, and a diverse array of virulence factors. *Frontiers in Microbiology,* 8, 2485.

Kiu, R. & Hall, L. J. (2018). An update on the human and animal enteric pathogen Clostridium perfringens. *Emerging Microbes & Infections,* 7, 1-15.

Kulshrestha, S. (1974). Effect of period of incubation and pH on the production of beta and epsilon toxins by Clostridum welchii types B and C. *Indian Journal of Animal Sciences*. 43, 987-990.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227, 680-685.

Lobato, F. C., Lima, C. G., Assis, R. A., Pires, P. S., Silva, R. O., Salvarani, F. M., Carmo, A. O., Contigli, C. & Kalapothakis, E. (2010). Potency against enterotoxemia of a recombinant Clostridium perfringens type D epsilon toxoid in ruminants. *Vaccine,* 28, 6125-6127.

Lyerly, D. M. a. W., T.D. (1991). Toxin of anaerobes. In: Anaerobic microbiology. *Oxford University Press,* 163-181.

Minami, J., Katayama, S., Matsushita, O., Matsushita, C. & Okabe, A. (1997). Lambda-toxin of Clostridium perfringens activates the precursor of epsilon-toxin by releasing its N-and C-terminal peptides. *Microbiology and immunology,* 41, 527-535.

Morcrette, H., Bokori-Brown, M., Ong, S., Bennett, L., Wren, B. W., Lewis, N. & Titball, R. W. (2019). Clostridium perfringens epsilon toxin vaccine candidate lacking toxicity to cells expressing myelin and lymphocyte protein. *npj Vaccines,* 4, 32.

Pawaiya, R. S., Gururaj, K., Gangwar, N. K., Singh, D. D., Kumar, R. & Kumar, A. (2020). The challenges of Diagnosis and Control of Enterotoxaemia caused by Clostridium perfringens in small ruminants. *Advances in Microbiology,* 10, 238-273.

Phukan, A., Dutta, G.-N., Daube, G. & Das, B.-C. (1997). Characterization of Clostridium perfringens isolates from goats. *Indian Veterinary Journal,* 74.

Pulotov, F. K., Nazarova, O., Akhmadov, N. & Karimzoda, A. (2021). Development of polyvalent toxoid Clostridium perfringens against anaerobic enterotoxaemia in young cattle and small ruminants. *E3S Web of Conferences*, EDP Sciences, 282, 04009.

Revitt-Mills, S. A., Rood, J. I. & Adams, V. (2015). Clostridium perfringens extracellular toxins and enzymes: 20 and counting. *Microbiology Australia,* 36, 114-117.

Rood, J. I., Adams, V., Lacey, J., Lyras, D., Mcclane, B. A., Melville, S. B., Moore, R. J., Popoff, M. R., Sarker, M. R. & Songer, J. G. (2018). Expansion of the Clostridium perfringens toxin-based typing scheme. *Anaerobe,* 53, 5-10.

Rood, J. I., Mcclane, B. A., Songer, J. G. & Titball, R. W. (1997). The clostridia: molecular biology and pathogenesis, *Academic Press*. San Diego London Boston.

Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V. & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature protocols,* 1, 2856-2860.

Singh, D. D., Pawaiya, R. S., Kumaresan Gururaj, K. G., Gangwar, N. K., Mishra, A. K., Dimple Andani, D. A., Singh, M. K., Saket Bhushan, S. B. & Ashok Kumar, A. K. (2018). Molecular detection of Clostridium perfringens toxinotypes, enteropathogenic Escherichia coli, rotavirus and coronavirus in diarrheic fecal samples of neonatal goat kids. *Veterinarski Arhiv,* 88, 1-20.

Sutton, G. (1952). The use of enterotoxaemia vaccine. *Journal of the South African Veterinary Association,* 23, 185-186.

Uzal, F. A., Kelly, W., Morris, W., Bermudez, J. & Baison, M. (2004). The pathology of peracute experimental Clostridium perfringens type D enterotoxemia in sheep. *Journal of Veterinary Diagnostic Investigation,* 16, 403-411.

Uzal, F. A. & Songer, J. G. (2008). Diagnosis of Clostridium perfringens intestinal infections in sheep and goats. *Journal of Veterinary Diagnostic Investigation,* 20, 253-265.

Worthington, R. & Mülders, M. (1977). Physical changes in the epsilon prototoxin molecule of Clostridium perfringens during enzymatic activation. *Infection and Immunity,* 18, 549-551.

Worthington, R., Mulders, M. S. & Van Rensburg, J. (1973). Enzymatic activation of Clostridium perfringens epsilon prototoxin and some biological properties of activated toxin. *Onderstepoort Journal of Veterinary Research*, 40, 151-154.

5 CHAPTER FIVE: SAFETY AND EFFICACY OF THE RIFT VALLEY FEVER AND PULPY KIDNEY (RVF/PK) COMBINATION VACCINE IN SHEEP

Matome Selina Matsiela [1,2], **Thandeka Khoza** [2*] **and Nobalanda Mokoena**[1*]

1. Onderstepoort Biological Products (Pty. Ltd), 100 Old Soutpan Road, Onderstepoort 0110, Pretoria South Africa.

2. Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg campus), Scottsville, 3209, KwaZulu-Natal, South Africa.

Nabalanda Mokoena: nobalanda@obpvaccines.co.za, khozat1@ukzn.ac.za

+27 12 522 1550 or 033 260 6281

Abstract

Periodic vaccinations of ruminants against the Rift Valley Fever (RVF) disease are often implemented during seasons of heavy rainfalls, to prevent the spread of the virus within susceptible hosts. This approach results in low vaccination coverage against RVF, leading to recurrent disease outbreaks in endemic regions. In order to enhance vaccination rates against RVF, this study developed an aluminium hydroxide ($Al(OH)_3$) adjuvanted bivalent vaccine that combines an inactivated form of the RVFV with the epsilon toxoid, offering protection against both RVF and pulpy kidney (PK) diseases. The combination vaccine was formulated at 1:1 ratio of the inactivated RVFV Smithburn (SB) and epsilon toxoid (ETX) at final concentrations of 6×10^6 PFU/mL and 10^2 MLD_{50}/mL, respectively, with 50% (w/w) $Al(OH)_3$ gel adjuvant. Safety and efficacy of the RVF/PK combination vaccine candidate was evaluated in merino sheep, and the monovalent vaccines (inactivated RVF-SB and PK-ETX) were included as control groups. In addition, the commercially available inactivated RVF and PK vaccines were included as positive controls. The adjuvanted phosphate buffered saline (PBS) served as a placebo formulation for the negative control group. The vaccines were administered through subcutaneous (S/C) route at two doses, D0 (primary vaccination) and D21 (booster injection). All vaccine formulations were safe for use in sheep, as indicated by absence of clinical signs for both RVF and PK infections. No adverse reactions on site of injection, pyrexia nor mortalities were recorded related to vaccine administrations. The combination vaccine induced antibody titres at an average of 1:36 and 5 IU/mL, as early as 14 days post-booster injection against RVFV and epsilon toxin, respectively. These antibody titres were maintained throughout the trial and were comparable to those of the monovalent form of the vaccine, and the positive control groups. Futhermore, no antibody response was observed for all animals vaccinated with placebo formulation as anticipated. The results indicate that the RVF/PK combination vaccine is safe and efficacious against both RVFV and epsilon toxin antigens in target animal species. These findings suggest that the RVF/PK combination vaccine can be used for prophylactic immunization of small ruminants against RVF and PK infections, as well as provide a sustainable vaccination strategy for ruminants in endemic regions to improve vaccination coverage against RVF.

Keywords: Pulpy kidney disease, Rift Valley Fever, epsilon toxin, aluminium hydroxide gel adjuvant.

5.1 Introduction

The Rift Valley Fever Virus (RVFV) and epsilon toxin produced by *Clostridium perfringens* type D present a significant threat to the livestock industries, resulting in substantial economic losses from severe disease outbreaks (Jemal *et al.*, 2016, Kwaśnik *et al.*, 2021). Infections by RVFV and the epsilon toxin can effectively be managed through systemic vaccination of ruminants. However, vaccination against RVF is typically conducted periodically, in response to emergency warnings of possible disease outbreaks (Fao and Africa—El Niño, 2015). Periodic vaccination of livestock against RVF is implemented as a result of the irregular outbreak patterns of the disease in endemic regions, which render regular vaccination campaigns economically impractical (Dungu *et al.*, 2018). Although this practice has widely been used, the emergency vaccination campaigns against RVF has proven to be unsustainable, leading to low vaccination coverage in affected regions (Elfadil *et al.*, 2006, Dungu *et al.*, 2013, Mroz *et al.*, 2017, Dungu *et al.*, 2018). Several reports of inconsistent vaccination of livestock against RVF were obtained across Africa and the Arabian Peninsula, with most countries failing to implement vaccination programs due to the epizootic nature of RVF or lack of reported cases in the field (Dungu *et al.*, 2018). In other cases reported, the vaccines administered during disease outbreaks failed to induce protective immune response on the anticipated time and the animals get infected by the pathogen before protective immunity against the disease was induced (Dungu *et al.*, 2013). This might raise concerns to the quality of the vaccine used for immunisation of animals, rather than looking into the vaccination schedule. Therefore, an alternative effective vaccination regime of the RVF is required. In contrast, regular vaccination of ruminants against the epsilon toxin infections is a common practice (Jemal *et al.*, 2016, Abdolmohammadi Khiav and Zahmatkesh, 2021). The development of a combined RVF/PK vaccine has the potential to improve vaccination coverage of RVF by providing immunity against both diseases.

The combination vaccine approach has been widely used by farmers for prophylactic immunisation of ruminants against various diseases. These include the peste des petits ruminants and sheep/goat pox diseases, chlamydia arbotus and vesicular stomatitis virus infections, and diseases caused by Clostridial organisms such as botulism and black quarter (Alexander and Haig, 1951, House *et al.*, 2003, Fakri *et al.*, 2015). This vaccination strategy is effective in targeting multiple disease-causing pathogens, which results in substantial economic losses in affected regions and provides improved vaccination coverage against more than one disease with a single injection. The use of combination vaccines further offer significant benefits such as simplified vaccination programmes for farmers and fewer injections, reduced level of animal stress and labour from animal caretakers during vaccination (Martinod, 1996). Manufacturing and formulation of combination vaccines requires thorough

optimisation while ensuring the required dosage to elicit the desired immune response from the monovalent vaccine components is maintained. The following factors are taken into account during the development of combination vaccines: (i) demonstration that the combination product does not compromise the safety and immunogenicity of the individual monovalent component (ii) the monovalent vaccines should be demonstrated to be safe and immunogenic prior use in formulation of combination product, (iii) vaccine components should be stable and compatible for formulation of the combined product (since some viruses may suppress immunity of other antigens), and (iv) selected products should have aligned vaccination regime and hosts or target specie (Provost and Perreau, 1978). The recommendation for administration of the PK vaccine in small ruminants typically falls between the ages 4-5 months. This vaccine presents an ideal option for inclusion in a combination vaccine with the RVFV. This study demonstrated the safety and immunogenicity of the RVF/PK combination vaccine in sheep.

5.2 Materials and Methods

5.2.1 Preparation of Al $(OH)_3$ gel adjuvant

The Al $(OH)_3$ gel adjuvant was prepared according to the method described by Nail *et al.* (1976) with modification. Initially, a 37.69% (w/v) ammonium aluminum sulfate (Merck Millipore, Germany) and 3.65% (w/v) ammonium sulfate (Merck Millipore, Germany) were separately dissolved in WFI at a temperature of 70 °C using a high-speed homogenizer (D500 Wiggens, China) at 13 000 rpm for 15 minutes. After cooling to 22 ±3 °C, the solutions were combined together to a 1:1 ratio, followed by the addition of ammonia solution (Minema Chemicals, SA) to a final concentration of 12.5% (v/v) and continuously mixed at 13 000 rpm for 15 minutes. The pH of the adjuvant was measured and recorded (with an acceptable range of 6.0-8.0). The adjuvant was then subjected to the washing process using WFI by first allowing the gel to sediment and replacing the supernatant with WFI. This process was repeated until the conductivity value was within the acceptable range of 300-350 µs/cm. Once the desired conductivity was reached, the adjuvant was allowed to sediment, followed by determining its percentage Pack Cell Volume (%PCV, acceptable at 50%), achieved through continuous wash process with WFI. Finally, the pH of the adjuvant was measured and recorded (with an acceptable range of 6.0 to 8.0), and adjusted temperature to ±80 °C and mixed by stirring at 13 000 rpm final speed for 15 minutes. The adjuvant was stored at 4 °C until required for vaccine formulation.

5.2.2 Production of the monovalent inactivated RVF-SB vaccine pilot batch

The RVFV-SB strain was propagated in BHK-21 cells (Clone 13, Lot number 09/007) according to the method previously described by Smith *et al.* (2019). The cells were maintained in Glasgow's minimum essential medium (GMEM), supplemented with 5.3 mg/mL amphotericin B (Sigma Aldrich, USA), 0.1 mg/mL streptomycin (Sigma Aldrich, USA), 100 U/mL penicillin (Sigma Aldrich, USA), and 10% (v/v) bovine serum (Cell Sera, Australia) in Corning® 850 cm^2 Polystyrene roller bottles (Corning® Incorporated Life Sciences, USA). The roller bottles were incubated at 37 °C on a rolling apparatus set at 0.5 rpm. The confluent monolayer of BHK-21 cell culture were infected with RVFV-SB and further incubated at 37 °C. The infected monolayer of cells was monitored daily for cytopathic effect (CPE) using the Leica DM IL LED inverted light microscope (Leica microsystems, Germany). The virus was harvested when 95-100% virus CPE was reached, by dislodging the infected monolayer of cells from the flask surface. The harvested virus culture was stored at 4 °C and the titre was determined using Plaque Forming Units (PFU) method (Smither *et al.*, 2013). Briefly, the confluent monolayer of Vero cell culture was prepared in 12-well plates. The monolayer of cells was infected (in triplicates) with

0.5 mL of ten-fold serial dilutions of the RVFV-SB culture prepared in serum-free GMEM media. The plates were then incubated for 1-hour at 37 °C in a humidified incubator containing 5% CO_2. Following 1-hour incubation, all plate wells were covered with 2 mL of 1:1 overlay (prepared from mixing 1% (w/v) agarose powder for molecular biology (Condalab, Spain) in 50% (v/v) Earle's buffered solution and 50% (v/v) serum-free GMEM media). The plates were further incubated at 37 °C in a humidified incubator with 5% CO_2 for 6 days. Following 6 days of incubation, a 1% (v/v) neutral red stain was added into each well and the plates were re-incubated at 37 °C for 24 hours before plaques were counted on day 7 to determine the virus titre. The RVFV culture, with a minimum titre of 1 x 10^6 PFU/mL was inactivated with 0.5 mM of binary ethylenimine (BEI) for 24 hours at 37 °C with continuous stirring at 150 rpm. Following 24 hours of virus inactivation, residual BEI in the virus culture was immediately neutralised by adding 1 M sodium thiosulphate pentahydrate to a final concentration of 10% volume of BEI used to inactivate the culture. Complete inactivation of the RVFV culture was validated using the Real Time Cell Analysis (RTCA) assay, using Vero cell culture adhered on 16 well E-plates in accordance with the protocol described by Moetlhoa *et al.* (2021). The complete inactivated RVFV-SB pilot batch was combined with $Al(OH)_3$ adjuvant at final concentration of 50% (w/w) at room temperature and stirred using a high-speed homogenizer at 13 000 rpm for 10-15 minutes. The adjuvanted inactivated RVF-SB vaccine was tested for sterility and quality control tests (including; centrifugation test, visual observation of the vaccine aspect following 24 hours incubation at 23 °C ±2 °C, 4 °C and 37 °C).

5.2.3 Production of the monovalent epsilon toxoid PK (PK-ETX) vaccine

A 20 L pilot batch of the epsilon toxin was produced through fermentation of the *C. perfringens* type D strain ET663, in a 20 L C-plus bioreactor (Sartorius, Germany) at 37 °C ± 2 °C, 100 rpm overnight, under anaerobic conditions with nitrogen purging for the first 2-hours of the fermentation process. At the end of a fermentation process, a sample was taken for optical density, purity test, pH analysis and detection of the epsilon toxin as described in chapter 4, section 4.3.3 and 4.3.4. The culture supernatant was analysed on 12.5% (w/v) Sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) gel for detection of the epsilon toxin. The toxin concentration was further quantified using the enzyme linked immunosorbent assay (ELISA) and radial immunodiffusion (RID) methods as described in chapter 4, section 4.3.8.2 and section 4.3.8.1, respectively.

