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Establishment of *in vitro* persistent infection of the foot-and-mouth disease virus SAT 2 serotype and comparative viral genome analysis during acute and persistent infections

By

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DECLARATION

I, Linda Nocaka (student number 20629606), declare that this MSc dissertation entitled "**Establishment of *in vitro* persistent infection of the foot-and-mouth disease virus SAT 2 serotype and comparative viral genome analysis during acute and persistent infections**" is my original work, which has not been submitted by myself for any degree at this university or any other university. The use of other people's work obtained from various sources was carefully acknowledged and referenced accordingly. I, therefore, declare that I did not use previous work by another student or any other person to claim as my own.

Signature: _____ Date: 14 November 2024

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ABSTRACT

The foot-and-mouth disease virus (FMDV) causes a very contagious disease that poses a significant threat to livestock globally. Persistent infections have been reported in ruminants that fail to clear the infection. These infections can create viral reservoirs and may be transmitted to other susceptible animals. This ongoing transmission may result in the development of new and more virulent strains not protected by existing vaccines. To date the mechanisms associated with persistent infections are poorly understood. Differential genomic variations during acute and persistent infection in FMDV have been reported in some serotypes, however, they are yet to be investigated in Southern African Territories (SAT) serotypes. Therefore, this study aimed to establish *in vitro* persistent infection of the SAT2 virus, a serotype responsible for most FMDV outbreaks in South Africa, in Baby Hamster Kidney (BHK-21) cell lines. Additionally, this study sought to compare genomic variations of FMDV SAT 2 serotype from persistently- and acutely infected cell cultures.

The BHK-21 cell lines were treated with ammonium chloride (NH_4Cl) to emulate persistent infection *in vitro*. To determine NH_4Cl-induced cytotoxicity, the cell cultures ($1x10^6$ cells/mL) were treated with varying concentrations of NH_4Cl (10 mM, 15 mM, and 20 mM). The cytotoxic effect of NH_4Cl on the BHK-21 cell line was found to be dose-dependent, with higher concentrations, especially at 20 mM, leading to reduced cell viability.

Persistent infection was established by infecting $1x10^5$ cells/mL BHK-21 cells with SAT2 virus at a multiplicity of infection (MOI) of 0.001 and treating with varying NH_4Cl concentrations (10 mM, 15 mM, and 20 mM). Persistent infection was achieved with 15 mM NH_4Cl, for up to 24 h. ‘Persistent infection’ cell cultures exhibited less signs of cytopathic effects (CPE) compared to cultures that were not treated with NH_4Cl (representing acute infection), from which CPE was already observed at 24 h. Furthermore, qPCR analysis based on the FMDV 3D gene showed inhibition of virus RNA replication in ‘persistent infection’ cell cultures, with Cq values ranging from 25 to 34, compared to 15–25 from ‘acute infection’ cell cultures, indicating successful establishment of persistent infection.

Subsequently, FMDV RNA was extracted from ‘persistent’ and ‘acute’ infection cell cultures for comparative genome sequence variation analysis. Overall, the analysis revealed a greater number of mutations in acutely infected cells compared to persistently infected cells, with most mutations (deletions and insertions) localized to the non-structural proteins. The number of mutations increased with time, indicating ongoing viral adaptation in both acutely and persistently infected cells.

The study demonstrates that NH_4Cl at 15 mM effectively induces persistent SAT 2 FMDV infection in BHK-21 cells by inhibiting viral replication, reducing CPE, and slowing viral adaptation, thereby supporting the study's aim to model persistent infection for this serotype. Genomic variation analysis also highlights the differences between acute and persistent infections, showing suppression of viral mutations during persistent infections due to inhibition of viral replication by NH_4Cl. The successful establishment of FMDV persistent infection *in vitro* will allow investigations of mechanisms associated persistent infection, thus a better understanding of its role in the epidemiology of FMDV. This is critical in the effective control of FMD.

Keywords: Foot–and–mouth disease virus, persistent infections, acute infections, ammonium chloride, BHK-21 cells, SAT 2 serotype, genomic mutations

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LIST OF ABBREVIATIONS

ARC	Agricultural Research Council
BHK-21	Baby hamster kidney cells
BSL-3	Biosafety Level 3
CPE	Cytopathic effect
Cq	Quantification cycle
DALRRD	Department of Agriculture Land Reform and Rural Development
FBS	Fetal bovine serum
FMD	Foot–and–mouth disease
FMDV	Foot–and–mouth disease virus
GMEM	Glasgow Minimum essential medium
Hpi	Hours post infection
LAH	Lactalbumin hydrolysate
MEM	Minimum essential medium
NH_4Cl	Ammonium chloride
NH_4+	Ammonium
NSPs	Non-structural proteins
qPCR	Quantitative real-time PCR
QC	Quality control
SAT	Southern African Territories
SNP	Single nucleotide polymorphism
SPs	Structural proteins
TAD	Transboundary Animal Diseases
TPB	Tryptose phosphate broth
UTR	Untranslated region
VGM	Virus growth medium

WRLFMD	World Reference Laboratory for FMD

LIST OF SYMBOLS

cm	Centimetre
mL	Millimetre
µL	Microlitre
PFU	Plaque forming units
Mm	Millimolar
rpm	Revolutions per minute
Nm	Nanometre
Δ	Delta

CHAPTER 1

1 INTRODUCTION

1.1 Background

Foot-and-mouth disease (FMD) is caused by the foot-and-mouth disease virus (FMDV) and is an infectious disease that poses a threat to cloven-hoofed animals such as cattle, sheep, pigs, and goats. The FMDV is mainly endemic in many Middle East countries, Asia, South America, and Africa, where it causes a strain on the economy (Ramirez-Carvajal et al., 2018). According to Phologane et al., in southern African countries, African buffalos serve as primary reservoirs of the FMDV. Although they are not primarily responsible for most outbreaks, they may play a role in infection of domestic cloven-hoofed animals, especially in wildlife-livestock interface areas (Phologane et al., 2008, Jori and Etter, 2016). In southern African countries, livestock are one of the most precious assets, with the sector contributing up to 80% to the African Agricultural Gross Domestic Products (Erdaw, 2023). However, local and international trade of animal products is affected due to FMD outbreaks, leading to major economic loss to farmers, while threatening food security for the affected countries (Maree et al., 2014, Eschbaumer, 2016).

The spread of FMD is prevented by various measures such as restricting the movement of animals when there is an outbreak, culling infected animals, intensive disease surveillance and routine vaccination usually conducted every six months (Peta et al., 2021). Although vaccines against the virus are available, they do not provide protection across different serotypes as immunity is serotype- and strain-specific (Ramirez-Carvajal et al., 2018). Moreover, the immunity with currently used vaccines is short-lived (Paton et al., 2018).

1.2 Problem statement

The control of FMDV is undermined by the capability of FMDV to cause persistent infections in some ruminants, which then serve as carriers and a risk to the spread of disease to other populations. Due to the high FMDV rate of mutation, the ongoing transmission during persistent infection can result in genetic and antigenic variations,

leading to the emergence of new and more virulent strains not protected by the existing vaccines. Mechanisms involved in FMDV persistent infections are not known, and *in vitro* models for their study are not available for all serotypes. Moreover, the mutations associated with persistent infections, particularly in SAT serotypes, remain poorly understood.

1.3 Aim and objectives

1.3.1 Study aim:

To establish persistent infections in Baby hamster kidney-21 (BHK-21) cells infected with FMDV SAT 2 serotype and compare genomic variations between viruses from acute and persistent infection cell cultures.

1.3.2 Objectives:

- *To determine ammonium chloride cytotoxicity in the BHK-21 cell line.*
- *To establish persistent infection with FMDV SAT 2 serotype in BHK-21 cells.*
- *To determine the viral cytopathic effect and infectivity in BHK-21 cells acutely and persistently infected with FMDV SAT 2 serotype.*
- *To generate whole genome sequences of FMDV SAT 2 serotype from acutely and persistently infected BHK-21 cell cultures, for identification of sequence variations/mutations associated with acute and persistent infections.*

1.4 Benefits of the study

This study will provide,

- A protocol for establishing persistent infection with FMDV SAT2 serotype in BHK-21 cell line, for future studies.
- Information on FMDV SAT 2 genomic variations determined during acute and persistent infections *in vitro,* which can be used for *in vivo* studies to understand the effects of mutations in persistent FMDV infections.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Aetiology

Foot-and-mouth disease (FMD) is a serious disease caused by the foot-and-mouth disease virus (FMDV), which belongs to the *Aphthovirus* genus of the *Picornaviridae* family. The FMD virus is spread in cloven-hoofed animals by direct or indirect contact. The FMD virus has seven serotypes which include A, O, Asia-1, C, and three Southern African Territories (SAT 1, SAT 2, and SAT 3), with multiple topotypes resulting from genetic and antigenic variations (Ramirez-Carvajal et al., 2018). These serotypes exhibit distinct genetic characteristics, leading to differences in antigenicity and cross-protection (Childs et al., 2022, Jamal and Belsham, 2013). Genomic analysis has shown that FMDV undergoes multiple recombination events, contributing to the virus' ability to persist and circulate in different geographic regions (Jamal et al., 2020). While genomic variations between FMDV serotypes play a crucial role in understanding the virus' evolution and pathogenesis (Jamal and Belsham, 2013), they can also compromise efforts toward disease control, especially when they result in antigenic diversity.