5.2.3.1 Downstream bioprocessing of the epsilon toxin

The *C. perfringens* type D culture, with the epsilon toxin at minimum concentration of 4.5 mg/mL and 17.9 TCP/mL, was clarified by centrifugation at 4500 x *g*, and at 4 °C for 45 minutes. Trypsin at final concentration of 0.032 mg/mL was added to the clarified supernatant and incubated at 37 °C with stirring at 100 rpm for 150 minutes for activation of the epsilon toxin. Toxicity of the epsilon toxin was evaluated in mice using mouse median lethal dose (MLD_{50}/mL) assay (Mokoena *et al.*, 2017). The active epsilon toxin, with a minimum titre of 10^2 MLD_{50}/mL was inactivated with 0.7% (v/v) formalin at 37 °C with continuous stirring at 100 rpm for 48 hours. Following inactivation, the culture was further clarified using the normal flow filtration (NFF) process, fitted with 0.3 µm SupaSpun II DOE + PE Gaskets filter (National Separations (Pty) Ltd, SA) and 0.45 + 0.2 µm Sartopore 2 IV double layered membrane filter (Sartorius, Germany). This procedure was conducted to remove remaining bacterial cells in the culture supernatant. Following clarification, the inactivated epsilon toxin was filtered, followed by concentration and buffer exchanged to PBS using Pellicon Single-Pass Tangential Flow Filtration (TFF) (Merck Millipore, Germany), fitted with Pellicon 3 Mini cassette Biomax 10 kDa A screen 0.11 m2 (Merck Millipore, Germany). The 10 kDa membrane filter was selected for filtration of the epsilon toxin since the size of toxin is more than 50% larger than the pore sizes of the membrane, and allows for retention of the toxin during the filtration process. Filtration experiments were conducted at room temperature under sterile conditions. Following filtration process, the antigen was analysed on SDS-PAGE for detection of the epsilon toxin to ensure that it was maintained throughout the filtration process. The final concentration of the epsilon toxin was determined by ELISA and RID assays as previously described in Chapter 4, sections 4.3.8.1 and 4.3.8.2, respectively. In addition, the residual formalin concentration was also evaluated in the antigen after buffer exchange. According to the Department of Agriculture, Land Reform and Rural Development (DALRRD) Act 36 of 1947, the residual formalin in the final vaccine product should be ≤0.05% for animal safety and welfare. Therefore, in this study residual formalin of the epsilon toxin was evaluated following the buffer exchange process, and compared with the crude culture before filtration process. The procedure was conducted according to the method described in the European pharmacopoeia (Commission, 1998). Briefly, a 4 mL Nash reagent (prepared as described in Annexture A) was mixed with 4 mL WFI in 5 mL glass centrifuge tubes. The epsilon toxin obtained before and after buffer exchange was added to the Nash reagent+WFI mixture at a volume of 10 µL and gently mixed by vortex. The formalin standard solution (OBP, SA) and PBS were included as positive and negative controls, respectively. The tubes were incubated in a 37 °C waterbath (LaboTech, SA) for 45 minutes. Following incubation, absorbance of the samples was analysed using Genesys 20 spectrophotometer model 4001/4 (Thermofisher

Scientific, USA) at 416 nm wavelength. Percentage value for residual formalin was calculated using formula indicated below:

% Residual Formalin = mean absorbance of antigen sample ÷ mean absorbance of the formalin standard solution x 0.186

5.2.3.2 Toxicity analysis of the epsilon toxin in mice

The validation of the complete cleavage and inactivation of the epsilon toxin was conducted in adult mice. To confirm complete activation of the toxin, a group of mice (n=4, per test sample) was administered intravenously with 0.2 mL of the 10-fold serial diluted trypsinised-*C. perfringens* type D culture supernatant. Trypsin-untreated *C. perfringens* type D culture supernatant was included as a negative control. Mice were daily monitored for 3-days following toxin administrations, and toxicity was measured by mortalities recorded per dilution. Similarly, to determine complete inactivation of the toxin, mice were injected with the formalin inactivated- *C. perfringens* type D culture supernatant. Formalin-free *C. perfringens* type D culture supernatant and PBS were included as negative and positive controls, respectively. The mortalities recorded in mice after 3-days post injection indicate incomplete inactivation of the epsilon toxin. On the other hand, the survival of mice injected with the inactivated toxin confirms its complete inactivation.

5.2.4 Formulation of the RVF/PK combination vaccine

The monovalent inactivated RVF-SB and PK-ETX vaccines were formulated using the inactivated RVFV-SB antigen at a minimum titre of 1 x 10^6 PFU/mL and the epsilon toxoid (10^2 MLD_{50}/mL), individually combined with 50% w/w Al $(OH)_3$ gel adjuvant. The vaccine formulations were conducted under sterile conditions using the high-speed homogeniser at 13 000 rpm for 10 ±5 minutes. The RVF/PK combination vaccine was aseptically formulated by combining the individual inactivated RVF-SB and the PK-ETX formulations, at a 1:1 ratio. The formulated vaccines were tested for sterility and quality control tests (including; centrifugation test, visual observation of the vaccine aspect after 24 hours incubation at 23 °C ±2 °C, 4 °C and 37 °C) to evaluate effective formulation of each vaccine.

5.2.5 Animal housing and care

Large animal model

Safety and efficacy of the RVF/PK combination vaccine was evaluated in merino sheep (4-6 months) were procured from Langfontein farm, SA. The animals were pre-screened for antibodies against RVFV and the epsilon toxin using serological assays before the trial could commence. The serum neutralisation test (SNT) was conducted in-house at OBP and sample sera were also sent to Agricultural Research Council at the Onderstepoort Veterinary Institute for detection of the RVFV-IgM and IgG antibodies using ELISA. Toxin neutralisation assay was conducted in mice, according to Lobato *et al*. (2010), for detection of antibodies against the epsilon toxin (Lobato *et al.*, 2010). Animals that tested free from RVFV and the epsilon toxin antibodies were transported to OBP, SA and housed in stable 155 and acclimatized for a period of 14 days before used for the clinical trial. The sheep were housed in stables provided with controlled environmental conditions at ambient temperature (20–25 °C), and lights were switched off at night to mimic the natural conditions. The stables were cleaned and wood shavings covering the floor replaced once a week. Animals were ear tagged on the right hand for identification. Animal handling was conducted by trained animal technicians according to the standard operating procedures at the clinical department at OBP. Rectal temperatures were daily recorded for the duration of the trial, starting at 4 days before vaccination of the animals. Since sheep are the natural habitat for *C. perfringens* type D, the animals were gradually introduced to Lucerne grass and access to ad libitum clean water so that the gut flora can adapt and not cause any infections.

Experimental animal models

Guinea pigs aged 8-14 weeks and weighing 2.0-2.8 kg were obtained from OBP (Experimental animal Unit). These animals were utilized to assess the safety and efficacy of the pulpy kidney component for the RVF/PK combination vaccine. A total of 48 guinea pigs were randomly selected and divided into six groups, each comprising of eight animals. The animals were housed in clean cages containing Corncob™ bedding. The animals were placed in groups of four (with each treatment group divided by two) and fed Epol rabbit chow and clean water supplemented with Vitamin C ad libitum. The animals were labelled according to the vaccine administered. Adult CD-1 mice of mixed gender, mass of 20 ±2 g and aged 8-12 weeks old were obtained from OBP, SA. Mice were used for assessing toxicity of the epsilon toxin and for toxin neutralisation test. The animals were housed in groups of four in clean cages containing Corncob™ bedding. The cages were labelled according to the treatment received, and mice were fed capsule feed and JCW Pet food mice chow and clean water ad libitum. The animals

were placed at OBP, experimental animal unit in a controlled environmental condition, with temperature and humidity of 22 ± 2 °C and 50%, respectively.

5.2.6 Safety and efficacy of the RVF/PK combination vaccine

The safety and immunogenicity of the RVF/PK combination vaccine was assessed in both experimental animals, using guinea pigs, and target animal models with merino sheep. The target animal species were selected for a general evaluation of the vaccine's safety, as it is intended for use in small ruminants. In this study, merino sheep were also utilized for the evaluation of neutralizing antibodies against the RVFV. Merino sheep were divided into six groups containing eight animals each. The animals in each treatment group were injected with the RVF/PK combination vaccine and the individual monovalent vaccine components (animal groups outlined on Table 5.1). The commercial OBP inactivated RVF and alum-precipitated PK vaccines were included as positive controls. The PBS formulated with Al $(OH)_3$ at 1:1 ratio was used as a placebo vaccine for negative control. The vaccines at 1 mL/dose were administered into animals using 18G x 1 ½'' (1.2x40 mm) needle on inner thigh via the subcutaneous route (S/C) on days 0 and 21. Animal rectal temperatures were recorded twice a day (mornings and afternoons) for a period of 14 days post each vaccination. Possible clinical reactions for RVF and PK induced by vaccines such as fever, nasal discharge, loss of appetite, weakness, and bloody diarrhoea and swelling at site of injection were monitored and recorded for the duration of the study. The RVF/PK combination vaccine was further evaluated for its safety and potency (for the PK component) in guinea pigs. Similar to treatment of the target animal species, the monovalents of the combination vaccine were included, as well as the positive and negative controls (Table 5.1). The vaccine was administered in animals using the sterile hypodermic needle 21 G x 1 ½'' (0.8 x 40 mm) and the 3 mL Luer slip syringe at 1 mL/dose S/C at the back of neck on days 0 and 21. The animals were monitored daily for clinical observations and adverse reactions on site of injection for 21 days post primary and secondary vaccination.

Table 5. 1: Safety and efficacy of the RVF/PK combination vaccine

Group	No. of animals	Vaccine	Dose of administration	Days of vaccination	Route of administration
A	8	Inactivated RVF-SB vaccine	1 mL	D0, D21	S/C
B	8	Commercial inactivated RVF vaccine	1 mL	D0, D21	S/C
C	8	PK-ETX vaccine	1 mL	D0, D21	S/C
D	8	Commercial PK vaccine	1 mL	D0, D21	S/C
E	8	RVF/PK combination vaccine	1 mL	D0, D21	S/C
F	8	Placebo	1 mL	D0, D21	S/C

5.2.7 Blood collection

Blood samples were obtained from each participating sheep and guinea pigs at various time points during the study, using 5 mL vacuum gel blood collection yellow top tubes. Blood was collected from merino sheep on days 0, 7, 14, 21, 28, 35, 42, 49, 56, and 63. In the guinea pigs, blood samples were obtained on days 0, 35, 42, 49, and 63, with a collection volume of 2-3 mL. The blood collected was centrifuged at 2500× *g* using a J6-MI centrifuge, with swinging bucket rotor JS-4.2 (Beckman Coulter, USA) at 4 °C for an hour. The serum samples were collected into 2 mL sterile cryovials and heated at 56 °C in a waterbath (LaboTech, SA) for 30 minutes. The heat inactivated serum samples were stored at − 20 °C until utilised for SNT (OIE Terrestrial manual, 2018), and IgM/IgG ELISA (Ellis *et al.*, 2014), and toxin neutralisation tests for detection of antibodies against the RVFV and epsilon toxin.

5.2.8 Evaluation of IgM/IgG antibody immune response using ELISA

The ELISA assay for detection of IgM and IgG antibodies against the RVFV was utilised to determine the levels of IgM and IgG RVFV antibodies in sheep sera at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI). The assay was conducted in accordance with the procedure described by Ellis *et al*. (2014). The value of the net absorbance at $OD_{(450\ nm)}$ was expressed

as S/P% of the positive control, and all serum samples with S/P% of ≥7 were considered positive (Ellis *et al.*, 2014).

5.2.9 Evaluation of the neutralising antibody immune response against the RVFV

The SNT assay was used to detect the presence of specific neutralising antibodies in serum of vaccinated sheep. The antibody levels measured by this test are mostly induced against the Gn/Gc glycoproteins of RVFV and afford protection in infected and vaccinated animals. The SNT method was performed as described by Frey and Liess (1971). Briefly, the heat inactivated serum samples were serially diluted two-fold using serum free GMEM cell culture media. Positive and negative anti-RVFV serums, were included as controls. The live RVF Smithburn virus antigen was added in each microplate well (50 µL) at a final concentration of 100 $TCID_{50}$/mL. After 1-hour incubation of the diluted sera with the virus at 37 °C in a humidified CO_2 incubator, 100 µL of Vero cells (1,00 x 10^4 viable cells/mL) were added in 96-well microplates followed by incubation under same conditions. The RVFV Smithburn neutralisation antigen was also back-titrated in three ten-fold dilutions (10^{-1} to 10^{-3}) using the $TCID_{50}$ protocol. Cells non-treated with the virus were included. Plates were incubated at 37±2 °C with a humidified 5% CO_2 atmosphere. Back titration plate was monitored daily for observation of CPE under light microscope on the 10^2 sample dilution before the test samples may be evaluated for RVFV-neutralisation. Antibody titres were expressed as the reciprocal of the serum dilution that inhibited 50% of virus-induced CPE.

5.2.10 Neutralising antibody immune response against the epsilon toxin

The toxin neutralisation assay was conducted in mice to measure the levels of anti-epsilon toxin units (IU/mL) detected in sera obtained from guinea pigs. The procedure was modified from the method described by Lobato *et al.* (2010), for detection of neutralising antibody titres against the epsilon toxin. Briefly, ten-fold sera dilutions (10^{-1} to 10^{-2}) were initially prepared in saline buffered solution (pH 7.4 ±2) and used in preparations of the anti-epsilon toxin antibodies with concentrations of 1 IU/mL, 2.5 IU/mL, 5 IU/mL, 10 IU/mL, 20 IU/mL and 33.3 IU/mL (Table 5. 2). The standard concentrations of the neutralising anti-epsilon antibodies were pre-determined at the Quality Control section (OBP, SA), using the standard commercial anti-epsilon toxin antibody serum (NIBSC, UK). The epsilon toxin antigen at final concentration of 5.55 µg/mL was added in sera dilutions to a volume of 1 mL (Table 5. 2). The standard anti-epsilon antibody serum and saline buffered solution were included as positive and negative controls, respectively. The reaction mixtures were incubated at 37 °C for 30 minutes. Following incubation, the dilutions were each injected in adult CD-1 mice (n=4) intravenously at 0.2

mL/dose. Mice were monitored for a period of 24 hours following injections, and dead or live mice were recorded. Dead mice represent non-neutralisation of toxin, while live mice represent neutralisation of toxin. The level of antitoxin units of the test sera was measured based on the dilution factor as indicated on Table 5. 2. Concentration of antibodies ≥5 IU/mL was considered to be potent for protection of sheep against PK according to the European Pharmacopoeia of 1998 (Commission, 1998, Pharmacopoeia, 2008).

Table 5. 2: Preparation of serum dilutions for toxin neutralisation test in mice. The positive and negative controls were included.

	Antibody titre (IU/mL)	**33.3**	**20**	**10**	**5**	**2.5**	**1**
				1.Test sera			
	Saline (mL)	0.4	0.9	0.8	0.6	0.2	0.8
	Test serum (mL)	0.6 (10^{-2})	0.1 (10^{-1})	0.2 (10^{-1})	0.4 (10^{-1})	0.8 (10^{-1})	0.2
	Epsilon toxin (1 mL)	1	1	1	1	1	1
				2. Anti-epsilon toxin serum (positive control)			
	Saline (mL)	0.4	0.9	0.8	0.6	0.2	
	Positive control serum (mL)	0.6 (10^{-2})	0.1 (10^{-1})	0.2 (10^{-1})	0.4 (10^{-1})	0.8 (1/10)	
	Epsilon toxin (mL)	1	1	1	1	1	
				3. Epsilon toxin control			
	Dilutions	1:1	1:2	1:4	1:8		
Step 1	Saline	1	1	1	1		
Step 2	Epsilon toxin (mL)	1	1	1 (1:2)	1 (1:4)		
Step3	Final addition of saline (1 mL)	1	1	1	1		

Key: 10^{-1} and 10^{-2} represent dilution of serum sample; 1:2 and 1:4 represents the dilution of the standard toxin

5.2.11 Statistical analysis

Statistical analysis was conducted to compare the significant difference of the antibody immune response induced in sheep by the inactivated RVF, and RVF/PK combination vaccine. A two-way analysis of variance (ANOVA) followed by a Bonferroni's post-test was utilised to determine the difference in antibody immune response over time. GraphPad prism version 5.0 software for Windows was utilised for statistical analysis at a significance level of 5% ($p < 0.05$).