Certain ruminants play a crucial role in both spreading and maintaining FMDV in Southern Africa. African buffalo can carry the virus for up to 5 years (Condy et al., 1985), cattle for more than 2 years (Hedger, 1972, Bertram et al., 2018), and in sheep and goats for around 8 months (Burrows, 1968). Persistent infection can also contribute to genomic variations as the virus can continue to mutate. According to Maree et al, FMDV persistent infections in South Africa are a concern due to their impact on livestock and trade (Maree et al., 2014).

The FMD virus can be cultured *in vitro*, allowing the study of various aspects including its replication dynamics, host-virus interactions, immune responses, and the development of vaccine strategies (Childs et al., 2022, Hassan, 2016, Park et al., 2021). *In vitro* cultures have also been used in genomic and persistent infection studies for various FMDV serotypes (Han et al., 2018, Huang et al., 2011b, Kopliku et al., 2015).

2.2 Structure of the foot-and-mouth disease virus genome

After the virus has infected the host, it then enters the host cells by endocytosis and replicates its genome using the host cell's machinery (Li et al., 2021). The FMD virus has a diameter of around 25-30 nm and consists of a capsid-covered single-stranded, positive-sense RNA genome with a size of about 8.5 kilobases in length (Jamal and Belsham, 2013, Tuthill et al., 2010). The viral genome has a single open reading frame that has a 5' untranslated region (UTR) upstream and a short 3' untranslated region (UTR) downstream, with a poly-(A) tail at the end (Figure 2.1) (Clarke et al., 1987, Tuthill et al., 2010). The open reading frame is translated into a polyprotein that contains the Lpro, and P1, P2, and P3 regions (Roberts and Belsham, 1995). In turn, the polyprotein is cleaved to produce four structural proteins, VP1, VP2, VP3, and VP4, and eight non-structural proteins, 2B, 2C, 3A, 3B, 3D, Lpro, 2A, and 3Cpro (Figure 2.1). The structural proteins form the outer shell of the virus and are responsible for the binding of the virus, entry into host cells, and immune recognition; while non-structural proteins are involved in viral replication and modulation of host cell functions (Beck et al., 1983, Gao et al., 2016, Jamal and Belsham, 2013, Malik et al., 2017).

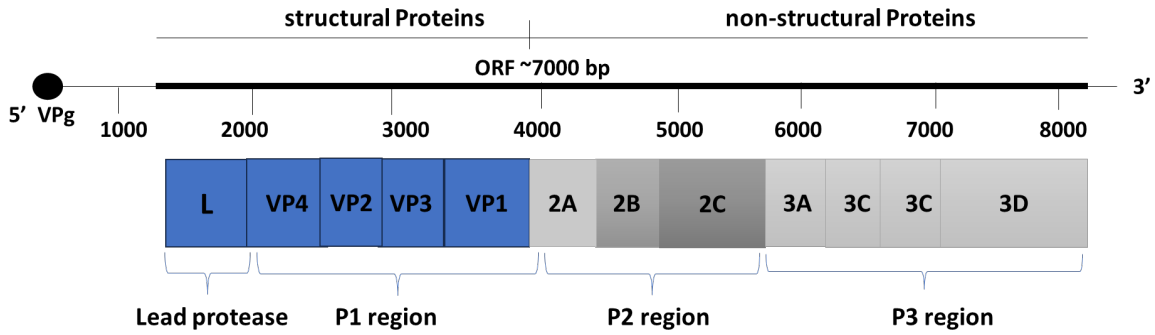


Figure 2.1: Genome structure of foot-and-mouth disease virus.

2.3 Foot-and-mouth disease virus capsid

The FMDV capsid is a critical component of the virus and protects the viral RNA and facilitates virus entry into host cells. The capsid is bound by electrostatic interactions, and weak bonds between the inter-pentameric subunits (Acharya et al., 1989, Porta et al., 2013). Additionally, it comprises 60 copies of each of the four structural proteins

(VP1 to VP4) arranged into 12 pentameric subunits, which are crucial intermediates for capsid assembly and disassembly as shown in Figure 2.2 (Martín-Acebes et al., 2011, Vázquez-Calvo et al., 2014). The capsid assembly involves the multimerization of precursor subunits that encapsidate a single copy of the viral RNA genome (Newman et al., 2021). The heat shock protein 90 is also essential for FMDV capsid precursor processing and assembly of capsid pentamers, highlighting the intricate cellular machinery involved in capsid formation (Newman et al., 2018).

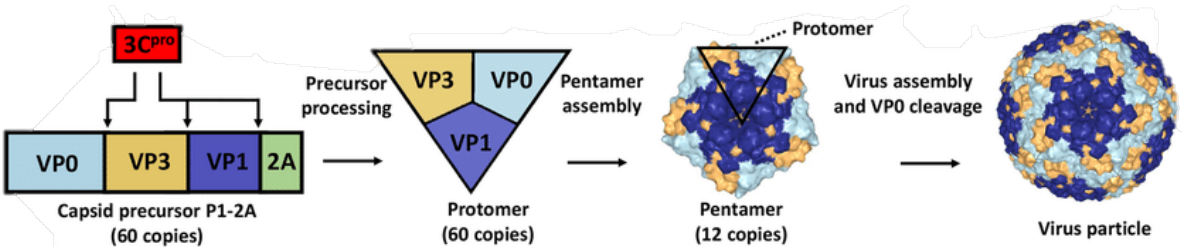


Figure 2.2: The process of foot-and-mouth disease virus capsid assembly (Kristensen and Belsham, 2019).

2.4 Effect of acidic pH on foot-and-mouth disease virus stability

One notable aspect of the FMDV is its susceptibility to acidic conditions. The FMDV capsid is highly sensitive to acidic pH, leading to the dissociation of the capsid into pentameric subunits and the release of viral RNA, which is vital for initiating virus replication (Yuan et al., 2017). Foot-and-mouth disease virus relies on the acidic pH environment in the endosomes for the transportation of viral RNA into the cytosol. The acidic nature of the endosome compartments causes the virus to uncoat and release its genetic material into the cytosol, which is crucial for the initial steps of virus infection, (Suomalainen and Greber, 2013, Vázquez-Calvo et al., 2012). The stability of the FMD virus capsid is modulated by specific amino acid substitutions, such as the replacement of histidine with tyrosine, which increases the capsid's resistance to acidic conditions (Vázquez-Calvo et al., 2014). At neutral pH, the amino acid side chains located at the interaction sites between pentameric subunits are charged, destabilising the virus' capsid (López-Argüello et al., 2019). The FMD virus inactivation resulting from exposure to a low pH environment renders the virus unable to infect and cause disease (Newman et al., 1973).

Lysosomotropic drugs like chloroquine and ammonium chloride (NH_4Cl) hinder this process, inhibiting FMDV replication *in vitro* (Curry et al., 1995, Carrillo et al., 1985). Hence, NH_4Cl is the most used lysosomotropic agent in FMDV research to achieve persistence in cell cultures (Huang et al., 2011a). Ammonium chloride neutralizes the acidic pH within the endo-lysosome compartments of the host cells (Dabydeen and Meneses, 2009); consequently, the virus is unable to uncoat, while its binding or entry into the cell remains unaffected (Baxt, 1987b, Curry et al., 1995, Martín-Acebes et al., 2010). Therefore, by treating the infected cells with NH_4Cl, the normal viral replication process can be disrupted and the effects on viral persistence and host-virus interactions over an extended period can be studied (Zhang et al., 2013b).

2.5 Pathogenesis of foot-and-mouth disease virus

The pathogenesis of FMDV has specific steps of infection, depending on the virus type and host. Generally these steps are described as “pre-viremia”, “viremia” and “post-viremia” (Arzt et al., 2010, Rodríguez-Habibe et al., 2020).

2.5.1 Pre-viraemia

Pre-viraemia represents the early phase of infection, occurring before the virus is detectable in the bloodstream. During this phase, the virus follows a specific sequence of events within the host, as described below.

- *Foot-and-mouth disease virus entry into a host*

The FMD virus infection is acquired when uninfected and infected animals are in direct contact (Paton et al., 2018). Initially, the virus infects and replicates at primary replication sites, which may vary depending on the host (Arzt et al., 2011). In cattle, the virus initially replicates in the epithelial cells of the pharyngeal tonsils, followed by replication in pneumocytes (Arzt et al., 2010). In pigs, the oropharynx/ oropharyngeal tonsils within the upper gastrointestinal tract are considered the primary site of infection for FMDV (Stenfeldt et al., 2014, Stenfeldt et al., 2016, Alexandersen et al., 2002). The virus is transmitted through air as tiny droplets of saliva spread from infected animals, although high infectivity levels have also been found in droplets of milk and faeces. (Barlow, 1972). Another form of host infection is through body fluids via nose-nose contact; this can happen when animals feed on or share a contaminated environment. The infection can also occur through injury to the skin; this was observed

after some cattle were inoculated with contaminated needles, which manifested clinical symptoms thereafter (Colenutt et al., 2020, Burrows et al., 1971). Generally, cattle and sheep are easily infected by FMDV (Colenutt et al., 2020, Rodríguez-Habibe et al., 2020) On the other hand, pigs are considered to be less vulnerable to aerosol infection (Kitching and Alexandersen, 2002). Pigs can contract FMDV through the consumption of contaminated products, close proximity to infected animals, or exposure to a contaminated environment (Pacheco et al., 2012).

- *Foot-and-mouth disease virus entry into cells*

After entering a host, FMDV must be established in the host cells. Thus, viral proteins interact with cell surface receptors to cause receptor-mediated endocytosis, which allows FMDV particles to enter the cells (Figure 2.3). The FMD virus uses αv integrins such as αvβ6 as receptors (Berryman et al., 2005); according to (O'Donnell et al., 2008), it uses at least four αv integrins for mediating infection. Once bound to integrin receptors, the viral entry into the cells is via a clathrin-mediated endocytosis pathway and forms clathrin-coated pits (Figure 2.3). These pits then develop into clathrin-coated vesicles, which enter the early endosome. In this acidic environment, the virions uncoat, releasing viral RNAs into the cell cytoplasm (McMahon and Boucrot, 2011). In some cases, the virus binds to heparan sulfate receptors; in this bound form, it enters the cell by caveolin-mediated endocytosis and is then transported to endosomes where the viral genomic RNA is released (Figure 2.3) (Berryman et al., 2012, Li et al., 2023b, O'Donnell et al., 2008, Rodríguez-Habibe et al., 2020).

- *Foot-and-mouth disease virus replication*

When the FMDV is in the endosome, the intracellular acidic endosomes trigger the capsid disassembly and the translocation of the virus RNA into the cytosol (Baxt, 1987a, Berryman et al., 2005). This leads to the disintegration of the virus into 12 pentameric units, releasing the viral RNA as illustrated in Figure 2.2 (Stenfeldt et al., 2014, Cavanagh et al., 1978). The released infectious genomic RNA (gRNA) serves as a messenger RNA (mRNA) which initiates the translation of the virus genome, following the cleavage of the genome-associated viral protein (VPg), bound to the 5’ end (Ambros and Baltimore, 1978). The Internal Ribosome Entry Site region of the FMDV initiates translation in a cap-dependent manner, resulting in a single polypeptide. The breaking down of certain eukaryotic initiation factors through

proteolysis simplifies the process (Martínez-Salas and Ryan, 2010). The long open-reading frame is translated into a polyprotein, which breaks down into smaller peptides (Ascione and Woude, 1971). This intricate process facilitates the replication and spread of FMDV within the host.

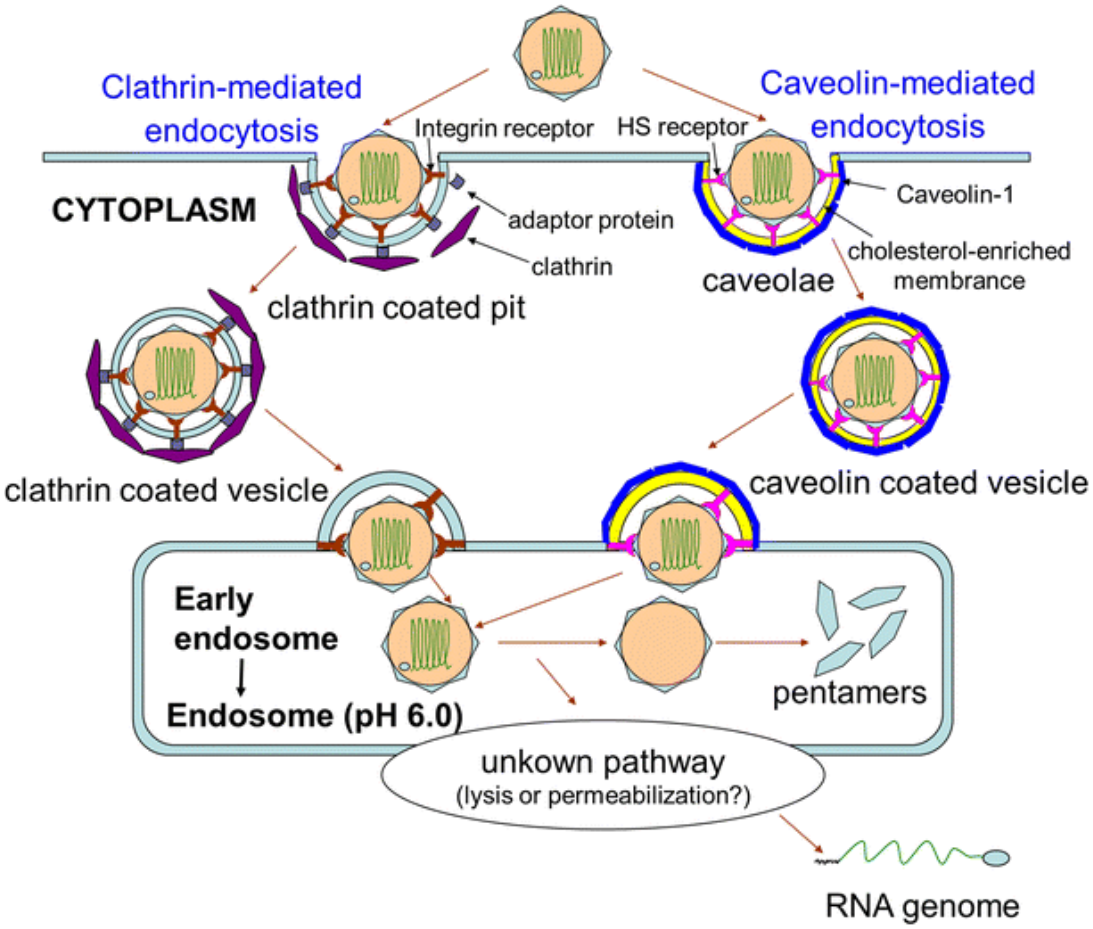


Figure 2.3: An illustrative model of foot-and-mouth virus entry pathways into host cells (Han et al., 2015).

2.5.2 Viraemia

The FMDV viraemia phase is characterized by the presence of the virus in the bloodstream. Viremia is accompanied by a significant increase in the viral load in pulmonary tissues and a reduction in the virus found nasopharyngeal tissues (Arzt et al., 2010). During viraemia, humoral immunity also initiates the clearance of the virus (Li et al., 2021).

- *Incubation period*

The incubation period for FMDV ranges from 2 to 14 days, which is the period between exposure to the virus and the onset of clinical symptoms (Grubman and Baxt, 2004, Kitching and Alexandersen, 2002, Sellers and Forman, 1973). During this phase, the virus replicates within the host's body, but the animal does not show any noticeable signs of illness (Baron et al., 1996). This makes it challenging to detect and control the disease during this stage, as infected animals can spread the virus to others without notice by the owners (Orsel et al., 2009).

2.5.3 Post-viraemia

The post-viraemia/convalescence stage involves the clearance of clinical symptoms and could potentially lead to a persistent infection. The host's immune system gets activated, particularly the humoral immune response, which then contributes to the clearance of the virus, and the affected animal develops immunity against subsequent infections with the same serotype of FMDV (Arzt et al., 2011, O'Donnell et al., 2014)

- *Foot-and-mouth disease acute infection*

Following the incubation period is the acute infection phase, during which the virus becomes active and starts shedding, causing the clinical symptoms (Baron et al., 1996). Acute infection with FMDV typically occurs within the first 2-3 days post-infection, which is when most animals start showing clinical symptoms (Sei et al., 2016). The acute phase is marked by rapid viral replication and spread between susceptible animals (Rai et al., 2021). In cattle, it has been shown that they remain immunocompetent during FMDV acute infection (Windsor et al., 2011).

- *Clinical manifestations of foot-and-mouth disease*

Some of the clinical symptoms that are notable during the acute phase include fever, vesicular lesions in the mouth and feet, lameness, excessive saliva or drooling, reduced milk production, and reduced appetite (Alexandersen et al., 2003, Donaldson and Sellers, 2000, Paton et al., 2018). Blisters can be found on the mouth, nose, oral cavity, hooves, and teats as shown in Figure 2.4 (Berry, 1901, Chakraborty et al., 2014, Thomson et al., 2003a). The rupture of these vesicles leads to profound lameness and a notable reduction in voluntary movement and feeding. This leads to weight reduction and a decline in milk yield (Ruhweza, 2014). Foot-and-mouth disease

is considered a threat to the livestock industry owing to its highly infectious nature and rapid dissemination. Consequently, the implementation of accurate biosecurity measures and strategic vaccination protocols is imperative for the control and prevention of FMD outbreaks (Pattnaik et al., 2012).