5.3 Results

5.3.1 Production of the pilot monovalent inactivated RVF Smithburn vaccine

The live attenuated RVFV-SB was propagated in BHK-21 cell culture. The harvested virus culture with a titre of 6 x 10^6 PFU/mL was obtained and inactivated with 0.5 mM BEI. To determine complete inactivation of the RVFV-SB, Vero cell culture was infected with the inactivated virus. Figure 5.1 represents the typical Vero cells growth kinetics before and after infecting with the inactivated RVFV-SB. The Cell Index (CI) showed exponential growth curve of the cells infected with inactivated virus, indicating the absence of virus infection and thus complete inactivation. This trend was similar to that of non-virus infected Vero cells. Although there was a slight difference observed in CI values between cells infected with the inactivated virus and cells only control, this was attributed to the media components contained in the inactivated virus culture. The inactivated virus contained neutralized BEI reaction mix whereas the cells only controls were topped up with fresh GMEM media which promoted cell growth. In contrast, Vero cells infected with the live RVFV-SB showed a gradual decrease in the CI value from 50 to 104 hours of incubation, indicating CPE formation on infected cells. Following successful inactivation, RVF-SB vaccine was formulated with $Al(OH)_3$ gel adjuvant to a final concentration of 50% (w/w) at room temperature and under sterile conditions. The RVF-SB was formulated to a 1 x 10^6 PFU/dose, similar to the OBP-commercial inactivated RVF vaccine. The formulated inactivated RVF-SB was free from bacterial and fungal contamination as confirmed during the period of observation after incubation on BTA, soy, and thioglycolate media for 14 days (data not shown).

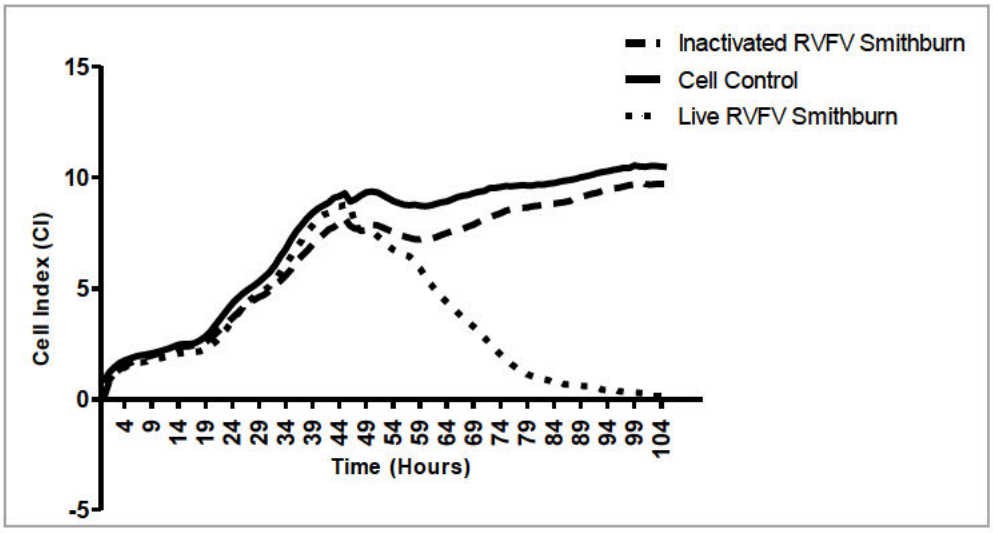


Figure 5. 1: Validation of the inactivation of rift valley fever virus (RVFV) Smithburn using real time cell analysis (RTCA) assay. Growth profile of Vero cells infected with the inactivated and live RVFV Smithburn was recorded.No cell death was recorded in Vero cell culture infected with the inactivated RVFV Smithburn as indicated by the stationary cell index following treatment with the inactivated virus. The live RVFV induced a decline in cell index showing cell death following treatment with the virus. Vero cell culture was included as positive control.

5.3.2 Production of the pilot monovalent epsilon toxoid

The epsilon toxin was produced by fermentation of the *C. perfringens* type D under anaerobic conditions and the harvested culture was assayed for level of the epsilon toxin using SDS-PAGE. Figure 5.2 shows successful expression of the epsilon toxin as indicated by a visible protein band with molecular weight of 35 kDa. The protein concentration was confirmed to be 31.2 TCP/mL and 17.11 mg/mL by RID and ELISA assays, respectively. The expressed epsilon toxin was cleaved by trypsin at final concentration of 0.032 mg/mL to obtain a matured active and antigenic toxin. Figure 5. 2 also shows complete cleavage of the toxin, as indicated by a reduced in size of the prototoxin from 35 kDa to 29 kDa, as well as the absence of residual prototoxin following activation. Prior to vaccine formulation, the activated epsilon toxin was inactivated with 0.7% (v/v) formalin. Complete inactivation of the toxin was confirmed by injection of CD-1 adult mice with the neat/undiluted formalinised epsilon toxoid. No mortalities nor signs of distress were observed in mice injected with the formalinised epsilon toxoid, instead the animals remained live following 48 hours of injections. Similar observations were made in mice injected with PBS, suggesting complete inactivation of the epsilon toxin (Table 5.3). On the other hand, mice injected with the negative control (non-formalinised culture supernatant) induced mortalities in injected adult CD-1 mice (Table 5.3).

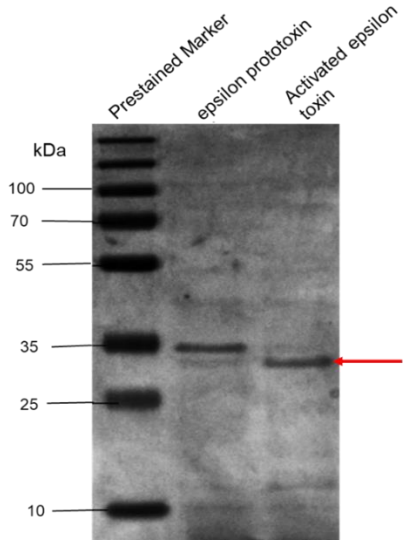


Table 5. 3: Evaluation of complete inactivation of the epsilon toxin in adult CD-1 mice.

Sample culture	Live/dead
Formalin treated-epsilon toxin	√√√√
Non-formalin treated epsilon toxin	††††
PBS	√√√√

Key: † (dead mice), √ (live mice)

Figure 5. 2: Assessing complete activation of the epsilon prototoxin after treatment of the toxin with trypsin to produce a complete activated mature epsilon toxin. The epsilon prototoxin was treated with 0.032 mg/mL trypsin at 37 °C for 150 minutes, with continuous stirring. The arrow points a successfully cleaved epsilon toxin as represented by a protein band with molecular weight of 29 kDa.

5.3.3 Downstream bioprocessing of the epsilon toxoid used for formulation of the vaccine

The complete inactivated epsilon toxoid was assessed for the level of residual formalin in the *C. perfringens* type D culture supernatant. The residual formalin is acceptable at a concentration of 0.05% and below for vaccine formulation. This stage of antigen processing is crucial given that the epsilon toxin antigen will be used in vaccine formulation with the BEI-inactivated RVFV-SB. Furthermore, the final formulated combination vaccine requires to comply with the regulatory measures. After assessment, the final formalin concentration was determined to be at 0.2%, indicating non-compliance with the regulatory requirements. Multiple washes of inactivated epsilon toxoid with PBS using the TFF system, removed excess residual formalin with the final preparation consisting of 0.001% formalin which is suitable for formulation with the inactivated RVFV-SB for the RVF/PK combination vaccine. The concentration of the epsilon toxoid was monitored during the removal of formalin following inactivation. Figure 5.3. A demonstrate the retention of the epsilon toxoid following each was of the TFF process. Table 5.4 also showed a low level of protein decrease after the TFF process, indicating that the conditions used for washing of the antigen are suitable. These results were also consistent with the SDS-PAGE analysis of the samples collected in the washing steps, which showed minimal epsilon toxin loss detected in the permeate after one wash cycle (Figure 5.3 B). The RVF/PK combination vaccine was formulated using a 1:1 ratio of the inactivated RVFV-SB and the epsilon toxoid, combined with the $Al(OH)_3$ gel adjuvant at a final concentration of 50% (w/w). The

formulated vaccine was incubated at 23 °C ±2 °C, 37 °C, and 4 °C, and no separation in formulated components after 24 hours of incubation (data not shown). The formulated vaccine products were stored at 4 °C until used.

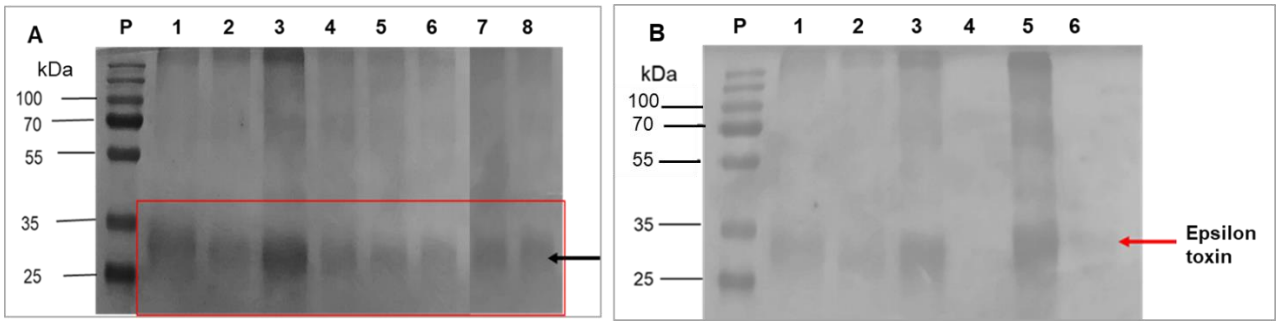


Figure 5. 3: SDS-PAGE analysis of the epsilon toxoid after filtration process. (A) The epsilon toxoid was maintained throughout the filtration process. P: Pre-stained protein marker, 1: Epsilon toxoid before NFF process, 2: Epsilon toxoid after clarification using NFF, 3: The 10x concentrated epsilon toxoid, 4-8: Epsilon toxoid after the 1st, 2nd, 3rd, 4th and the 5th wash with PBS, respectively. **(B)** The epsilon toxoid both in the retentate and permeate after TFF process. P: Pre-stained protein marker, 1: Epsilon toxoid before clarification with NFF system, 2: Epsilon toxoid after clarification with NFF, 3: The 10x concentrated epsilon toxoid in the retentate culture after TFF process, 4: Epsilon toxoid in the permeate of culture after TFF process, 5: The 100x concentrated epsilon toxoid in the retentate, 6: The 100x concentrated epsilon toxoid detected in permeate.

Table 5. 4: Assessing the concentration of the epsilon toxin using direct ELISA and RID assays after the filtration process.

Sample	RID (TCP/mL)	ELISA (mg/mL)
C. perfringens type D culture supernatant after inactivation	28.89	15.12
PK after clarification	24.88	12.61
10x concentrated toxin	35.77	18.69
Epsilon toxin after buffer exchange	20.80	8.94

5.3.4 Safety of the RVF/PK combination vaccine in sheep

Safety of the RVF/PK combination vaccine was evaluated in merino sheep. The animals were subdivided into six groups, with each group containing eight animals. Merino sheep were immunised with the RVF/PK combination vaccine, and monovalent components of the vaccine were included. The commercial inactivated RVF and alum-precipitated PK vaccines served as positive controls. The $Al(OH)_3$ adjuvanted PBS served as a placebo vaccine formulation (negative control). Rectal temperatures of the animals were recorded 4 days prior to commencement of the trial to establish the animals state of health prior vaccination, and 14 days post primary (day-0) and secondary (day-21) vaccinations. No clinical signs of the RVF or PK infections, nor reaction on site of injection were recorded following vaccination. The mean temperature records of the six participating animal groups are indicated in Figure 5.4. The animal group immunised with the RVF/PK combination vaccine have recorded rectal temperatures ranging from 39-40.1 °C, indicating a slight increase of the normal sheep physiological temperature range of 38.9-39.9 °C. This temperature spike was recorded at 40.1 °C after 6-days of booster inoculations and had only lasted for a single day. Similarly, animals in the positive control groups obtained temperature ranging from 39-40.2 °C. Specifically, the animal group immunised with the commercial OBP-PK vaccine recorded the highest temperature of 40.2 °C following 6 and 10-days of both primary and booster vaccinations. The temperature spike of 40.1 was also recorded in animal group immunised with the commercial OBP-formalin inactivated RVF vaccine 9-days after booster injections (Figure 5.4). The placebo formulation had also resulted in increased temperature spikes in injected merino sheep at 3 and 19-21 days after booster injections. These results clearly indicate that the high temperatures recorded within the animal groups were not caused by vaccine administrations, as pyrexia typically result in consistent high temperature records that last for upto 4-5 days (Kroeker *et al.*, 2020). In addition to safety of the vaccine, two mortalities were recorded in animals immunised with the commercial OBP-inactivated RVF vaccine, one in those injected with the commercial OBP-PK vaccine, and one in the group immunised with the inactivated RVF-SB vaccine during the trial. The animals were diagnosed with haemonchosis, a blood disease caused by intestinal parasitic worms (*Haemonchus contortus*) (as confirmed by post mortem reports; Annexture B). This infection was reported to be common in small ruminants (Arsenopoulos *et al.*, 2021). The affected animals showed clinical signs of weakness, loss of appetite, passing of worms in stool, and reduced mobility with paralysed front limbs. The animals showing these clinical signs of infection were treated with anti-biotics (Duplocillin; 4.0 ml/100 kg) or systemic anti-inflammatory drugs (Flunixin meglumine; 6.0 mg/kg) and monitored every 2-3 hours daily for 2-3 days following treatment. Some animals died 1-2 days after the onset of infection, and some were euthanized with pentobarbital sodium 100-150mg/kg

prior to post mortem analysis. A total number of four mortalities were recorded in sheep involved in the study, which did not result from vaccinations.

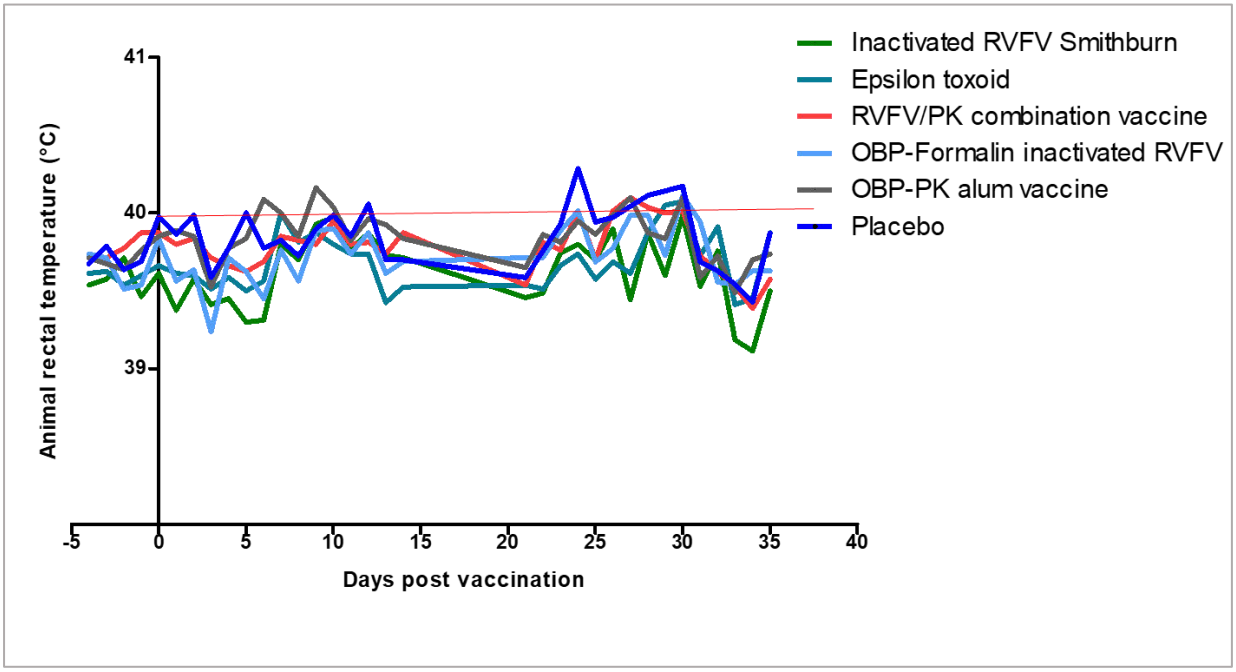


Figure 5. 4: Rectal temperature records obtained in merino sheep that were vaccinated with the test vaccines. Merino sheep were vaccinated with the RVF/PK combination vaccine. The inactivated RVFV and the epsilon toxoid monovalent vaccines were included. The OBP commercial inactivated RVFV and PK vaccines served as positive controls. The vaccines demonstrated safety in injected animals, no pyrexia was recorded throughout the trial.