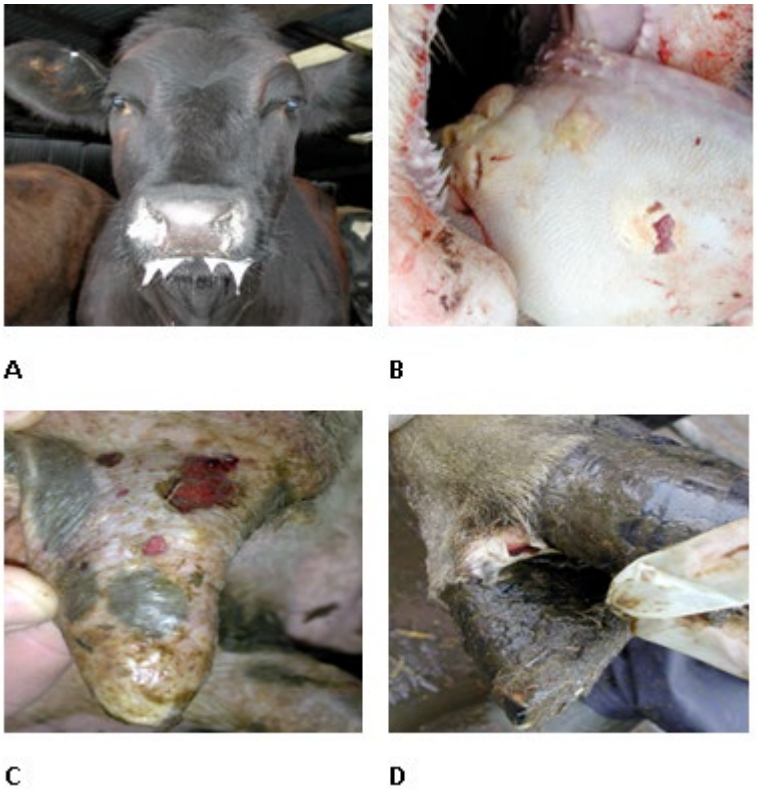


Figure 2.4: Symptoms of foot-and-mouth disease in cattle; excessive salivation (A), vesicles in the tongue (B), blisters on teats (C), and blisters on hooves (D) (Scott, 2017).

2.6 Foot-and-mouth disease virus persistent infection

Persistent infection is a prolonged asymptomatic FMDV infection among ruminant species, which occurs around day 28 post-infection (Sutmoller and Gaggero, 1965, Stenfeldt et al., 2016). One of the biggest challenges in controlling FMDV is its capability to induce persistent infections in ruminants; the virus can persist regardless of the animal's vaccination status (Eschbaumer, 2016). However, pigs do not experience persistent infection and are capable of eliminating the infection within four weeks (Kitching and Alexandersen, 2002). However, persistent infections can lead to long-term infections in some animals such as cattle and African Buffalo (*Syncerus caffer*) (Thomson et al., 2003b, Vosloo et al., 1996). Animals that continue to carry infections for an extended period may spread the virus to naïve animals. However, the

evidence of the latter under natural conditions is currently limited (Stenfeldt et al., 2016). The transmission of FMDV to disease-free animals, following direct intranasopharyngeal exposure in cattle and intraoropharyngeal exposure in pigs, highlights the potential risk posed by carriers in perpetuating outbreaks (Arzt et al., 2018).

In southern Africa, African buffalo (*Syncerus caffer*) serve as reservoirs for FMD SAT-type viruses, which are undergoing constant evolutionary adaptations during persistent infections in these animals (Vosloo et al., 1996, Bastos et al., 2001, Bastos et al., 2003, Ayebazibwe et al., 2010). Consequently, the SAT-type FMDV genome evolves due to mutations acquired by persistent infections, occurring especially in African buffalo (Cortey et al., 2019). Hence, African buffalo are not only reservoirs for SAT-type viruses for livestock, but they also contribute to antigenic variations of these viruses, which complicates disease management through immunization (Malirat et al., 1994). As they are the main reservoir of the virus, African buffalo hardly show any clinical symptoms and the persistent infection in these animals can be detected even after several years (Ayebazibwe et al., 2010, Cortey et al., 2019, Phologane et al., 2008, Vosloo et al., 1996). Although African buffalo are reservoirs of FMDV but the evidence of transmission to livestock and contribution to outbreaks is rare (Omondi et al., 2020). Moreover, the transmission from carrier buffaloes depends on factors such as direct contact and environmental conditions, making them less efficient in transmitting the virus to cattle (Jori and Etter, 2016).

Foot-and-mouth disease persistent infections continue to be a threat to South African livestock due to FMDV's high genetic variability and quasispecies nature (Wright et al., 2011, Ramirez-Carvajal et al., 2018). Foot-and-mouth disease virus exhibits genetic diversity due to its high mutation rate in its RNA genome (Domingo and Holland, 1997). This leads to the formation of quasispecies, allowing it to adapt rapidly to immune pressures, thereby causing challenges to vaccine efficacy (Sardanyés et al., 2024). Some studies found that even minor mutations during persistent infections in the viral genome could significantly affect the virus's virulence and transmissibility (Yang et al., 2020). This sometimes leads to outbreaks, further complicating the disease control strategies (Cottam et al., 2006). Persistent infections continue to be a

significant problem due to their potential for widespread outbreaks and challenges to vaccine development, hence it is important to address these issues.

2.7 Foot-and-mouth disease virus epidemiology in Southern Africa

The FMD virus epidemiology is different in Southern African countries because of the three serotypes (SAT 1, 2, 3). Each of these serotypes has different topotypes depending on the location (Ludi et al., 2014). Understanding the serotype/topotype distribution is essential for understanding the epidemiology of FMDV (Lycett et al., 2019).

The World Organisation of Animal Health (WOAH) listed South Africa as an FMD-free zone where vaccination is not practiced (WOAH, 2010). The first outbreak of FMDV since 2001 occurred in January 2019 causing the country to be removed from the list of FMDV-free zones by the WOAH (Sirdar et al., 2021, Bruckner et al., 2002). In January 2023, according to a Department of Agriculture, Land Reform and Rural Development (DALRRD) report, there were a total of 195 outbreaks in the country, 185 open outbreaks, and 10 solved outbreaks. The FMDV SAT 2 and SAT 3 serotypes were responsible for outbreaks in Limpopo, Gauteng, North West, Free State, Mpumalanga and Kwa-Zulu Natal provinces (DALRRD, 2023). In January 2024, DALRRD reported 163 open outbreaks and 52 solved. As of September 2024, there are currently 175 open outbreaks, with 23 recent outbreaks from the Eastern Cape province. Currently, Limpopo province has no open outbreaks and has remained outbreak-free since 29 August 2023. Mpumalanga, Gauteng, North West, Northern Cape, and Western Cape are currently considered FMD-free provinces. Samples from the outbreaks were submitted to the Agricultural Research Council-Transboundary Animal Diseases (ARC-TAD) Biosafety Level 3 (BSL3) laboratory and infections with FMDV SAT 3 and SAT 2 serotypes were confirmed (DALRRD, 2024). Control measures implemented during the outbreaks include restriction of animal movements, keeping all provinces with outbreaks under quarantine, buying of animals exclusively from legal sources, vaccination and controlled slaughter (DALRRD, 2024).

2.7.1 Foot-and-mouth disease virus Southern African Territories (SAT) serotypes and topotypes distribution

The FMD virus serotypes distribution is based on specific geographical locations. There are currently seven geographical pools according to the World Reference Laboratory for FMD (WRLFMD). Geographical pools offer information on the serotypes that are circulating in specific geographic regions. The map in Figure 2.5 shows the different serotypes and how they are distributed in different pools (Pinto, 2017). Relevant to this study is the distribution of the SAT serotypes.