5.3.5 Efficacy of the RVF/PK combination vaccine

Immunogenicity of the test vaccines were evaluated in sheep by detection of antibody immune response in serum collected at 7-days interval from 0 to 63 days post vaccination. IgM and IgG antibodies against RVFV were detected using ELISA assay. Figure 5.5 and Figure 5.6 represents the IgM and IgG antibody titres, respectively, as expressed by S/P%. Serum samples obtaining antibody titre ≥7 S/P% are positive. SNTs were used as a confirmatory test for IgG positivity. The neutralizing antibody titres of ≥ 1:4 when using SNT method are indicative of seroconversion and ≥ 1:16 is considered sufficient to confer protection against the RVF disease as per OBP internal standard operating procedures. The antibody immune response of the RVF/PK combination vaccine against the epsilon toxin was evaluated in guinea pigs. Sera from vaccinated guinea pigs was collected on days 0, 35, 42, 49, and 63 for detection of the neutralising antibodies against the epsilon toxin as per the protocol described by Lobato *et al.* (2010). Neutralising antibody detection against the epsilon toxin

was determined in mice using the toxin neutralisation assay. The anti-epsilon toxin concentrations detected at ≥5 IU/mL were indicative of seroconversion against PK infection.

5.3.5.1 Determination of the IgM antibody levels against RVFV

A total number of 48 merino sheep were involved in the study. The animals were subdivided into six groups with each containing eight animals. The antibody immune response in sheep vaccinated with RVF/PK combination vaccine were compared with the individual monovalent component vaccines. The commercial inactivated RVF and OBP-PK vaccines were included as positive controls. Placebo vaccine was included as a negative control. Figure 5.5 represents the mean IgM antibody titres induced in each group of the vaccinated animals. The results have shown that the animal groups vaccinated with the monovalent inactivated RVF-SB and commercial inactivated RVF vaccines induced higher IgM antibody responses of 20 S/P% and 10 S/P%, respectively, after 7 days of primary injection (Figure 5.5). The IgM antibody titres were detected at a lower titre of 2 S/P% and 3 S/P% for both the inactivated RVF-SB vaccine and commercial inactivated RVF, respectively, post 21 days of primary vaccination. Seven days following booster vaccination, the IgM antibody titres were detected at an increased level of 6 S/P% for the inactivated RVF-SB and 2 S/P% for commercial inactivated RVF vaccine. These antibody titres were maintained up to 14 days post booster vaccination. The IgM antibody titres detected in serum of sheep vaccinated with the RVF/PK combination vaccine were presented at a negative concentration level, on days 7 (5 S/P%) and 14 (4 S/P%) post primary vaccination. After 21 days of primary vaccination, no antibody levels were detected in animal group vaccinated with the RVF/PK combination vaccine. However, after second dose of vaccination, the antibody levels were induced at a low 2 S/P% in vaccinated sheep. The animal groups vaccinated with the monovalent PK-ETX, commercial OBP PK, and the placebo vaccines did not obtain any levels of IgM antibody titres against RVFV throughout the study (Figure 5.5). A high significant difference was observed (***$P < 0.001$) between the IgM antibody titres in sheep vaccinated with the inactivated RVF-SB and RVF/PK combination vaccine 7 days post vaccination. Data sets presented no significant difference ($P > 0.05$) between the IgM antibody titres on day 0 and 14 onwards post vaccinations.

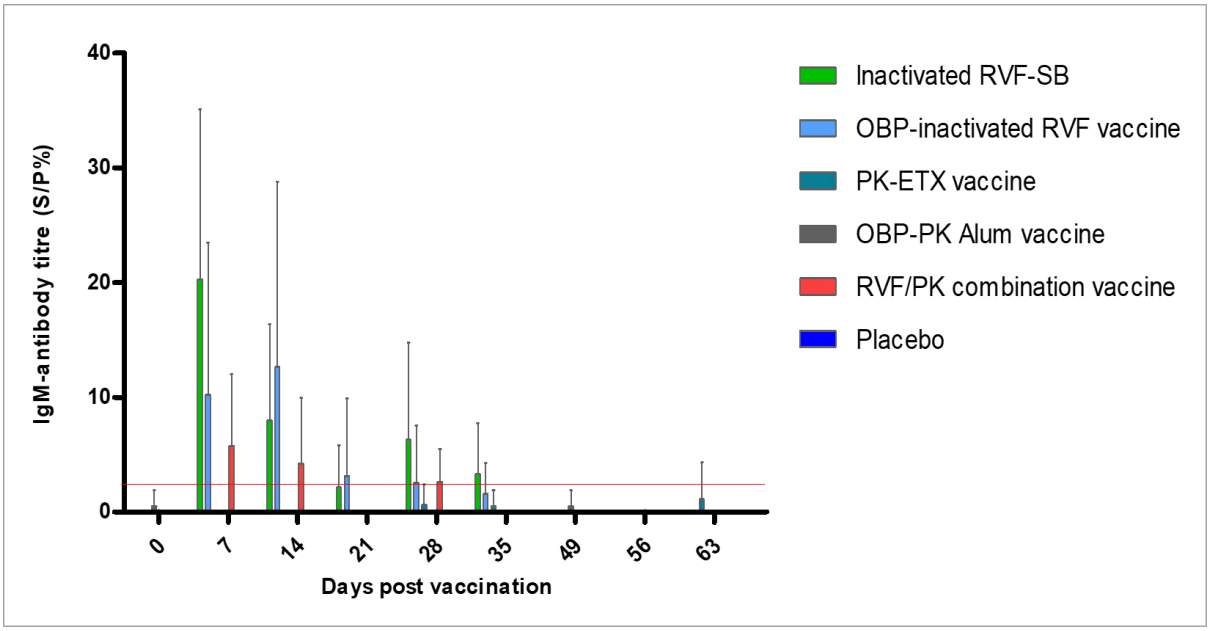


Figure 5. 5: The mean IgM antibody immune response against RVFV obtained in sheep vaccinated with the RVF/PK combination vaccine. IgM antibody titres against the RVFV nucleoprotein were measured using ELISA. Serum samples were collected from sheep injected with inactivated RVF/PK combination and monovalent vaccines at 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post vaccination. The commercial OBP-inactivated RVF vaccine and the placebo were respectively included as positive and negative controls. Data sets are presented as the mean ± SEM in the same treatment. The red solid line represents the minimum threshold cut-off of the positive antibody titre post animal vaccinations. Antibody titres above the threshold cut-off are indicative of a positive immune response.

5.3.5.2 IgG antibody immune response against RVFV

Figure 5. 6 represents the mean IgG antibody titres detected in sheep vaccinated with the RVF/PK combination vaccine, as well as the monovalent vaccine components. Animal group vaccinated with the inactivated RVF-SB vaccine obtained a mean IgG antibody titre of 8 S/P% after 7 days of primary vaccination. The antibody titres were shown to increase gradually from day 14 up to day 35 (14 days post booster vaccination), reaching maximum concentration of 79 S/P% at day 35 (Figure 5. 6). The IgG antibody immune response was maintained at a positive level for duration of the study in animal groups vaccinated with the inactivated RVF-SB. The RVF/PK combination vaccine induced positive IgG antibody titres at concentration of 17 S/P% after 14 days of primary vaccination. The antibody titres detected in sheep vaccinated with the RVF/PK combination vaccine were shown to increase from day 21 up to day 35, reaching a mean maximum peak of 88 S/P% after 14 days of booster vaccination (Figure 5.6). The antibody titres detected in sheep vaccinated with the RVF/PK combination vaccine were maintained at a positive level for the duration of the study. The commercial OBP inactivated RVF vaccine induced positive level of IgG antibody titres from 14 days of primary vaccination at a mean

concentration of 14 S/P%. The antibody titres have gradually increased from day 21 up to day 35, reaching a maximum mean concentration of 98 S/P% after 14 days post booster vaccination (Figure 5.6). The antibody levels were maintained at positive level for the duration of the study. The inactivated RVF-SB and RVF/PK combination vaccines induced IgG response that was comparable to the commercial inactivated RVF vaccine and remained above the threshold throughout the duration of the trial. A low level of anti-RVFV antibody immune response was observed in sheep vaccinated with the PK-ETX, OBP PK, and placebo vaccines on day 63. This observation has only been detected on a single day of the study and it was therefore considered as invalid, due to cross-contaminations during the ELISA test preparations. (Figure 5.6). There was no significant difference (P>0.05) between the IgG antibody titres induced in sheep by the inactivated RVF-SB and RVF/PK combination vaccine throughout the study.

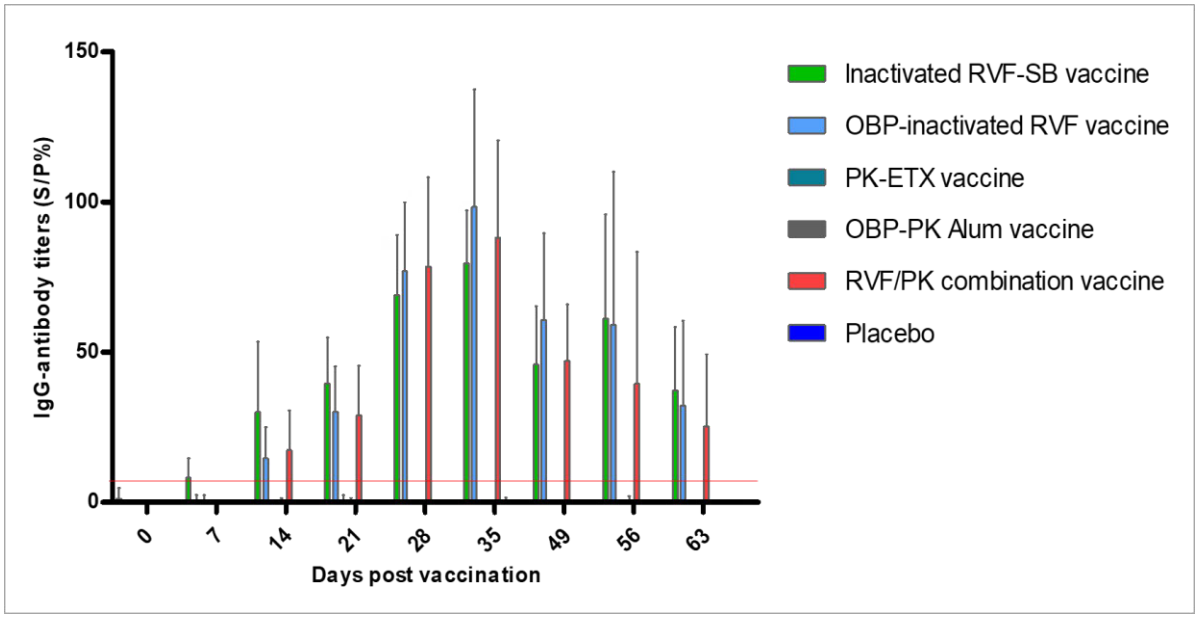


Figure 5. 6: The mean IgG antibody titres detected in vaccinated sheep using competitive ELISA. IgG antibody titres against the RVFV nucleoprotein were measured using ELISA. Serum samples were collected from sheep injected with inactivated RVF/PK combination vaccine and the monovalent vaccines at 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post vaccination. The commercial OBP-inactivated RVF and the placebo vaccines were respectively included as positive and negative controls. Data sets are presented as the mean ± SEM in the same treatment. The red solid line represents the minimum threshold cut-off of the positive antibody titre post animal vaccinations. IgG-antibody titres above the threshold cut-off are indicative of a positive immune response.

5.3.5.3 Neutralising antibody immune response against the RVFV

Neutralising antibody titres against RVFV were measured in serum collected from sheep using SNT tests. The results were expressed as reciprocal of the serum dilution which neutralised 50% of the RVFV. The animal group vaccinated with the inactivated RVF-SB vaccine induced neutralising antibody titres of 1:8 following 7 days of primary vaccination. The antibody titres increased at exponential level, reaching maximum mean concentration of 1:98 after 21 days of booster vaccination (Figure 5.7). The neutralising antibody titres in animal group vaccinated with the inactivated RVF-SB vaccine remained at a neutralising level for duration of the study. Similarly, the animal group vaccinated with the RVF/PK combination vaccine obtained neutralising antibody titres against RVFV at concentration of 1:6 after 7 days of primary vaccination. The antibody titres were detected at 1:51 after 14 days of primary vaccination, and had decreased to 1:12 after day 21 of primary vaccination (Figure 5.7). The neutralising antibody titres were shown to increase again following booster vaccination to a concentration of 1:40, reaching a maximum peak antibody level of 1:88 after 28 days of booster vaccination. The animal group vaccinated with the commercial OBP inactivated RVF vaccine obtained neutralising antibody titres against RVFV at concentration of 1:9 after 7 days of primary vaccination. The neutralising antibody titres induced by this vaccine were shown to increase at exponential level from day 14 to 56 (28 days post booster vaccination), reaching maximum level of 1:213 at day 56 (Figure 5.7). The antibody titres were maintained at a neutralising level throughout the study. No detectable neutralising antibody titres were obtained in animal groups vaccinated with the PK-ETX, OBP commercial PK and placebo vaccines (Figure 5.7). There was no significant difference ($P>0.05$) between the neutralising antibody titres induced in sheep vaccinated with the inactivated RVF-SB and the RVF/PK combination vaccine throughout the study.

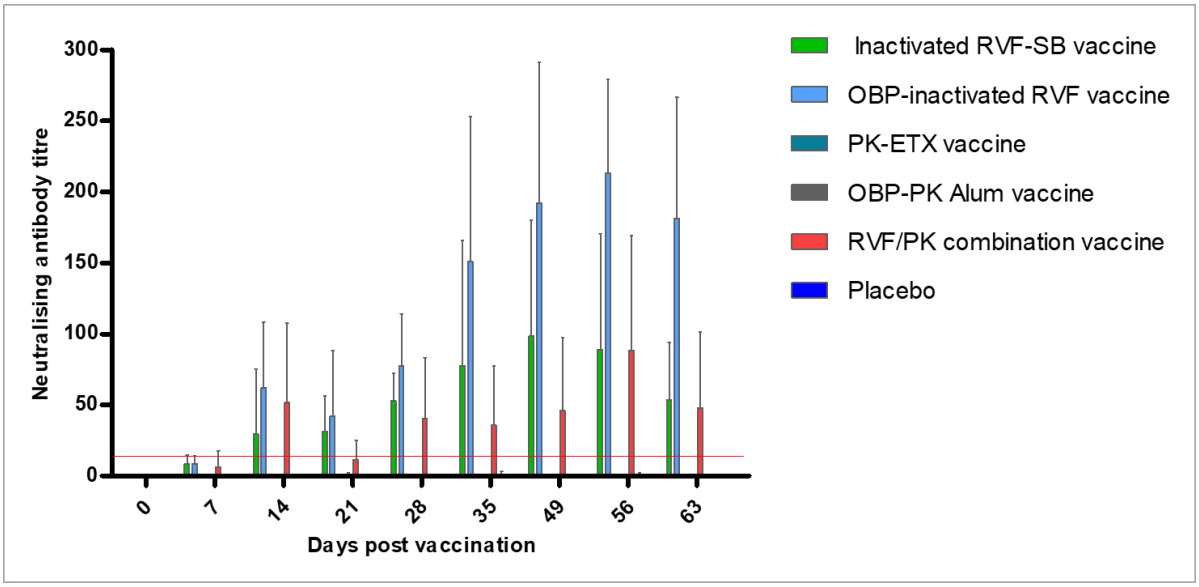


Figure 5. 7: The mean RVFV-neutralising antibody titres detected in sheep injected with the inactivated RVF/PK combination vaccine. Neutralizing antibody titres were measured using serum neutralization test for day 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 post vaccination. The individual inactivated RVF-SB and the PK-ETX monovalent vaccines were included. The commercial OBP-inactivated RVF vaccine and PK vaccines served as positive controls. Placebo vaccine was included as the negative control Error bars represent standard error of the mean (SEM). The red solid line represents the minimum threshold cut-off of the positive antibody titre post animal vaccinations. Neutralising-antibody titres above the threshold cut-off are indicative of a positive immune response.