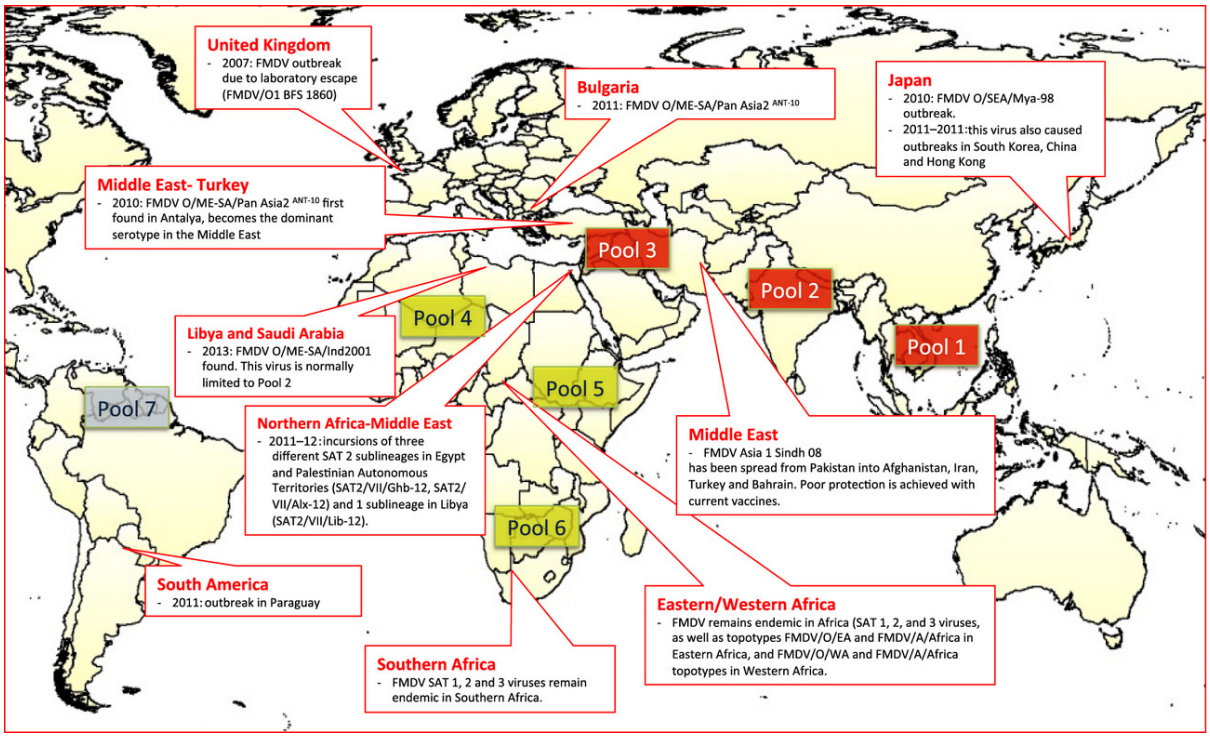


Figure 2.5: Geographic distribution of foot-and-mouth disease virus serotype-based pools. Notably, Pool 6 exhibits the occurrence of serotypes endemic in Southern Africa, including SAT 1, SAT 2, and SAT 3 (Pinto, 2017).

- *Southern African Territories 1 (SAT 1)*

Serotype SAT 1 predominantly occurs in Sub-Saharan Africa, it does not typically occur in the Middle East, Asia, South America, and Europe (Vosloo et al., 2002). SAT 1 has a total of 13 topotypes (I to XIII) identified by sequencing the FMDV VP1 region (weblink: WRLFMD). According to a study by Fana et al. (2021), SAT 1 was responsible for several outbreaks in Southern Africa, between 2014 and 2018. Together with the other two SAT serotypes, SAT 1 has caused 30 out of 33 recorded FMDV outbreaks. This serotype was specifically responsible for 6 of the 30 outbreaks

(Fana et al., 2021). According to WOAH, there are no recent outbreaks of FMDV associated with SAT 1 in South Africa.

- *Southern African Territories 2 (SAT 2)*

Serotype SAT 2 is mostly found in West Africa (Senegal and Ethiopia) and South Africa, causing the most of outbreaks in the latter (Hall et al., 2013) (Blignaut et al., 2020). This serotype exhibits greater diverse lineages than any SAT serotype, with a total number of 14 topotypes (Bastos et al., 2003). In South Africa, outbreaks associated with the SAT 2 serotype have been reported in several provinces, including recent outbreaks in Limpopo (November 2019), Mpumalanga (March 2020), Kwa-Zulu Natal (May 2021), and Free State (August 2022) provinces (DARRLD, 2023).

- *Southern African Territories 3 (SAT 3)*

Serotype SAT 3 is primarily found in sub-Saharan Africa, where it is also responsible for FMD outbreaks. It is known to occur in most Southern African Development Community countries (Vosloo et al., 2002). This serotype has fewer topotypes and caused the least number of outbreaks compared to other SAT serotypes (Fana et al., 2021). Based on real-time qPCR results, solid-phase competitive Enzyme-linked immunosorbent assay (ELISA) and non-structural Protein ELISA, SAT 3 was shown to be responsible for the recent outbreak in Limpopo province (March 2022) a previous FMDV-free zone in South Africa, as well as in North-West, Gauteng, and Mpumalanga provinces of South Africa (in March 2022, July 2022, and August 2022 respectively) (DARRLD, 2023).

2.8 Foot-and-mouth disease virus *in vitro* infection

During the 1950s, Frenkel pioneered the establishment of a large-scale *in vitro* culture for virus multiplication using natural host tissue (Frenkel and Kapsenberg, 1954). Following the implementation of Frenkel's system, subsequent developments in tissue culture methods were established, leading to numerous laboratories exploring the production of FMDV in primary cells (Mowat et al., 1969, Polatnick and Bachrach, 1964). *In vitro* cultures have been used in various applications including amplifying the virus to produce large quantities of the virus for vaccine development studies (Lalosević et al., 2008, Park et al., 2021). *In vitro* studies have also played a huge role in understanding interactions between host cells and FMDV (O'Donnell et al., 2014).

Thus, *in vitro* cell cultures can be useful in the initial stages of proving hypotheses that can later be confirmed *in vivo,* especially because *in vivo* studies can be expensive. Since the use of live animals should be limited, as an ethical consideration*, in vitro* studies are recommended for exploratory studies where possible.

2.8.1 The use of BHK-21 cells for foot-and-mouth disease virus *in vitro* infection

BHK-21 cells originated from the kidneys of five Syrian hamsters that were part of litter number 21 (Hernandez and Brown, 2010). Due to their susceptibility to many different viruses, BHK-21 cells have been proven to be useful for the growth of various viruses (Amadori et al., 1997). Accordingly, BHK-21 cell monolayers have also been found to be suitable for FMDV propagation. Several potential antiviral compounds and methods have been evaluated *in vitro* using BHK-21 cells (Younus et al., 2016). They can also be used to amplify large-scale virus for vaccine manufacturing and development (Shahiduzzaman et al., 2016). The use of BHK cells as an *in vitro* model system for the study of persistent FMDV infection has been reported and it mimics a frequent type of FMDV infection observed in nature (de la Torre et al., 1985). The use of BHK-21 cells to establish FMDV persistent infection *in vitro* can be useful in understanding some mechanisms of this poorly understood phase of infection, as reported in various studies (Huang et al., 2011b, Herrera et al., 2008). Moreover, some studies have used BHK-21 cells to investigate changes in the host cell transcriptome to understand the host cellular response to FMDV persistent infection (Han et al., 2018). Furthermore, Chang and colleagues established double-stable BHK-Tet-SARS-N cell lines that can be used in the control of SARS-CoV using BHK-21 cell lines (Chang et al., 2010). Hence, BHK-21 cells have been useful in studying host-virus interactions, understanding of viral infections, viral replication, and pathogenesis (de la Torre et al., 1985, de la Torre et al., 1988, Han et al., 2021, Rieder et al., 2005, Saravanan et al., 2011).

2.8.2 Foot-and-mouth disease virus persistent infection in *in vitro* cell culture

de la Torre et al, successfully established the first *in vitro* model of a cell line with persistent infection for FMDV serotype C (de la Torre et al., 1985). Persistent infection was accomplished by cultivating BHK-21 or IBRS-2 cells that endured cytolytic infections triggered by FMDV. These cells were infected using natural isolates that

were obtained from animals during the persistent phase. In 1988, de la Torre established that cells and the virus undergo co-evolution during FMDV persistent infection, leading to resistance to infection by FMD virus (de la Torre et al., 1988, Han et al., 2018).

The BHK-21 cell lines, which are the most frequently used *in vitro* cultures, have been extensively used to establish persistent FMDV infections and investigate the mechanisms underlying viral persistence using serotype O (de la Torre et al., 1985, Martín Hernández et al., 1994, Huang et al., 2011b). Various cell lines, such as Madin-Darby bovine kidney (MDBK), IBRS-2 (Ibaby Rat Shwann-2), Lung Fibroblast of Baby kidney (LFBK), and Porcine Kidney-15 (PK-15), have also been utilized to establish persistent infections with FMDV, and to study the mechanisms responsible for viral persistence (Kopliku et al., 2015). Recent studies using *in vitro* cell cultures have revealed how FMDV interacts with host cells during persistent infection, including the viral replication kinetics, immune responses, and factors influencing the establishment and maintenance of persistent infections (Perez-Martin et al., 2022, Yao et al., 2008).