5.3.5.4 Neutralising antibody immune response against the epsilon toxin

The efficacy of the RVF/PK combination vaccine was evaluated in guinea pigs to determine the anti-epsilon toxin units generated by the vaccine. The commercial OBP-PK and the inactivated RVF vaccines served as positive controls. The adjuvanted PBS was included as negative control. The vaccine monovalent components were included as controls. The sample sera were collected in all participating animals on days D0, D35, D42, D49 and D63. The neutralising antibody immune response against the epsilon toxin were determined using toxin neutralisation assay was performed in mice. The results presented in Table 5. 5 had shown that the RVF/PK combination vaccine induced anti-epsilon toxin antibodies at an acceptable level of 5 IU/mL, 14-days post-secondary vaccination (Table 5. 5). The antibody titres were maintained at the neutralising margin and had increased to 10 IU/mL on day 63 after 42 days of secondary vaccination. The antibody titres obtained in animals vaccinated with the RVF/PK combination vaccine were at an acceptable range for regulatory approval of candidate enterotoxaemia vaccines according to the Europpean Pharmacopoeia (Commission, 1998, Pharmacopoeia, 2008). On the other hand, the antibody immune response detected in guinea pigs vaccinated with the monovalent PK-ETX vaccine were at higher concentrations of 10 IU/mL 14-days

post-secondary injection (Table 5.5). The antibody titres were maintained, reaching a peak of 20 IU/mL on day 63, 42 days post-secondary injection. Guinea pigs vaccinated with the OBP commercial PK vaccine had obtained low neutralising antibody titres of 2.5 IU/ml after 14 days of secondary injection, and the antibody titres were maintained, reaching a potency level of 5 IU/mL following 42 days of booster injection (Table 5.5). The animal groups vaccinated with the placebo formulation, the inactivated RVF-SB and the commercial OBP-inactivated RVF vaccines had not induced any level of antibody immune response throughout the experiment. The positive control group had obtained neutralising antibody titres of 10 IU/mL against the epsilon toxin during the performance of the assay, whilst the negative control group recorded mortalities in the 1st and 2nd dilutions as expected.

Table 5. 5: Neutralising antibody titres against epsilon toxin, detected in sheep vaccinated with the inactivated RVF/PK combination vaccine after 28 days booster inoculations.

Day of bleed	RVF/PK combination vaccine	PK-ETX vaccine	OBP- alum PK vaccine	Placebo vaccine
Day 0	0	0	0	0
Day 35	5	10	2.5	0
Day 42	5	10	2.5	0
Day 49	5	10	2.5	0
Day 63	10	20	5	0

5.4 Discussion

The livestock industry experiences an ongoing threat of the RVF and PK disease outbreaks, which result in severe economic impact in affected regions. Despite the availability of several commercial vaccines used for protection of ruminants against these diseases, the vaccines are associated with a number of drawbacks that affect their safety and efficacy in injected animals (Dungu *et al.*, 2018, Alkan *et al.*, 2023). A significant amount of research has been conducted towards development of the novel RVF vaccines for use in livestock (Warimwe *et al.*, 2016, Faburay *et al.*, 2017, Dungu *et al.*, 2018, Alkan *et al.*, 2023). However, none of these efforts have focused on developing improved vaccination strategies for the disease. This study developed the RVF/PK combination vaccine, which has the potential to enhance vaccination coverage of RVF in regions where the disease is endemic. Combination vaccines, which incorporate inactivated antigens, requires use of high concentrations of monovalent product. This is because the vaccine is formulated in combination with other antigens, particularly in the instance of toxoid vaccines, the formulation ratio must not impede the efficacy of the other antigen being formulated with, while also ensuring a satisfactory vaccine shelf-life. In this study, the epsilon toxoid was utilised at a concentration of 10^2 MLD_{50}/mL and inactivated with formalin to ensure its safety before formulation with the inactivated RVFV-SB. Similar concentrations were used for formulation of the monovalent inactivated PK-ETX vaccine. The epsilon toxoid at concentration of 10^2 MLD_{50}/mL was also shown to induce protective immune response in vaccine through the commercial OBP-PK vaccine. Complete inactivation of the epsilon toxin with formalin was achieved after 48 hours of treatment, as confirmed by no mortalities nor abnormalities observed in injected mice. Formalin has widely been used as an inactivating agent of pathogens for vaccine production in Pharmaceutical industries (Glenny and Hopkins, 1923, Delrue *et al.*, 2012). Studies have confirmed that inactivated toxoids are associated with a risk of residual formaldehyde being present in the final product which affect its safety and efficacy (Byrne and Smith, 2000, Bokori-Brown *et al.*, 2014). According to Act 36 of 1947 (DALRRD), residual formalin in the final vaccine product should not exceed 0.05%. In this study residual formalin in the final inactivated epsilon toxoid was initially detected at 0.2%, indicating non-compliance with the regulatory requirements. Therefore, the inactivated epsilon toxoid was subjected to filtration system to remove excess residual formalin for toxoid preparation. TFF system was previously utilised as an effective scalable purification method to reduce 60% of the uncomplexed linear polyethylenimine concentration in the plasmid DNA nanoparticle formulation, without changing the size and morphology of the nanoparticles (Liu *et al.*, 2021). The residual formalin was reduced to 0.001% in the *C. perfringens* type D culture supernatant. Furthermore, culture impurities contained in broth media were removed. However, a marginal reduction in the epsilon toxin concentration was

detected after the filtration process, indicating that a low amount of the toxin passed through the filtration membrane into the permeate. These losses are standard in downstream processes. Therefore, a quality assessment was conducted to ensure that the concentration of the toxoid used for vaccine formulation still falls within the required specifications. The PK-ETX vaccine with the final epsilon toxoid concentration at 6.3 mg/mL was formulated with $Al(OH)_3$ at 50% (w/w). The monovalent inactivated RVF-SB vaccine was formulated at 1 $x10^6$ PFU/dose was formulated with with $Al(OH)_3$ at 50% w/w.

The inactivated RVF-SB and the PK-ETX monovalent vaccines, were combined in a 1:1 ratio for formulation of the RVF/PK combination vaccine. This formulation ratio was selected in order to ensure that the potency of each antigen in the combined vaccine is not compromised. The $Al(OH)_3$ adjuvant was selected as a delivery system for this vaccine due to its ability to stimulate antibody immune response (Ulanova *et al.*, 2001). This adjuvant also has the capacity to interact with the antigen in the formulation and slowly release the antigen to lymphocytes and antigen-presenting cells at the site of injection (Lindblad, 2004). The $Al(OH)_3$ has been widely utilised in the formulation of inactivated vaccines for over 90 years. It has been demonstrated that $Al(OH)_3$ provides a good safety profile for the inactivated RVF-SB vaccine and enhances a significant level of neutralising antibody immune response in vaccinated sheep (chapter 3, section 3.4.4.3). Several studies have shown that neutralising antibodies offer protection against both RVF and PK diseases (Silva *et al.*, 2018, Wright *et al.*, 2020). Given that alum adjuvant is also used for formulation of the commercial OBP-PK vaccine, the $Al(OH)_3$ was deemed suitable for use in combination with the two antigens. Following formulation, the RVF/PK combination vaccine was subjected to quality control evaluation and was demonstrated to be free from contaminations with homogeneous emulsions, demonstrating effective formulations.

Safety and efficacy of the RVF/PK combination vaccine was evaluated in merino sheep following inoculations at day 0 (primary vaccination) and day 21 (secondary vaccination). The vaccines were demonstrated to be safe for use in sheep and induced no clinical signs of infections against both RVF and PK diseases in vaccinated animals, or adverse reactions on site of inoculations. Although some vaccinated animal groups displayed temporary temperature reactions lasting for one or two days, these were not considered to be pyrexia resulting from vaccination. Animal pyrexia which results from infection is known to be indicated by fever reactions which lasts between 4-5 days (Kroeker *et al.*, 2020). The potential cause of the temperature spikes may have been due to intestinal worm infections. The trial was aimed at evaluating the safety and efficacy of the combination vaccine against the RVFV and epsilon toxin. Consequently, the animals could not be vaccinated against the PK disease

nor dewormed before the trial began. This posed a risk to the animals leading to clinical conditions caused by the intestinal worms in the form of haemonchosis disease. A total of four mortalities were recorded due to this condition, as confirmed by post mortem reports for each animal death. The safety data of the RVF/PK combination vaccine had demonstrated that the vaccine is safe for use in merino sheep following primary and booster administrations.

The antibody immune response against the RVF were monitored in sheep at 7-day interval following primary and secondary vaccination. The RVF/PK combination vaccine induced neutralising IgM antibody immune response against RVFV from 7-14 days of primary vaccination as anticipated. The antibody titres were not detected at 21 days post primary vaccination, however, after 7 days post-secondary vaccination the neutralising IgM antibodies were detected and lasted up to day 14. As discussed previously in chapter 3, this is the typical nature of the IgM antibodies as they are short-lived and decrease in concentrations due to the accumulation of the IgG antibodies in blood circulation (Matsiela *et al.*, 2023). The inactivated RVF-SB vaccine induced the highest levels of the anti-RVFV-IgM antibody titres, detected after 7 days of primary vaccination and were maintained until 14 days post-secondary-vaccination, followed by the OBP-inactivated RVF vaccine. In addition, there was a high significant difference (***$P < 0.001$) observed between these antibody titres in sheep injected with the inactivated RVF-SB and RVF/PK combination vaccine. This observation is common since monovalent vaccines contain a single antigen at higher concentration, resulting in higher IgM antibody production than when formulated as a combination with other competitive antigens (Morefield *et al*., 2008). The IgM antibodies induced by the OBP-inactivated RVF vaccine in sheep were maintained until day 35 (14 days post-secondary vaccination). This may have occurred due to the progressive accumulation of the IgG antibodies in the vaccinated animals which have reached a peak on day 35 post injections. The RVF/PK combination vaccine induced positive anti RVFV-IgG antibody titres following 14 days post primary vaccination, and the antibodies were maintained at a high level throughout the study. The IgG antibodies induced by the RVF/PK combination vaccine were comparable to those elicited by both the monovalent inactivated RVF-SB vaccine and the commercial OBP-inactivated RVF vaccine. Statistical analysis of the immune response induced by the monovalent inactivated RVF-SB and RVF/PK combination vaccine also confirmed a non-significant difference ($P > 0.05$). Similar findings were observed for detection of the RVFV-neutralising antibody titres in sheep vaccinated with the RVF/PK combination vaccine. The neutralising antibodies against RVFV were detected after 14 days of primary vaccination and were maintained at a neutralising level throughout the trial. The highest neutralising antibody titres were observed in animals vaccinated with the OBP-inactivated RVF vaccine, followed by those injected with the monovalent inactivated RVF-SB as

expected. The neutralising antibody levels induced by both vaccines were comparable, with variation in titre due to the different vaccine strains used in their vaccine formulations. The Smithburn strain utilised in the combination product resulted in lower levels of neutralising antibodies when compared to the inactivated RVF-SB and the OBP-inactivated RVF vaccines. This may be attributed to combination of the virus vaccine with the epsilon toxoid. However, the data also showed no significance of difference ($P>0.05$) between the neutralising antibody titres induced in sheep by the inactivated RVF-SB and RVF/PK combination vaccine throughout the study. Furthermore, it was also demonstrated that the RVF/PK combination vaccine induces neutralising antibody immune response against RVFV which may be efficient for protection of animals against the disease. Sheep vaccinated with the PK-ETX vaccine, OBP-PK alum vaccine and placebo formulations obtained low titres of the IgM antibodies, and no RVFV-neutralising antibodies were obtained as anticipated. Toxin neutralisation test was also conducted in mice to evaluate the anti-epsilon toxin antibodies induced in guinea pigs following vaccination with the RVF/PK combination vaccine. The neutralising antibody concentrations induced by the combination vaccine were detected at 5 IU/mL following 14 days post-secondary vaccination of guinea pigs. The antibody titres detected were characterised with an overall marginal detection of 5 IU/mL, and have increased to 10 IU/mL on day 63. These levels of antibody concentrations were confirmed to be effective for protection of ruminants against infections with the epsilon toxin by the Code of Federal Regulations (CFR) and the European Pharmacopeia (Commission, 1998, Pharmacopoeia, 2008). The CFR and the European Pharmacopoeia recommends the epsilon toxoid vaccine potency of 2.5 IU/mL and 5 IU/mL, respectively, for protection of ruminants against PK disease. The neutralising antibody titres induced in guinea pigs by the RVF/PK combination vaccine were within the specification range by both the CFR and the European Pharmacopeia. Furthermore, the study conducted by Silva *et al.* (2018) also demonstrated that the level of antibodies induced in experimental animals against the Clostridial toxoids are equivalent to the ones induced in target animal species(Silva *et al.*, 2018). The data obtained in this study confirms the efficacy of the RVF/PK combination vaccine in sheep. The anti-epsilon toxin antibodies induced by the monovalent epsilon toxoid in vaccinated guinea pigs were detected at higher concentrations of 10 IU/mL 14 to -35 days post-secondary vaccination. On day 63 the neutralising antibody titres against the epsilon toxin were obtained at a peak of 20 IU/mL. The antibody levels were higher than those generated by the RVF/PK combination vaccine, as expected. This may be attributed to the varied level of antigen formulation of the two products and the presence of another antigen in the combination vaccine. The positive control group, vaccinated with the OBP-commercial PK vaccine, has obtained antibody titres at 2.5 IU/mL concentrations below the threshold cut-off of 5 IU/mL. The vaccine only elicited marginal antibody titres, obtaining 5 IU/mL on day 63, following 42 days of booster vaccination. The animal groups

vaccinated with placebo formulation, the OBP-inactivated RVF, as well as the inactivated RVF-SB vaccines had not induced any level of anti-epsilon toxin antibody titres as anticipated. The data collected suggests that the RVF/PK combination vaccine elicits a desirable antibody immune response against both RVFV and epsilon toxin, meeting the regulatory requirements for both vaccine products. This indicates that the vaccine may be used for prophylactic immunization of small ruminants against RVF and PK. Additionally, the vaccine may provide an alternative vaccination strategy for ruminants in endemic regions to improve vaccination coverage against RVF.

5.5 Conclusions

Safety and efficacy of the RVF/PK combination vaccine was evaluated in sheep and guinea pigs following subcutaneous vaccinations with 2-doses (1 mL/dose). The vaccine was shown to be safe in sheep and guinea pigs, with no observed adverse reactions or signs of infection at the injection site. The RVF/PK combination vaccine induced neutralizing antibodies against both RVFV and the epsilon toxin, at levels considered to be protective for small ruminants against both diseases. The animals vaccinated showed an immune response against both antigens and may be further challenged with a virulent RVFV strain to confirm the vaccine's efficacy against RVF.

5.6 References

Abdolmohammadi Khiav, L. & Zahmatkesh, A. (2021). Vaccination against pathogenic clostridia in animals: A review. *Tropical Animal Health and Production,* 53, 284.

Alexander, R. & Haig, D. (1951). The use of egg attenuated bluetongue virus in the production of a polyvalent vaccine for sheep. A. Propagation of the virus in sheep. *Onderstepoort Journal of Veterinary Science,* 25, 3-15.

Alkan, C., Jurado-Cobena, E. & Ikegami, T. (2023). Advancements in Rift Valley fever vaccines: a historical overview and prospects for next generation candidates. *npj Vaccines,* 8, 171.

Arsenopoulos, K. V., Fthenakis, G. C., Katsarou, E. I. & Papadopoulos, E. (2021). Haemonchosis: A challenging parasitic infection of sheep and goats. *Animals,* 11, 363.

Bokori-Brown, M., Hall, C. A., Vance, C., Da Costa, S. P. F., Savva, C. G., Naylor, C. E., Cole, A. R., Basak, A. K., Moss, D. S. & Titball, R. W. (2014). Clostridium perfringens epsilon toxin mutant Y30A-Y196A as a recombinant vaccine candidate against enterotoxemia. *Vaccine,* 32, 2682-2687.

Byrne, M. P. & Smith, L. A. (2000). Development of vaccines for prevention of botulism. *Biochimie,* 82, 955-966.

Commission, E. P. (1998). European pharmacopoeia. *(No Title),* 3rd Ed. Maisonneuve, 561.

Delrue, I., Verzele, D., Madder, A. & Nauwynck, H. J. (2012). Inactivated virus vaccines from chemistry to prophylaxis: merits, risks and challenges. *Expert Review of Vaccines,* 11, 695-719.

Dungu, B., Donadeu, M. & Bouloy, M. (2013). Vaccination for the control of Rift Valley fever in enzootic and epizootic situations. *Vaccines and Diagnostics for Transboundary Animal Diseases*. Karger Publishers.

Dungu, B., Lubisi, B. A. & Ikegami, T. (2018). Rift Valley fever vaccines: current and future needs. *Current Opinion in Virology,* 29, 8-15.

Elfadil, A., Hasab-Allah, K., Dafa-Allah, O. & Elmanea, A. (2006). The persistence of Rift Valley fever in the Jazan region of Saudi Arabia. *Revue Scientifique et Technique-Office International des Epizooties,* 25, 1131-1136.

Ellis, C. E., Mareledwane, V. E., Williams, R., Wallace, D. B. & Majiwa, P. A. (2014). Validation of an ELISA for the concurrent detection of total antibodies (IgM and IgG) to Rift Valley fever virus. *Onderstepoort Journal of Veterinary Research,* 81, 1-6.

Faburay, B., Labeaud, A. D., Mcvey, D. S., Wilson, W. C. & Richt, J. A. (2017). Current status of Rift Valley fever vaccine development. *Vaccines,* 5, 29.

Fakri, F., Ghzal, F., Daouam, S., Elarkam, A., Douieb, L., Zouheir, Y., Tadlaoui, K. & Fassi-Fihri, O. (2015). Development and field application of a new combined vaccine against Peste des Petits Ruminants and Sheep Pox. *Trials in Vaccinology,* 4, 33-37.

Fao, O. & Africa—El Niño, W. (2015). increased risk of Rift Valley fever—Warning to countries. *EMPRES WATCH,* 34.

Glenny, A. & Hopkins, B. E. (1923). Diphtheria toxoid as an immunising agent. *British Journal of Experimental Pathology,* 4, 283.

House, J. A., House, C., Dubourget, P. & Lombard, M. (2003). Protective immunity in cattle vaccinated with a commercial scale, inactivated, bivalent vesicular stomatitis vaccine. *Vaccine,* 21, 1932-1937.

Jemal, D., Shifa, M. & Kebede, B. (2016). Review on pulpy kidney disease. *Journal of Veterinary Science and Technology,* 7, 361.

Kroeker, A., Babiuk, S., Pickering, B., Richt, J. & Wilson, W. (2020). Livestock Challenge Models of Rift Valley Fever for Agricultural Vaccine Testing. *Frontiers in Veterinary Science*; 7: 238.

Kwaśnik, M., Rożek, W. & Rola, J. (2021). Rift Valley fever–a growing threat to humans and animals. *Journal of Veterinary Research,* 65, 7-14.

Lindblad, E. B. (2004). Aluminium adjuvants—in retrospect and prospect. *Vaccine,* 22, 3658-3668.

Liu, H.-W., Hu, Y., Ren, Y., Nam, H., Santos, J. L., Ng, S., Gong, L., Brummet, M., Carrington, C. A. & Ullman, C. G. (2021). Scalable purification of plasmid DNA nanoparticles by tangential flow filtration for systemic delivery. *ACS Applied Materials & Interfaces,* 13, 30326-30336.

Lobato, F. C., Lima, C. G., Assis, R. A., Pires, P. S., Silva, R. O., Salvarani, F. M., Carmo, A. O., Contigli, C. & Kalapothakis, E. (2010). Potency against enterotoxemia of a recombinant Clostridium perfringens type D epsilon toxoid in ruminants. *Vaccine,* 28, 6125-6127.

Martinod, S. (1996). Combination vaccines for cattle. *The Bovine Practitioner*, 36-39.

Matsiela, M. S., Naicker, L., Khoza, T. & Mokoena, N. (2023). Safety and immunogenicity of inactivated Rift Valley Fever Smithburn viral vaccine in sheep. *Virology Journal,* 20, 221.

Moetlhoa, B., Naicker, L., Hayeshi, R., Grobler, A., Mokoena, N. B. & Mawadza, C. (2021). Application of a real-time cell analysis system in the process development and quantification of Rift Valley fever virus clone 13. *Access Microbiology,* 3.

Mokoena, T., Chakauya, E., Crampton, M., Weyers, B., Tselanyane, M., Tsekoa, T. & Chikwamba, R. (2017). Evaluation of plant-produced Clostridium perfringens type D epsilon toxoid in a vaccine against enterotoxaemia in sheep. *Onderstepoort Journal of Veterinary Research,* 84, 1-7.

Morefield, G. L., Tammariello, R. F., Purcell, B. K., Worsham, P. L., Chapman, J., Smith, L. A., Alarcon, J. B., Mikszta, J. A. & Ulrich, R. G. (2008). An alternative approach to combination vaccines: intradermal administration of isolated components for control of anthrax, botulism, plague and staphylococcal toxic shock. *Journal of Immune based Therapies and Vaccines,* 6, 1-11.

Mroz, C., Gwida, M., El-Ashker, M., Ziegler, U., Homeier-Bachmann, T., Eiden, M. & Groschup, M. (2017). Rift Valley fever virus infections in Egyptian cattle and their prevention. *Transboundary and Emerging Diseases,* 64, 2049-2058.

Nail, S. L., White, J. L. & Hem, S. L. (1976). Structure of aluminum hydroxide gel I: initial precipitate. *Journal of Pharmaceutical Sciences,* 65, 1188-1191.

Pharmacopoeia, E. (2008). Clostridium perfringens vaccine for veterinary use. *Monograph,* 01, 0363.

Provost, A. & Perreau, P. (1978). Combined vaccines in veterinary medicine in the developing countries. *Developments in Biological Standardization,* 41, 349-360.

Silva, R. O. S., Duarte, M. C., Junior, C. a. O., De Assis, R. A., Lana, A. M. Q. & Lobato, F. C. F. (2018). Comparison of humoral neutralizing antibody response in rabbits, guinea pigs, and cattle vaccinated with epsilon and beta toxoids from Clostridium perfringens and C. botulinum types C and D toxoids. *Anaerobe,* 54, 19-22.

Smith, M. R., Schirtzinger, E. E., Wilson, W. C. & Davis, A. S. (2019). Rift Valley fever virus: Propagation, quantification, and storage. *Current Protocols in Microbiology,* 55, e92.

Smither, S. J., Lear-Rooney, C., Biggins, J., Pettitt, J., Lever, M. S. & Olinger Jr, G. G. (2013). Comparison of the plaque assay and 50% tissue culture infectious dose assay as methods for measuring filovirus infectivity. *Journal of Virological Methods,* 193, 565-571.

Ulanova, M., Tarkowski, A., Hahn-Zoric, M. & Hanson, L. Å. (2001). The common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infection and Immunity,* 69, 1151-1159.

Warimwe, G. M., Gesharisha, J., Carr, B. V., Otieno, S., Otingah, K., Wright, D., Charleston, B., Okoth, E., Elena, L.-G. & Lorenzo, G. (2016). Chimpanzee adenovirus vaccine provides multispecies protection against Rift Valley fever. *Scientific Reports,* 6, 20617.

Wright, D., Allen, E. R., Clark, M. H., Gitonga, J. N., Karanja, H. K., Hulswit, R. J., Taylor, I., Biswas, S., Marshall, J. & Mwololo, D. (2020). Naturally acquired Rift Valley fever virus neutralizing antibodies predominantly target the Gn glycoprotein. *Iscience,* 23.

6 CHAPTER SIX: CONCLUDING REMARKS

6.1 General study outcome

Vaccination of livestock against the spread of Rift Valley Fever (RVF) is a crucial aspect of disease control. Current RVF vaccination programs are not sustainable and result in low vaccination coverage. This poses the risk of virus exposure to susceptible animals in both endemic and non-endemic regions. The aim of this study was to develop a safe and efficacious RVF and pulpy kidney (PK) combination vaccine, since vaccination against PK is commonly being practiced in the field. This novel combination vaccine will help improve vaccination coverage of RVF in endemic regions. The study was initiated by developing a safe and immunogenic monovalent inactivated RVF vaccine using the Smithburn (SB) live attenuated strain. The RVFV-SB strain was selected for use in order to mitigate the potential safety hazards associated with the use of virulent RVFV isolates for formulation of inactivated vaccines. The utilization of such isolates poses a risk of infection to production technologists and laboratory personnel during the manufacturing process. Additionally, the live attenuated RVF-SB vaccine has been known to cause teratogenic effects in vaccinated pregnant animals, and thus its use is restricted solely to non-pregnant animals. Chapter 3 focused on improving the safety profile of the SB strain for use in livestock. The live attenuated RVFV Smithburn was inactivated with 0.5 mM BEI for 24 hours at 37 °C with stirring at 100 rpm. The complete inactivation of the virus was confirmed using RTCA system which monitored cell growth kinetics of Vero cell culture incubated with the inactivated RVFV in comparison with the cell only control. The complete inactivated RVFV-SB was formulated with aluminium hydroxide and Montanide™ gel-01 adjuvants at 50% w/w and 10% w/w final concentrations, respectively. The inactivated RVF-SB vaccine was demonstrated to be safe for use in sheep, with no clinical signs of infection, no mortalities nor reactions on site of injection. In addition, no increase in temperature was recorded in vaccinated animals, and they maintained their normal sheep temperature range of 38.9-39.9 °C. The inactivated RVF-SB formulated with aluminium hydroxide induced high neutralising antibody titres against RVF comparable with the response induced by the OBP-commercial inactivated RVF vaccine. The antibody titres induced by the inactivated RVF-SB formulated with Montanide gel-01 were detected at a lower concentration closer to the cut-off margin of neutralising antibody titre against RVF. The inactivated RVF-SB vaccine formulated with aluminium hydroxide gel adjuvant was therefore selected to be the best-suited formulation for use in combination with the epsilon toxoid.

The research was further focused on characterising growth pattern of *Clostridium perfringens* type D, assessed at both experimental and pilot scale for preparation of the monovalent epsilon toxoid (chapter 4). Bacterial growth kinetics was monitored at different anaerobic fermentation conditions to identify suitable conditions for cultivation of *C. perfringens* type D to achieve maximum production yields of the epsilon toxin. The fermentation process was conducted in yeast and potassium hydrogen phosphate (PYP) media at temperature of 37 ± 2 °C, pH of 7.2 and a stirring speed of 100 rpm. Three anaerobic conditions were evaluated for improved yields of the epsilon toxin, namely: (a) a closed system with no air flow, (b) a system supplied with nitrogen gas for the first two hours of the fermentation process, and (c) a continuous nitrogen gas supply throughout the fermentation process. Biomass production was monitored at 3-hour interval in relation to pH of the fermentation system. Most importantly, maximum yield production of the epsilon toxin was also monitored at 3-hour interval during the fermentation process. The results showed that maximum growth of the *C. perfringens* type D, obtaining an OD of 2.00 was detected after 9 hours of cultivation in anaerobic conditions supplemented with nitrogen purging. Following 12 hours of cultivation, the cells reached stationary phase and harvested following 21 hours of fermentation. The pH of the system was also shown to decrease immediately when cells entered an exponential phase. An improved yield of the epsilon toxin was obtained after 12-21 hours of fermentation under anaerobic conditions maintained with anaerobic gas supplied for first two hours of the fermentation process. The data obtained suggested that the organism expresses protein during the exponential growth phase and no protein is excreted when bacteria has reached stationary phase.

The epsilon toxin was secreted as an inactive form, with a molecular weight of 35 kDa. The protein was proteolytically cleaved using trypsin at final concentration of 0.032g/L to obtain an active and antigenic toxin with molecular weight of 29 kDa. Activation of the epsilon toxin resulted in its improved toxicity obtaining a titre of 10^2 MLD_{50}/mL. The activated epsilon toxin was then inactivated with 0.7% (v/v) formalin at 37 °C with continuous stirring at 100 rpm for 48 hours, to obtain a complete inactivation of the toxin. Following inactivation with formalin, the crude culture was clarified using centrifugation method at 4000 x g for 45 minutes to remove bacterial cells. For further clarification of the culture supernatant, normal flow filtration system, equipped with the 0.3 µm SupaSpun II DOE + PE Gaskets (AMAZON Filters LTD, England) and 0.45 + 0.2 µm Sartopore 2-IV double layered filter membranes was utilised. The epsilon toxin obtained in the culture supernatant was further processed through tangential flow filtration (TFF) system for exchange of buffers, to remove the broth media used for bacterial culture and replace it with phosphate buffered saline. This procedure was also adequate for reducing the concentrations of residual formalin in the final vaccine product, to meet

required specifications according to Department of Agriculture Land Reform and Rural Development (Act 36 of 1947). Chapter 5 evaluated safety and efficacy of the RVF and PK combination vaccine in merino sheep. Initially, the monovalent inactivated RVF Smithburn vaccine and the epsilon toxoid were produced and individually formulated with aluminium hydroxide at 50% (w/w) final concentration each. The vaccine formulations were combined at 1:1 ratio to achieve the RVF/PK combination vaccine in sterile conditions. The vaccine candidates were subjected to quality control measures before use in clinical trials and were found to be free from bacterial and fungal contaminations. Furthermore, the vaccines maintained a homogenous properties with a uniform emulsions, obtaining no separations of vaccine components after 24 hours incubation at 4 °C, 25 °C and 37 °C, suggesting effective formulation of the emulsions. The novel RVF/PK combination vaccine was evaluated for safety and efficacy in merino sheep after primary and booster administrations of the vaccine. The monovalent vaccine components were included as controls. The commercial OBP-inactivated RVF and alum-precipitated PK vaccines were included as positive controls, and placebo vaccine served as the negative control. The vaccine candidates were shown to be safe for use in sheep, inducing no adverse reactions on site of injections and no clinical signs against RVF nor PK infections in vaccinated animals. The humoral antibody immune response against the RVFV and the epsilon toxin were evaluated using the RVFV-IgM/IgG enzyme linked immunoassay and serum neutralisation tests. The positive response of the IgM antibody titres was detected 7 days post primary vaccinations, in animal groups vaccinated with the monovalent inactivated RVF-SB, the RVF/PK combination as well as the commercial inactivated RVF vaccines. The IgM antibodies declined from day 14 to day 21 as anticipated. This was due to a gradual accumulation of the IgG antibodies, which occurs from day-7 after injections onward to maintain a long-term immunity in vaccinated animals. The IgG antibody response were detected in sheep vaccinated with the inactivated RVF-SB 7 days following primary vaccination. Sheep vaccinated with the RVF/PK combination vaccine and the commercial inactivated RVF obtained positive response of the IgG at 14 days post primary vaccination, and these antibody titres were maintained throughout the trial. The RVF/PK combination vaccine elicited neutralising antibody titres against the RVFV at levels comparable to the monovalent inactivated RVF Smithburn and the OBP-commercial inactivated RVFV vaccines at 14 days following primary vaccination. Maximum detection of the neutralising antibody titres was obtained after 35 days of booster vaccinations of sheep with the RVF/PK combination vaccine and were maintained throughout the trial. Assessment of the vaccine potency against the epsilon toxin was conducted in guinea pigs according to the European Pharmacopoeia, which recommends the vaccination of guinea pigs with Clostridial vaccines, followed by determining the induced antibody concentrations in sera using the toxin neutralization test in mice. Guinea pigs were similarly vaccinated with the RVF/PK combination

vaccine, and the monovalent components of the vaccine were included as controls. The commercial OBP-inactivated RVF and the alum-precipitated PK vaccines were included as positive controls, and placebo as negative control. The animals were daily monitored for any clinical signs of RVF/PK infection following vaccine inoculations, and no signs of infections, reactions on site of injection, reduced mobility or mortalities were recorded in animals. Blood was collected from 14 days after booster vaccinations onwards (D35, D42, DD49, D63). Toxin neutralisation tests were conducted in mice to evaluate the concentrations of the neutralising-epsilon toxin antibodies obtained in sera collected from vaccinated guinea pigs. The RVF/PK combination vaccine induced neutralising antibody immune response against the epsilon toxin, and maximum antibody concentrations were detected on D63, after 42 days of booster vaccination. Higher concentrations of the anti-epsilon toxin antibodies were detected in animals injected with the monovalent pulpy kidney vaccine as anticipated. The results have shown that the RVF/PK combination vaccineis safe for use in merino sheep and guinea pigs, also inducing protective levels of antibody immune response. This vaccine candidate is suitable for use in regions where the RVF is endemic to help improve vaccination coverage of the disease, while protecting animals against PK infections.

6.2 Limitations to the study

6.2.1 Production of the epsilon toxin

The epsilon toxin was utilised at a similar dose to the currently registered pulpy kidney vaccine, which is 10^2 to 10^3 MLD_{50}/mL. The toxin was produced under anaerobic conditions maintained using nitrogen gas. Further optimisation of the production process is required to obtain higher yields of the toxin. During the fermentation process, the optimum epsilon toxin concentration obtained was ≤10^2 MLD_{50}/mL. It was observed that the toxin at a production concetration of 15 mg/mL (10^2 MLD_{50}/mL), induces antibody immune response at marginal levels for the recommended epsilon toxoid (5 IU/mL) when used in combination with the RVFV.

6.2.2 Challenge studies for RVF/PK combination vaccine

The challenge studies for the inactivated RVF Smithburn and the RVF/PK combination vaccine against the RVFV were not conducted due to the unavailability of a suitable biosafety level-3 (BSL-3) facility for conducting the trial. Challenge studies shall be conducted upon availability of the BSL-3 facility, in order to register dossier of this product.