2.8.3 *In vitro* cell reaction to foot-and-mouth disease virus persistent infection

During persistent infection in BHK-21 cells, infected cells are usually monitored for the distribution of intact virions, which are expected to be dispersed in the cytoplasm, an observation that is common during persistent infection (Huang et al., 2011a, Monaghan et al., 2004). Morphological changes such as cell rounding, detachment, and the formation of syncytia after infection, are also observed during FMDV persistent infections (Herrera et al., 2008). During persistent infection, cells that survive initial FMDV infection can re-establish infection when re-exposed to FMDV (García-Briones et al., 2006, de la Torre et al., 1988, de la Torre et al., 1985). Some studies have observed that persistently infected cells exhibit a decrease in replication rate as time progresses (Hägglund et al., 2020). Differentially expressed genes have also been identified from the comparison of acute and persistent FMDV infection, in BHK-21 cells infected with serotype O. The differentially expressed genes found are involved in specific functions such as blocking viral entry or activating cellular defence pathways, leading to reduced cell sensitivity to the virus, thus the establishment of persistent infection (Li et al., 2020).

2.9 Genomic variations in foot-and-mouth disease virus serotypes

Genomic variations between FMDV serotypes play an important role in understanding the virus' evolution, pathogenesis, and disease control. Foot-and-mouth disease virus serotypes and their variants have been shown to exhibit distinct genetic and antigenic characteristics, which may affect the efficiency of existing vaccines and pose challenges in the vaccine development that can provide broad protection (Childs et al., 2022). New viral variants often result from mutations, but may also arise from inter-serotype recombination (Carrillo et al., 2005). Multiple recombination events within FMDVs have been shown in various studies and they play an significant role in shaping the genetic landscape of FMDV, contributing to its adaptability to new environments, evading immune host responses, and establishing persistent infections (Jamal et al., 2020).

The high mutation rate of FMDV, characteristic of many RNA viruses, is caused by the lack of proofreading in FMDV's RNA polymerase (Domingo et al., 1996, Domingo and Holland, 1997, Steinhauer et al., 1992). This high mutation rate does not only affect vaccine efficacy and development, but also the development of accurate diagnostic assay since the standard assay may not detect all the circulating strains (El Bagoury et al., 2022).

Studying genomic variations require complete FMDV sequences from specific areas. Currently, such comprehensive data is limited, resulting in scarcity of regional molecular epidemiology data, affecting the development of efficient control measures (Stenfeldt et al., 2022). Therefore, increasing efforts to sequence and analyze FMDV genomes from various geographic locations is crucial for improving our understanding of viral evolution, diversity, and transmission dynamics, to help enhance global disease control strategies (Wong et al., 2020).

2.9.1 Foot-and-mouth disease virus mutations during persistent infections

Mutations in FMDV are key to the virus' survival during persistent infections, as they allow it to escape the host immune defences, while enabling the continuous replication and adaptation of the virus within the host (Stenfeldt et al., 2016, Stenfeldt and Arzt, 2020). Thus, these mutations play a crucial role in FMDV evolution, adaptation, and pathogenicity.

Several specific mutations have been identified that facilitate FMDV adaptation to host cells, particularly during persistently infection. For instance, the 83K mutation in the VP1 protein of serotypes O and SAT 2 has been associated with increased cellular adaptability (Ekanayaka et al., 2020). Mutations like these, particularly in the VP1 and VP3 proteins, have been observed in vaccine strains and play a crucial role in virus persistence within host cells (Hwang et al., 2021). Mutations in the VP1 and VP3 regions, identified in persistently infected cells, appear to affect the virus' ability to infect host cells. They impact the virus' interactions with host factors influencing replication and pathogenicity, illustrating the dynamic coevolution between host and virus (Zhang et al., 2013a). In addition to adaptation and interaction changes, mutations during persistent infections can affect FMDV's stability. Mutations in regions VP1 and VP2 in serotype O, such as N17D and D86H substitutions, have been reported to enhance acid stability, contributing to the virus' resistance to disassembly (Caridi et al., 2020, Yuan et al., 2020). Finally, persistent infection mutations can enhance immune evasion. For instance, amino acid substitutions in structural proteins, such as E82K, H88N, E131K, and V155A, alter the virus' surface electrostatic properties. These alterations reduce antibody recognition, allowing FMDV to evade immune responses more effectively during the persistent phase (Fish et al., 2020).

In conclusion, FMDV's ability to persist within the host is intricately linked to its capacity to accumulate mutations that enhance its survival and pathogenicity. These mutations not only contribute to antigenic variation and immune evasion but also influence the virus' interaction with host factors, ultimately playing a crucial role in the virus' persistence and evolution.

CHAPTER 3

3 METHODOLOGY

3.1 Ethical considerations

This study was conducted at the Agricultural Research Council-Transboundary Animal Diseases (ARC-TAD) BSL-3 laboratory and RNA samples were sequenced at Agricultural Research Council (ARC) Biotechnology Platform. Applicable ethics approvals were obtained before the study commenced including the ARC animal ethics (AEC 22.17), University of Pretoria (UP) research ethics (REC168-22) and the Section 20 of Animal Disease Act (35 of 1984) from the Department of Agriculture (former Department of Agriculture, Land Reform and Rural Development (DALRRD); reference 12/11/1/1(a)/3009(HP)). All approval letters are attached as part of appendices.

3.2 Study design

The summary of the study workflow is shown in Figure 3.1. Briefly, the SAT 2 FMD virus was propagated in BHK-21 cells and the viral titre calculated using a $TCID_{50}$ assay. Prior to establishing persistent infection with ammonium chloride (NH_4Cl), its cytotoxicity on BHK-21 cells was determined. Subsequently, acute and persistent infections of FMD SAT 2 virus were established in the BHK-21 cell line. RNA was extracted from FMDV-infected cell cultures for virus viability evaluation by qPCR, and for whole genome sequencing followed by sequence data analysis.

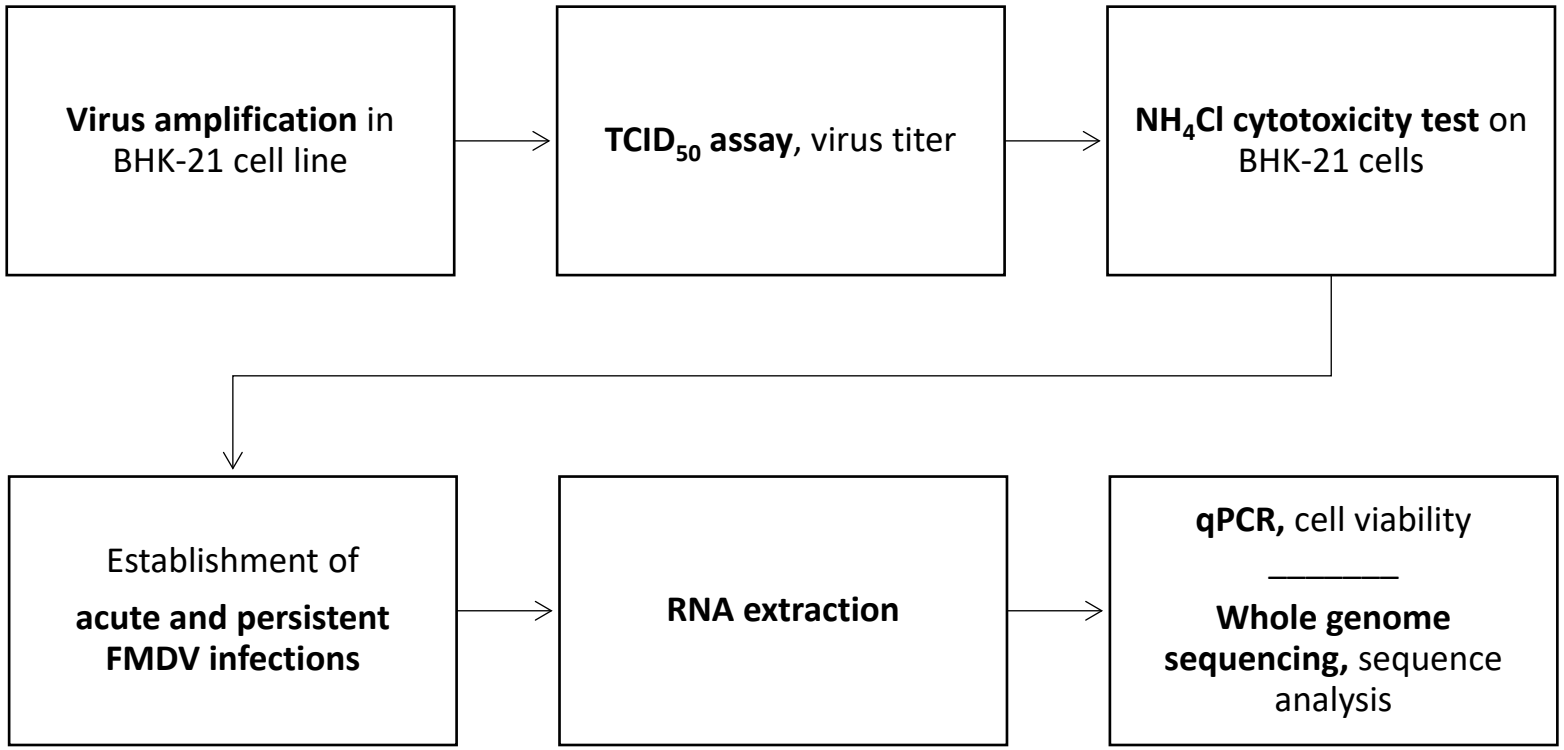


Figure 3.1: The schematic summary of the study workflow.