6.2.3 Use of naïve animals for PK vaccine

It was also difficult to conduct clinical trials for PK vaccine in the typical animal stables, since Clostridial organisms are gut flora and any daily activities of the animals can can create favourable conditions that trigger proliferation and growth of the *C. perfringens*, ultimately resulting in the production of lethal toxins, including epsilon toxin. Use of PK naïve animals to evaluate potency of the vaccine also created a study limitation as the animals were not dewormed in prevention for exposure to the disease, as a result exposing them to Haemonchus cortosus infection.

6.3 Recommendations

6.3.1 Optimisation of production of the epsilon toxin for use in the RVF/PK combination vaccine

To obtain high concentrations (≥10^3 MLD_{50}/mL) of the epsilon toxin, further validation of the production procedure is required. Alternatively, the toxin may be concentrated 100x using the TFF system fitted with a 10 kDa filtration membrane. Furthermore, subunit technology can be used to

evaluate the expression of the epsilon toxin and obtain a higher concentration of the toxin yields. Increasing the titre of the epsilon toxin may also enhance the neutralising anti-epsilon toxin antibody titres induced in sheep by the RVF/PK combination vaccine.

6.3.2 Challenge studies for the RVF/PK combination vaccine

In order to evaluate the efficacy of inactivated RVF Smithburn and RVF/PK combination vaccine against the RVFV, challenge studies should be conducted in sheep, in a protective BSL-3 facility. In addition, duration of immunity may further be analysed and recorded since long-term protective immunity is essential for future development of the RVF vaccines. The vaccine potency against the epsilon toxin may further be analysed in sheep, to compare the humoral antibody response elicited by the vaccine in guinea pigs with the concentrations obtained in sheep.

6.3.3 Use of naïve animals for PK vaccine

Undertaking clinical trials for PK studies in a breeding farm would be more advantageous, as it would ensure consistent environmental conditions for the animals, thus preventing their gradual exposure to the PK disease. Moreover, this environmental setup would enable the assessment of the vaccine's potency in sheep that have previously been confirmed to be naïve to the epsilon toxin.

7 ANNEXTURES

7.1 Annexture A: Buffer solutions used in the study

7.1.1 Buffered solutions

7.1.1.1 Saline buffered solution

The solution was prepared by dissolving 8.5 g/L sodium chloride (Merck Millipore) in WFI and adjusted to pH 7.4 ± 0.2. The solution was sterilised by normal flow filtration system fitted with the 0.45 + 0.2 µm Sartopore 2-IV double layered filters (Sartorius, Germany).

7.1.1.2 Phosphate buffered saline (PBS)

PBS was prepared from 8 g/L sodium chloride (Merck Millipore), 0.2 g/L potassium chloride (Merck Millipore), 0.15 g/L disodium hydrophosphate (Sigma Aldrich) and 0.2 g/L potassium dihydrogen phosphate (Merck Millipore), dissolved in WFI. The pH of the solution was adjusted to 7.4 ± 0.2 and sterilised by normal flow filtration system fitted with the 0.45 + 0.2 µm Sartopore 2-IV double layered filters (Sartorius, Germany).

7.1.1.3 Earles buffered solution

Earles buffered solution was prepared by dissolving 0.265 g/L calcium chloride (Sigma Aldrich, USA), 0.097 g/L magnesium sulfate (Sigma Aldrich, USA), 0.4 g/L potassium chloride (Sigma Aldrich, USA), 6.8 g/L sodium chloride (Sigma Aldrich, USA), 0.122 g/L sodium phosphate (Sigma Aldrich, USA), and 1.0 g/L D-glucose (Sigma Aldrich, USA) in WFI. The buffer was sterilised by NFF system fitted with the 0.45 + 0.2 µm Sartopore 2-IV double layered filters (Sartorius, Germany).

7.1.1.4 Neutral red

The neutral red stock solution was prepared by dissolving 1% (w/v) neutral red powder ($C_{15}H_{17}ClN_4$) (Merck Millipore, Germany) in WFI and stirred at 200 rpm for 1-hour at room temperature. The solution was filtered using Grade 2 Whatman filter paper (with 8 µm pore size) (Merck Millipore, Germany), and sterilised by autoclaving at 121 °C for 15 minutes. After cooling, the sterile neutral red solution was stored at room temperature and away from sunlight.

7.1.1.5 Nash reagent

Nash reagent was prepared from a mixture of 30% (w/v) ammonium acetate ($C_2H_7NO_2$) (Minema Chemicals, SA), 6% (v/v) acetic acid (Merck Millipore, Germany) and 2% (v/v) acetyl acetone for analysis (Merck Millipore, Germany) in WFI and stored at 4 °C and away from sunlight.

7.1.1.6 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffers

All buffers used in the SDS-PAGE protein analysis were prepared using chemicals procured from Sigma Aldrich and Merck Millipore (as indicated on Table 8. 1) that were dissolved in WFI.

Table 7. 1: Preparation of the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS buffers	Components
1.5 M Tris-HCl buffer (pH 8.8)	18.1% (w/v) Tris base (Merck Millipore) of molecular biology grade, dissolved in WFI and adjusted to pH 8.8 with 6 N HCl (Sigma Aldrich).
0.5 M Tris-HCl (pH 6.8)	6% (w/v) Tris base (Merck Millipore) dissolved in WFI, and adjusted to pH 6.8 with 6 N HCl (Sigma Aldrich).
10x Tris-Glycine SDS-PAGE running buffer (pH 8.3)	3% (w/v) Tris base (Sigma Aldrich), 14.4% (w/v) glycine (Sigma Aldrich), and 1% (w/v) SDS (Sigma Aldrich).
10% (w/v) ammonium persulfate	0.1 g ammonium persulfate (Sigma Aldrich) was dissolved in 1 mL WFI
125 mM Tris-HCl buffer (non-reducing treatment buffer), pH 6.8	4% (w/v) SDS (Sigma Aldrich), 25% (v/v) 0.5 M Tris-HCl, 20% (v/v) glycerol (Sigma Aldrich) and 0.002 (w/v) bromophenol blue (Merck Millipore)
Coomassie blue staining solution	0,1% (w/v) Coomassie blue R250 (Sigma Aldrich), 30% (v/v) methanol at 99.6% (v/v) (Sigma Aldrich), 5% (v/v) glacial acetic acid (Merck Millipore) and 65% (v/v) WFI
Destaining solution	30% (v/v) methanol (Sigma Aldrich) and 5% glacial acetic acid (Merck Millipore) and 65% WFI.

7.1.1.7 Buffers used for radial immunodifussion (RID) assay

All buffers used for radial immunodifussion assay were prepared using chemicals procured from Sigma Aldrich and Merck Millipore (as indicated on Table 8. 2) and were dissolved in WFI.

Table 7.2: Preparation of buffers for radial immunodifussion (RID) assay

Buffers	Components
Phosphate buffered saline (pH 7.15)	8.12 g/L sodium chloride (Merck Millipore), 0.994 g/L disodium phosphate (Merck Millipore) and 0.48 g/L potassium dihydrogen phosphate (Merck Millipore) dissolved in WFI.
Coomassie blue staining solution	5.0 g/L Coomassie brilliant blue R250 (Merck Millipore) in 45% (v/v) ethanol at 96% for general analytical application according to the European pharmacopeia (Merck Millipore), 10% (v/v) glacial acetic acid 99.7% (Merck Millipore) added in 45% (v/v) WFI.
Destaining solution	45 % (v/v) ethanol at 96% (Merck Millipore), 10% (v/v) glacial acetic acid at 99.7% (Merck Millipore) added in 45% (v/v) WFI.

7.1.1.8 Blocking solution used for western blot

The western blot blocking solution was prepared from dissolving a 3% (w/v) skim milk (Thermo Scientific™ Oxoid™, UK) dissolved in saline buffered solution, and added 0.1% (v/v) Tween 20 (Thermofisher Scientific, USA).

7.2 Annexture B: Consumables used for clinical trials

Luer slip-syringe locked with a needle size 27G x ½'' (0.40 x 13mm) (Neomedic Pty Ltd, SA) was used for sample administrations in mice.

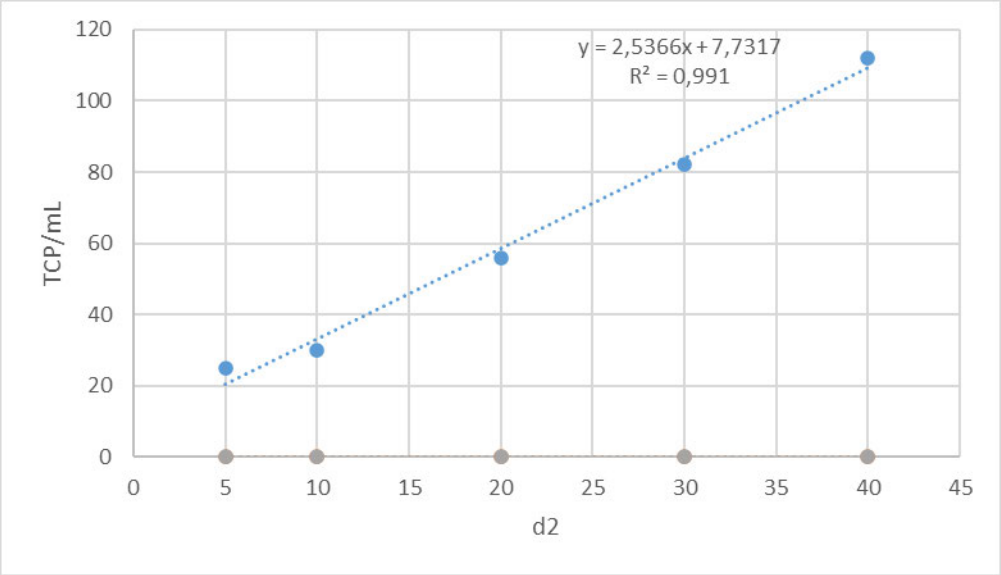
The sterile hypodermic needle 18G x 1 ½'' (1.2 x 40 mm) (Surgi Plus Medical, China) and the 3 mL Luer slip syringe (Surgi Plus Medical, China) were used for vaccine administrations in merino sheep. The BD Vacutainer Precision Glide needle 18G x 1.5'' (1.2 x 38 mm) (Beckton Dickinson and company, USA), and 5 mL vacuum gel blood collection tubes (Scientific Group, China) were used for blood collections. The 1 mL Luer slip-syringe locked with a needle size 27G x ½'' (0.40 x 13mm) (Neomedic Pty Ltd, SA) were used for mice injections. The sterile hypodermic needle 21 G x 1 ½'' (0.8 x 40 mm) (Surgi Plus Medical, China) and the 3 mL Luer slip syringe (Surgi Plus Medical, China) were used for vaccine administrations in guinea pigs. Venous blood collection needle 21 G x 1 ½'' (Scientific Group, China) and the 5 mL vacuum gel blood collection tubes (Scientific Group, China) were used for collection of blood from guinea pigs.

7.2.1.1 Other consumables used in the study

The *C. perfringens* epsilon toxin ELISA kit (ref: Bio K 268) (BioX-Diagnostics, Belgium), Pierce™ bicinchoninic acid (BCA) protein assay (BCA) kit (Thermofisher Scientific, USA), Invitrogen iBlot gel Transfer Stacks, Regular Nitrocellulose (Thermofisher Scientific, Israel)

8 APPENDICES

8.1 Appendix A: Standard linear of regression for quantification of the epsilon toxin or toxoid produced by *Clostridium perfringens* using Radial Immunodifussion assay.



Appendix A: Standard linear of regression for quantification of the epsilon toxin or toxoid produced by *Clostridium perfringens*using RID assay. This data was generated at Onderstepoort Biological Products, at the Research and Development section.

8.2 Appendix B: Pathology postmoterm reports provided by the University of Pretoria, and the onsite Veterinary report for the clinical trial.

Onderstepoort Biological Products SOC Ltd
Co Reg No. 2000/022686/06
Private Bag X07, Onderstepoort, 0110
Tel: +27 (0) 12 522 1500
Fax: +27 (0) 12 522 1591
www.obpvaccines.co.za

VETERINARY REPORT.

Date: 10/12/2023

Compiler: Dr MS Ncube – OBP Resident Veterinarian.

Trial: Safety and efficacy of the RVF/PK combination vaccine.

RE: Combined report for sheep number: P27, P46, P65, and P68.

The affected animals, mixed sex and 10 months average age, were part of a trial and their specific group was not vaccinated as per the requirements of the study. Due to their unvaccinated status against Pulpy kidney the animals could not be dewormed due to the pathological progression of enterotoxaemia. This subsequently led to the animals succumbing to a high worm burden and subsequently having to be put down at the discretion of the veterinary personnel.

The haemonchosis progression was relatively similar in all affected animals; general weakness, failure to stand, lying on the side, inappetence, pale mucous membranes and poor body condition score averaging 1.0-1.5 on a scale of 1-5.

On post mortem by independent specialist pathologists the animals were officially diagnosed with protein and energy malnutrition with moderate to severe abomasal haemonchosis. The blood smears indicated moderate erythrocytic polychromasia and anisocytosis (no Ehrlichia organisms were observed) while the faecal float showed Strongyle and Monezia-like eggs.

Among other findings, the blood was watery, there was serous fat and skeletal muscle atrophy, small and contracted spleen, hypoplastic rumen papillae, severe bone marrow atrophy, severe pulmonary oedema, centrilobular hepatic coagulative necrosis. Lesions found in the animals were consistent with protein and energy malnutrition which may have resulted in the observed anaemia and hypoproteinaemia. The Haemonchus worms in the abomasum exacerbated the anaemia and hypoproteinaemia. The animals then suffered from hypothermia and died.

OBP Veterinarian Signature: 

Date: 10 December 2023

S02970-23
Report Date
11 OCT 2023 14:43

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Pathology Laboratory

Accession Number: S02970-23

Owner: ONDERSTEPOORT BIOLOGIESE PRODUKTE
PRIVATE BAG X07
ONDERSTEPOORT, GP 0110

Received: 10/09/2023 **Finalized:** 10/11/2023
Sampled:

To: .
ONDERSTEPOORT BIOLOGIESE PRODUKTE
PRIVATE BAG X07
ONDERSTEPOORT, GP 0110

History: Under RVF/PK combination trial +/- 7d ago. Was found dead on morning of 07.10.2023. PM carried out 09.10.2023

Final Report

TEST REPORT: PATHOLOGY

NECROPSY WITH HISTO SA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Pathology Laboratory

Final Report **Accession Number:** S02970-23

TEST REPORT: PATHOLOGY

ANIMAL ID	P46
SPECIES	Ovine
BREED	Merino
SEX	Female Intact
AGE	4m
SPECIMEN DESC	Dead Animal
PM LAB TEST	Blood smear: 1+ Erythrocyte polychromasia and anisocytosis Faecal float: 4+ Strongylid-type ova, 3+ coccidian oocysts Brain smear: No heartwater colonies observed. Rumen pH 6
PM CHANGES	Advanced (severe autolysis and putrefaction with widespread pseudomelanosis, post mortem emphysema and haemoglobin imbibition). Interim > 48 hours. Rigor mortis absent.
GENERAL CHANGES	Body condition score 2/5 (Lean muscle mass and reduced fat reserves)
SPECIFIC CHANGES	Main finding: 4+ Haemonchus adult worms forming dense mats admixed with brown-tinged (when compared with rumen content) abomasal content. [MD Abomasitis with petechiation, mild, diffuse, acute with severe verminosis (haemonchosis) and parasite haematin] Associated findings: Anaemia, severe, with minimal regeneration (marked mucous membrane and carcass pallor with watery blood) Pulmonary oedema, moderate to severe, diffuse, acute
INCIDENTAL FINDINGS	Plastic yellow right ear tag “P46”.
DIAGNOSIS	Fatal anaemia due to acute haemonchosis
COMMENTS	The necropsy results reveal the animal died from anaemia due to a massive Haemonchus contortus (wireworm, haarwurm) burden. These specific nematodes attach to the abomasal wall and are haematophagic and a high worm burden is collectively capable of consuming large quantities of blood. Anaemia is fatal if severe enough (as in this case). It is suggested to contact a Veterinary Surgeon as soon as possible regarding treatment and effective control strategies as additional losses are possible if remedial measures are not taken.
PATHOLOGIST	Dr R Mawson

Dept. Paraclinical Sciences
University of Pretoria
P/Bag X04, Onderstepoort, 0110
Republic of South Africa
Tel:+27 529 8073 Fax:+27 12 529 8303
www.up.ac.za

S02984-23
Report Date
12 OCT 2023 15:25

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Pathology Laboratory

Accession Number: S02984-23

Owner: ONDERSTEPOORT BIOLOGIESE PRODUKTE
PRIVATE BAG X07
ONDERSTEPOORT, GP 0110

Received: 10/10/2023 **Finalized:** 10/12/2023
Sampled: 10/09/2023

To: .