3.3 Cells and viruses

Baby Hamster Kidney (BHK-21) clone 13 MH7450 cell line was provided by the ARC-TAD Tissue culture laboratory. Cell cultures were maintained in complete Glasgow's Minimum Essential Medium (GMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco™, Thermo Fisher, United States of America (USA)), 10% Tryptose Phosphate Broth (TPB), 3% Lactalbumin hydrolysate (LAH) and 1% Penicillin-streptomycin (Gibco™, Thermo Fisher, USA). The FMDV serotype SAT 2 KNP 1/10/2 MVS+2 20/06/2013, was obtained from the ARC-TAD biobank. SAT 2 virus was isolated in 2002 from buffalo in Kruger National Park, South Africa and is preserved at the ARC-TAD's research biobank. Prior to the experiments, the virus was passaged on a 80-90% confluent monolayer of BHK-21 cells and harvested when complete cytopathic effect (CPE) was reached (typically after 24 hours (h)).

3.4 Virus passage

Corning® 25 cm^2 tissue culture flasks (Merck, South Africa (SA)) (T25 flasks) containing a 90% confluent monolayer of BHK-21 cells were inoculated with SAT 2 virus KNP 1/10/2 MVS+2 20/06/2013 at a multiplicity of infection (MOI) of 0.223 and incubated for 1 h. Virus growth medium (VGM), prepared by supplementing Minimum Essential Medium (MEM) with 5% FBS, 5% TPB, 1.5% LAH and 1% Penicillin-Streptomycin, was added to the virus/cells mixture. The mixture was then incubated for 24 h until the cells reached complete CPE, observed as detachment of cells and formation of syncytia. Subsequently, the tissue culture flasks were centrifuged to separate the cells from the virus supernatant. The virus supernatant was transferred into 5 mL cryovial tubes and stored at -80 °C until used in an experiment.

All experiments were carried out in a biosafety cabinet. Before and after use, the surface of the cabinet was decontaminated by applying 2% citric acid solution, which was allowed to work for 20 minutes (min), then subsequently cleaned with 2% F10 and 70% ethanol.

3.5 Virus titrations

The SAT 2 virus stock was subjected to titration to determine the tissue culture infectious dose at 50% ($TCID_{50}$). To achieve this, 50 µL of VGM was added to 88

experimental wells in a 96-well plate, while 100 µL was added to the remaining eight wells as a control. Subsequently, 50 µL of 0,3x10^6 BHK-21 cells was added to each experimental well and 100 µL onto control wells. To create a working stock of SAT 2 FMDV, 500 µL virus was diluted with 4.5 mL VGM in 8 mL Nalgene™ Natural PPCO diagnostic bottles (Thermo Fisher Scientific™, SA) and mixed well. The diluted virus of 2x10^{-2} PFU/mL concentration was used as a working stock. Seven 8 mL diagnostic bottles were filled with 4.5 mL VGM and 500 µL of the diluted virus stock was transferred to the first bottle to start a series of dilutions to create full logs. Additionally, five bottles of half logs were prepared by transferring 1 mL of the diluted stock from full logs to diagnostic bottles containing 2.3 mL VGM. Then 50 µL of the serially diluted virus was added to experimental wells in 96-well plates. This experiment was done in using two 96-well plates to create two replicates for each test. Plates were incubated at 37 °C with 5% CO_2.

To monitor cytopathic effect (CPE) on the infected cells, 50 µL methylene blue was added to the plates at 72 hours post-inoculation (hpi) and incubated on the bench for 1 h and washed in running water. Clear wells were considered CPE positive, while wells that retained the blue stain indicated the absence of CPE. The $TCID_{50}$ of the virus stock used was determined at 1x10^{-5} PFU/mL.

3.6 Cytotoxicity of ammonium chloride on BHK-21 cells

A total of 1x10^6 cells/mL BHK-21 cells were seeded in T25 flasks. The cells were maintained in complete GMEM and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, allowing the cultures to reach 90% confluency. After 24 h, the culture medium was replaced with GMEM supplemented with different concentrations of NH_4Cl (10 mM, 15 mM and 20 mM). The treated cells were incubated under the same conditions as above. At 6 and 24 h post-treatment, the cells were observed under an Olympus CK2 inverted microscope (Thermo Fisher scientific, USA) to detect any signs of cytotoxicity changes such as cell rounding, detachment, lysis and cell death. Images were captured at each time point for comparative analysis.

The BHK-21 cell cultures without NH_4Cl treatment were maintained as an untreated or a negative control for this experiment.

3.7 Establishment of acute infection

A total of 1x10^6 cells/mL BHK-21 cells were seeded in T25 flasks, and maintained in complete GMEM supplemented with 10% FBS, 10% TPB, 3% LAH, and 1% Penicillin-Streptomycin, in a 37 °C incubator with 5% CO_2 for 48 h. When cells reached 90% confluency, they were washed with 1x PBS to prevent cell rapture/lysis. The 90% confluent BHK-21 cells were then inoculated with SAT 2 virus at a MOI of 0.001 in T25 flasks. Cells were incubated at 37 °C for 1 h in a shaker to allow for virus adsorption, subsequently, 5 mL VGM was added. Flasks were then incubated at 37 °C for 6, 24 and 48 hpi. At each time point, the medium was replaced to ensure sufficient nutrients for the cells, and the incubation was extended for another 6, 24, and 48 hpi, respectively. A 2-mL sample of cells was harvested at each time point for further analysis. BHK-21 cell cultures without FMDV infection was used as a negative control. Cells were also monitored under a microscope to observe the morphological changes caused by the FMD virus infection.

3.8 Establishment of persistent infection

Ammonium chloride (NH_4Cl) was used to mimic persistent infection. BHK-21 cells (1x10^6 cells/mL) were seeded in T25 flasks and incubated to 90% confluency as described in Section 3.7. Afterward, BHK-21 cells were inoculated with the SAT 2 virus at MOI of 0.001. Cells were incubated at 37 °C for 1 h in a shaker to allow virus adsorption. Subsequently, VGM supplemented with NH_4Cl, at either 10 mM, 15 mM, or 20 mM concentration, was added to different tissue culture flasks containing infected cells. The tissue culture flasks were incubated at 37 °C for 6, 24, and 48 hpi, and 2 mL of cells was harvested at each time point. The medium was changed and replenished with VGM without NH_4Cl for the 24- and 48- h cultures, as described in Section 3.7 above. Cells were also monitored under a microscope to observe the morphological changes caused by the FMD virus infection and NH_4Cl treatment. Ammonium chloride treatments were performed in two independent replicates. An uninfected-untreated BHK-21 cell culture was used as a negative control.

3.9 Cell viability in FMDV SAT 2 BHK-infected cells treated with or without ammonium chloride

Cell viability was determined to establish the cytolytic effect of the SAT 2 FMD virus on NH_4Cl- treated and untreated BHK-21 cells. Thus, 100 µL of either uninfected BHK-

21 cells (negative control), virus-infected BHK-21 cells (from acute infection), or virus-infected/NH_4Cl-treated BHK-21 cells (from persistent infection), harvested at 6, 24, and 48 hpi, was added to 100 µL Trypan Blue. A Luna-II™ automated cell counter (Logos Biosystems) was used to determine viability.

3.10 RNA extraction and quantification

Total RNA was extracted from acutely and persistently infected cells harvested at 6, 24 and 48 hpi, using TRIzol reagent (Invitrogen™, Thermo Fisher), following the manufacturer's protocol. Briefly, 500 µL of SAT 2 virus-infected BHK-21 cells (acute and persistent) was added to 500 µL TRIzol reagent and vortexed for 1 min, then incubated for 5 min at room temperature. Subsequently, 200 µL of chloroform was added and vortexed before incubation for 15 min at room temperature. The mixture was subjected to centrifugation at 12 000 rpm for 15 min and the clear supernatant with the RNA was transferred to a new tube. Isopropanol (500 µL) was added to the supernatant, which was then incubated at room temperature for 10 min and centrifuged for 10 min at 12 000 rpm. The nucleic acids were precipitated by adding 1 mL of 75% absolute ethanol to the supernatant and mixed vigorously by vortexing. The solution was then centrifuged for 5 min at 12 000 rpm and the supernatant discarded. The pellet was allowed to air dry prior to resuspending the RNA with 50 µL diethylpyrocarbonate (DEPC)-treated water and incubation at 60 °C for 15 min, while mixing by pipetting every 5 minutes to facilitate dissociation. The DNA-free™ Ambion™ kit (Thermo Fisher) was used to remove any potential DNA contamination on the RNA samples following the manufacture's protocol.