Final Report

TEST REPORT: PATHOLOGY

NECROPSY WITH HISTO SA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Pathology Laboratory

Final Report **Accession Number:** S02984-23

TEST REPORT: PATHOLOGY

ANIMAL ID	P65
SPECIES	Ovine
BREED	Merino
SEX	Female Intact
AGE	8m
SPECIMEN DESC	Dead Animal
PM LAB TEST	Blood smear: 1+ Erythrocyte polychromasia and anisocytosis. Faecal float: 3+ Strongylid-type ova and 1+ coccidian oocysts. Brain smear: No Heartwater colonies observed
PM CHANGES	Mild, with interim <24 hours. Rigor mortis absent.
GENERAL CHANGES	Cachexia (serous atrophy of fat in bone marrow, renal pelvic and cardiac storage sites with severe generalised muscle atrophy and hepatic atrophy)
SPECIFIC CHANGES	Main finding: 3+ Haemonchus adult worms with brown-tinged (when compared with rumen content) abomasal content. [MD Abomasitis with petechiation, mild, diffuse, subacute with moderate verminosis (haemonchosis) and parasite haematin] Associated findings: Anasarca, severe ("bottle jaw", with marked clear straw coloured fluid accumulations in all body cavities) Severe anaemia with minimal regeneration (watery blood, pale carcass, white mucous membranes) Pulmonary oedema, severe, diffuse, acute Nephrosis, moderate, cortical, subacute
INCIDENTAL FINDINGS	Yellow plastic right ear tag: "P65" Tapeworm cyst, abdominal
DIAGNOSIS	Protein-energy malnutrition (PEM) due to haemonchosis
COMMENTS	The principal finding is cachexia (severe fat and protein malnutrition). The changes in the liver and adipose tissue are consistent with these findings. The anasarca (fluid under the skin and in the body cavities) is due to severe protein loss. Finding the underlying cause for Protein Energy Malnutrition (PEM) requires a step-by-step analysis of all the factors that influence: 1. Feed intake (NUTRIENTS NOT GOING IN): such as gastrointestinal diseases or pain affecting lips, oral tissues, swallowing, oesophagus and gastrointestinal tract, social interactions and hierarchy (bullying) preventing access to food, management aspects affecting access to feed and water and musculoskeletal diseases affecting ability to get to food. 2. Feed quality (THE NUTRIENTS ARE NOT THERE): Management and storage practices resulting in feed deterioration, contamination, fungal and mould overgrowth, feed mixing errors, inadequate feed quality, etc. 3. Chronic infections, wasting diseases, and intestinal parasitism (NUTRIENTS ARE BEING LOST). In this case the cachexia will be due to a more chronic manifestation of haemonchosis resulting in protein loss and secondary PEM. These

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Pathology Laboratory

Final Report **Accession Number:** S02984-23

TEST REPORT: PATHOLOGY

specific nematodes attach to the abomasal wall and are haematophagic giving rise to blood and protein loss over time as well as contributing to a negative energy balance resulting in cachexia (as in this case). Both anaemia and hypoproteinaemia can be fatal if severe enough.

It is suggested to contact a Veterinary Surgeon as soon as possible regarding treatment and effective control strategies as additional losses are possible if remedial measures are not taken.

PATHOLOGIST Dr R Mawson

S03566-23
Report Date
06 DEC 2023 07:32

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Pathology Laboratory

Accession Number: S03566-23

Owner: ONDERSTEPOORT BIOLOGIESE PRODUKTE
PRIVATE BAG X07
ONDERSTEPOORT, GP 0110

Received: 12/04/2023 **Finalized:** 12/06/2023
Sampled:

To: .
ONDERSTEPOORT BIOLOGIESE PRODUKTE
PRIVATE BAG X07
ONDERSTEPOORT, GP 0110

Final Report

TEST REPORT: PATHOLOGY

NECROPSY WITH HISTO LA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Pathology Laboratory

Final Report **Accession Number:** S03566-23

TEST REPORT: PATHOLOGY

ANIMAL ID	P27
SPECIES	Ovine
BREED	Merino
SEX	
AGE	10m
SPECIMEN DESC	Dead Animal
PM LAB TEST	Blood smear: 1+ Erythrocyte polychromasia and anisocytosis. 3+ Leukopaenia Faecal float: 1+ Strongylid-type ova
PM CHANGES	Mild, with interim <24 hours. Rigor mortis absent.
GENERAL CHANGES	Cachexia (serous atrophy of fat in bone marrow, renal pelvic and cardiac storage sites with severe generalised muscle atrophy and hepatic atrophy)
SPECIFIC CHANGES	Main finding: 4+ Haemonchus adult worms with markedly brown-tinged (when compared with rumen content) abomasal content. [MD Abomasorrhagia, moderate to severe, with severe verminosis (haemonchosis)] Associated findings: Anasarca, moderate (subcutaneous oedema and moderate straw coloured fluid accumulations in all body cavities) Severe anaemia with minimal regeneration (watery blood, pale carcass, white mucous membranes) Pulmonary oedema, severe, diffuse, acute
INCIDENTAL FINDINGS	Tapeworm cyst, multiple, abdominal
DIAGNOSIS	Protein-energy malnutrition (PEM) due to haemonchosis
COMMENTS	The principal finding is cachexia (severe fat and protein malnutrition). The changes in the liver and adipose tissue are consistent with these findings. The anasarca (fluid under the skin and in the body cavities) is due to severe protein loss. Finding the underlying cause for Protein Energy Malnutrition (PEM) requires a step-by-step analysis of all the factors that influence: 1. Feed intake (NUTRIENTS NOT GOING IN): such as gastrointestinal diseases or pain affecting lips, oral tissues, swallowing, oesophagus and gastrointestinal tract, social interactions and hierarchy (bullying) preventing access to food, management aspects affecting access to feed and water and musculoskeletal diseases affecting ability to get to food. 2. Feed quality (THE NUTRIENTS ARE NOT THERE): Management and storage practices resulting in feed deterioration, contamination, fungal and mould overgrowth, feed mixing errors, inadequate feed quality, etc. 3. Chronic infections, wasting diseases, and intestinal parasitism (NUTRIENTS ARE BEING LOST). In this case the cachexia will be due to a more chronic manifestation of haemonchosis resulting in protein loss and secondary PEM. These specific nematodes attach to the abomasal wall and are haematophagic giving rise to blood and protein loss over time as well as contributing to a

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Pathology Laboratory

Final Report **Accession Number:** S03566-23

TEST REPORT: PATHOLOGY

negative energy balance resulting in cachexia (as in this case). Both anaemia and hypoproteinaemia can be fatal if severe enough.

It is suggested to contact a Veterinary Surgeon as soon as possible regarding treatment and effective control strategies as additional losses are possible if remedial measures are not taken.

PATHOLOGIST Dr R Mawson

Dept. Paraclinical Sciences
University of Pretoria
P/Bag X04, Onderstepoort, 0110
Republic of South Africa

Tel:+27 529 8073 Fax:+27 12 529 8303

www.up.ac.za

S03294-23
Report Date
21 NOV 2023 10:57

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Pathology Laboratory

Owner: ONDERSTEPOORT BIOLOGIESE PRODUKTE
PRIVATE BAG X07
ONDERSTEPOORT, GP 0110

Accession Number: S03294-23
Reference Number: OBP VACCINES

Received: 11/08/2023 **Finalized:** 11/21/2023
Sampled:

To: .

History: Experimental sheep which was not treated with ivermectin or vacc against PK when it arrived (the others are injected with overmectin and vaccinated). OBP says they dont have a tick problem (not dipped). This was given an inactivated RVF/PK vaccine (?) - and was not yet challenged.
Sheep p68 was on RVF/PK combination trial. It walked with difficulty (started 2 days ago) and ended up unable to stand up. It was euthenased this morning (8-11-2023). FULL PM is required.
Added telephonic history - it was breathing with difficulty. Dr Ngcube is in charge - he phoned back after Keneuoe Williams phoned in response to my email to ask if live vaccine or challenge had been used/done.

Final Report

TEST REPORT: PATHOLOGY

NECROPSY WITH HISTO SA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Pathology Laboratory

Final Report **Accession Number:** S03294-23

TEST REPORT: PATHOLOGY

ANIMAL ID	Sheep P68
SPECIES	Ovine
BREED	Merino
SEX	Male Castrated
AGE	8m
SPECIMEN DESC	Dead Animal
PM LAB TEST	Blood smear - watery thin blood, white cell numbers in normal range but neutrophils outnumber round cells. Faecal floatation (normal faecal pellets in colon) - 2-3+ positive for Haemonchus contortus eggs. Brain smear - negative for Ehrlichia ruminantium. Smear of pus from small lesion inside left corner of mouth (caudal to mandibulr rmolars) - numerous small bacterial cocco-bacilli and occasional filamentous rods. Digital images were taken prior to and during the necropsy.
PM CHANGES	Mild
GENERAL CHANGES	Emaciated sheep with short curved horns, wool covered with sawdust, mucus membranes white (marked anaemia). Serous atrophy of renal hilar fat and early atrophy of long bone marrow fat with multifocal hyperplasia; a small amount of pericardial and abdominal fat is still just recognisable as normal fat. Generalised hypoproteinaemic oedema (anasarca, hydropericardium, hydrothorax and ascites (mild-moderate)).
SPECIFIC CHANGES	3+ Haemonchosis (live worms in abomasum; 2-3+ positive faecal floatation for eggs). Small abscess caudal to left lower molars due to penetrating grass stalk. Forestomach mucosa devoid of papillae due to watery fine soupy yellow fibrous content (forestomachs small for age due to lack of roughage). Marked diffuse small intestinal atrophy.
HISTOPATHOLOGY	Bone marrow - peripheral serous atrophy of fat - still some fat-containing lipocytes centrally in shaft; peripheral poietic tissue is almost exclusively producing neutrophils; most megakaryocytes immature; minimal erythropoiesis was found. Thymus - diffuse moderate to marked atrophy (may be normal for age). Skeletal muscle - moderate variation in fibre diameter - occasional necrotic fibres and a few scattered swollen eosinophilic fibres often with central nuclei. Heart narrow fibres, occasional fibres are necrotic. Spleen - fairly bloodless; perifollicular blood contains many neutrophils; red pulp shows much extramedullary haemopoiesis. Mesenteric lymph node - reactive - expansion of interfollicular lymphoid tissue and atrophy of follicles; marked sinus histiocytosis and increased medullary sinus neutrophils. Small intestine sections - moderate mucosal and submucosal congestion with increased lymphocytes and plasma cells in the lamina propria of villi; one section showed intermittent crypts filled with proteinaceous fluid and some cellular debris; scattered neutrophils in lamina propris of this latter section. Liver - relatively bloodless and diffuse mild atrophy; mild portal lymphoplasmacytic triaditis and occasional bile duct hyperplasia.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Pathology Laboratory

Final Report **Accession Number:** S03294-23

TEST REPORT: PATHOLOGY

	Lung - diffuse mild-moderate congestion with low to medium protein oedema. Kidney - relatively bloodless- mild proximal convoluted tubular epithelial degeneration (increased granular eosinophilia of cytoplasm and occasional pycnotic nuclei); distal medulla- scattered mineralised casts. Cerebrum and cerebellum - mild perivascular oedema.
DIAGNOSIS	Cachexia due to protein, energy and roughage undernutrition, exacerbated by Haemonchosis. Concommitant oral abscess causing a neutrophilia and possible septicaemia/toxaemia.
COMMENTS	Thank you for the case. Nutrition and deworming need to be addressed as soon as possible if this sheep was in a group originating from the same place and under the same circumstances at the moment or there will likely be more losses. The report is now finalised.
PATHOLOGIST	Dr JH Williams

Dept. Paraclinical Sciences
University of Pretoria
P/Bag X04, Onderstepoort, 0110
Republic of South Africa

Tel:+27 529 8073 Fax:+27 12 529 8303

www.up.ac.za

OBP 2019/002

ONDERSTEPOORT BIOLOGICAL PRODUCTS

ANIMAL ETHICS COMMITTEE

PROJECT NUMBER: **OBP 2019/002**

PROJECT TITLE: **Inactivated RVF Smithburn Vaccine**

PROJECT LEADER: **Dr Nobalanda Mokoena**

DIVISION: **RDV**

CATEGORY: **Review**

SPECIES OF ANIMALS:					
	Ovine				
	20				

√ APPROVED	NOT APPROVED	CONDITIONAL APPROVED

Comments:

SIGNATURE: [redacted] DATE: 07/06/2019

CHAIRMAN ANIMAL ETHICS COMMITTEE

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit changes to the Animal Ethics Committee for approval before commencing with the experiment.

OBP 2021/014

ONDERSTEPOORT BIOLOGICAL PRODUCTS

ANIMAL ETHICS COMMITTEE

PROJECT NUMBER: **OBP 2021/014**

PROJECT TITLE: **Development of combination vaccine for prophylactic immunization of domestic ruminants against Rift Valley Fever and Pulpy Kidney.**

PROJECT LEADER: **Ms. Matome Matsiela**

DIVISION: **RDV**

CATEGORY: **Review**

SPECIES OF ANIMALS:

Adult Mice **2520**	**Guinea Pigs** **50**			

√ APPROVED	NOT APPROVED	CONDITIONAL APPROVED

Comments:

SIGNATURE: [redacted] DATE: 25/10/2021

CHAIRMAN ANIMAL ETHICS COMMITTEE

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit changes to the Animal Ethics Committee for approval before commencing with the experiment.

ANIMAL ETHICS COMMITTEE

PROJECT NUMBER: **OBP 2021/022.1**

PROJECT TITLE: **Development of combination vaccine for prophylactic immunization of domestic ruminants against Rift Valley Fever and Pulpy Kidney.**

PROJECT LEADER: Ms Matome Matsiela

DIVISION: **RDV**

CATEGORY: **Ammendment**

SPECIES OF ANIMALS:			
Ovine **64**			

APPROVED	NOT APPROVED	CONDITIONAL APPROVED
√	√	

Comments:

SIGNATURE: [redacted] DATE: 14/06/2023

CHAIRMAN ANIMAL ETHICS COMMITTEE

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit changes to the Animal Ethics Committee for approval before commencing with the experiment.

agriculture, land reform
& rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: [redacted]
Reference: 12/11/1/1/MG (2302)

Ms Matome Matsiela
100 Old Soutpan road
Onderstepoort
0110
E-mail: [redacted]

Dear Ms Matsiela,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application dated 2 February 2022, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed by any other act of the Republic of South Africa;
2. The study is approved as per the application and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to [redacted];
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. A registered waste removal company must dispose of the material generated from the study. Records must be kept for five years for auditing purposes;
4. Sheep to be used in this study must be obtained from a place where a no objection letter would have been obtained from the local state veterinarian. While rodents and lagomorphs must be purpose bred at OBP;
5. Only the strains of the Rift Valley fever virus and *Clostridium perfringens* type D bacteria producing the epsilon toxin, contained in the registered vaccine formulations shall be used in this study;

6. No animals should be challenged with either RVF virus or *Clostridium perfringens* bacteria ;
7. The rodents and lagomorphs to be used in this study shall be housed within the Research and Development Viral section at OBP and must be euthanised at the end of the study. Sheep shall be kept at OBP large animal facilities with biosecurity and vector control measures outlined in section 5.1 of the section 20-application form, until the completion of the study. At the end of the study these sheep should either be euthanised or properly identified and incorporated into OBP flock for research purposes only. Sheep used in this study must not enter the food chain;
8. Samples collected from the study animals will be further processed at the Research and Development Viral Section at Onderstepoort Biological Products;
9. Serum samples will be stored within the Research and Development Viral Section laboratory at Onderstepoort Biological Products under access control. Any further use or distribution of the stored samples must be authorised by the Director Animal Health;
10. If required, an application for extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Development of combination vaccine for prophylactic immunisation of domestic ruminants against Rift Valley Fever and Pulpy Kidney.

Researcher:	Ms Matsiela
Institution:	Onderstepoort Biological Products
Our ref Number:	12/11/1/1/MG (2302)
Your ref:	
Expiry date:	31 December 2024

Kind regards,

[redacted]

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2022 -02- 23

SUBJECT: **Development of combination vaccine for prophylactic immunisation of domestic ruminants against Rift Valley Fever and Pulpy Kidney**