A NanoDrop 1000 spectrophotometer (Thermo Fisher) was used to determine the integrity of the RNA. Samples with an absorbance ratio value of 1.7-2.0 at 260/280 nm were used. RNA was then inactivated using heat at 60 ºC for 20-30 min and samples were stored in an air-lock container which was decontaminated before being moved out of the BSL-3 lab following standard protocols.

3.11 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) targeting the FMDV 3D region was performed to determine the effect of NH_4Cl on FMDV yield. The RNA extracted from acutely- and persistently-infected cells, harvested at 6, 24 and 48 hpi was used as a template. The

GoTaq® Probe One-Step RT-qPCR system (Promega, USA) was used to prepare the amplification reactions following the manufacturer's instructions, with minor modifications. Each 20 µL reaction contained 10 µL of GoTaq® Probe Master Mix (1X final concentration), 0.4 µL of GoScript™ RT Mix (1X final concentration), 1 µL of forward primer (5'-ACTGGGTTTACAAACCTGTGA-3'), 1 µL of the reverse primer (3'-GCGAGTCCTGCCACGGA-5'), and 1 µL of probe (5'-TCCTTTGCACGCCGTGGGAC-3'), with each primer and probe at 0.1 µM final concentration. The probe was labelled with 6-carboxyfluorescein (FAM) at the 5' end and tetramethylrhodamine (TRITC) at the 3' end (Inqaba Biotec™, SA). Additionally, 2.5 µL (ranging from 300 ng to 3000 ng, depending on the sample) of RNA template was added, and the total reaction volume was adjusted to 20 µL with nuclease-free water. A CFX96 Touch Real-time PCR Detection system (Bio-rad, USA) was employed to detect viral RNA. One-Step reverse transcription PCR was performed at 45 °C for 15 min, and the amplification of the target fragment was done under the following these parameters, 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for the annealing step. Samples that had a quantitative cycle (Cq) value ≤35 were considered positive for viral RNA.

3.12 Library preparation for whole genome sequencing

Twelve RNA samples were submitted to the ARC Biotechnology platform for library preparation and whole genome sequencing (WGS), including persistent infections (BHK-SAT 2 cells treated with 10 mM, 15 mM and 20 mM NH_4Cl) and acute infections (BHK-SAT 2 cells without NH_4Cl) across all time points. Briefly, the library was prepared using the MGIEasy rRNA depletion kit (MGI Tech Co., Ltd, USA), according to the manufacturer's instructions to remove rRNA from the samples. This step was followed by adding fragmentation buffer to the rRNA-depleted RNA sample and incubation at 94 ºC for 8 min to generate fragments with a target size of approximately 150 bp. After fragmentation, cDNA was synthesized. The cDNA was subjected to a series of processes and purification such as end repair, A-tailing adapter ligation, and subsequently PCR amplification following the manufacturer's protocol. The library was then purified and quantified using a Qubit dsDNA HS Assay Kit (Invitrogen™, Cat. No. Q32852), and the fragment size was evaluated using an Agilent 2100 Bioanalyzer. Finally, the library was subjected to sequencing on an MGISEQ-200 sequencer.

3.13 Bioinformatics analysis

The quality of the respective FastQ files containing reads from each sample was checked using FastQC (available at: FastQC). Following quality assessment, paired-end reads were trimmed using Trimmomatic version 0.39 (Bolger et al., 2014) to remove low-quality bases and adapter sequences, thereby enhancing the accuracy of subsequent analyses. The trimming parameters were configured as recommended in the Trimmomatic GitHub repository.

De novo assembly was prepared using SPAdes version 4.0 to construct a reference genome as described by Prjibelski and colleagues (Prjibelski et al., 2020). Briefly, the reads from acute infection cultures at 6 hpi were combined to construct a consensus sequence by aligning overlapping regions. The process involved multiple stages of filtering and refinement to achieve an optimal and accurate reference genome. The contigs were obtained and mapped against a known FMDV SAT 2 reference genome sequence (accession number: AY593848) using NCBI BLAST, to determine their similarity.

Trimmed reads were aligned to the constructed reference genome using Burrow-Wheeler Aligner-Maximal Exact Match (BWA-MEM) tools (BWA version 0.7.17) (Li and Durbin, 2009). Samtools version 1.13 was used to convert sequence alignment/map (SAM) files to binary alignment/map (BAM) files. Variant calling was performed using BCF tools mpileup (Danecek et al., 2021). The variants were normalized using bcftools and annotated using SnpEff tool (Cingolani et al., 2012). The evolutionary relationships among the samples were determined using the Maximum Likelihood method. Prior to the construction of the phylogenetic tree, the model test analysis was performed on MEGA 11 (Tamura et al., 2021), to determine the suitable model for the genome sequence data under investigation. A Maximum likelihood tree was also constructed on MEGA 11.

CHAPTER 4

4 RESULTS

4.1 Cytotoxic effects of ammonium chloride on BHK-21 cells

The cells from the negative control (NH_4Cl-untreated BHK-21 cells) showed a healthy intact monolayer with no changes in morphology at both 6 and 24 h (Figure 4.1a). Meanwhile, cells treated with 10 mM, 15 mM, and 20 mM NH_4Cl showed some cytotoxic effects such as cell rounding and early signs of detachment, especially with 15 mM and 20 mM. Moreover, at 24 h post-treatment, the cultures exhibited significant morphological changes, with massive cell detachment observed across all concentrations. This detachment increased with NH_4Cl concentration, and the 20 mM-treated cells showed the most pronounced detachment and dissociation. A time-dependent effect of NH_4Cl was observed since there were no changes in cells at 6 h; whereas at 24 h, signs of cytotoxic effect such as detachment, dissociation, and cell death were more prominent.

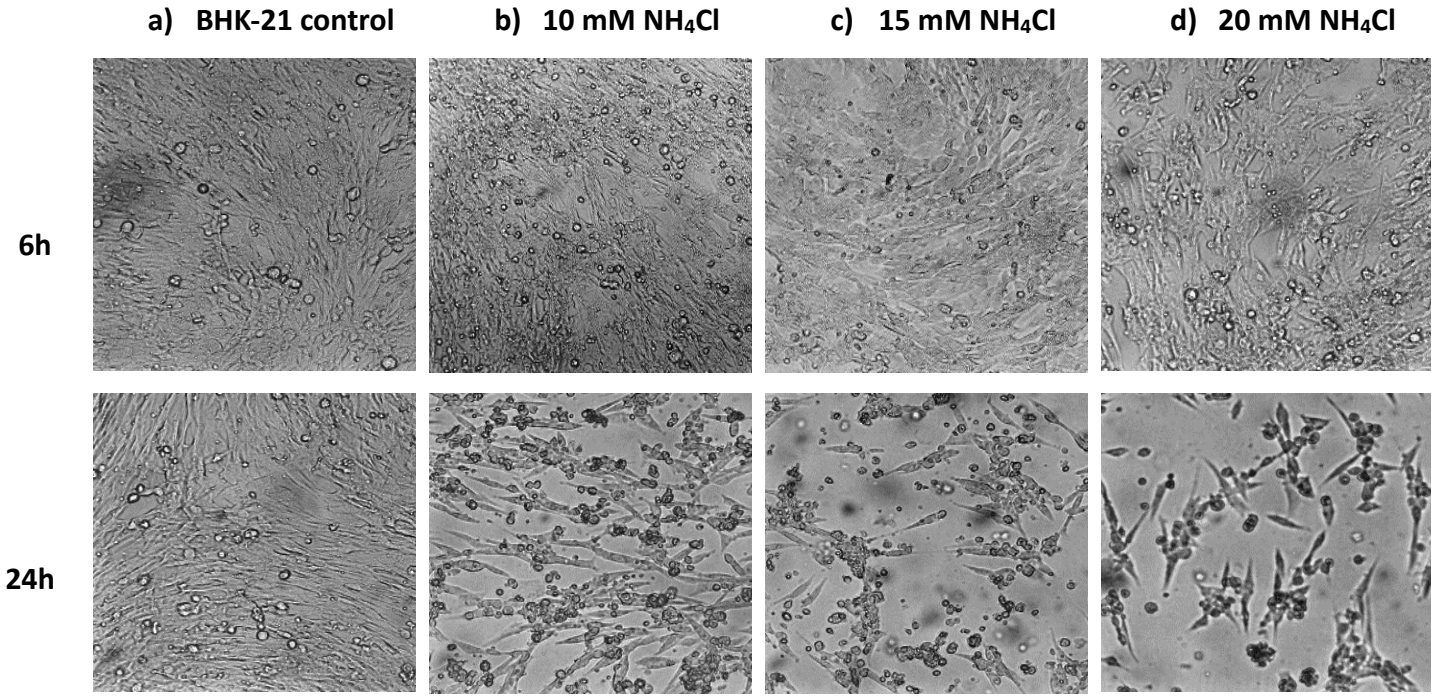


Figure 4.1: The cytotoxic effect of NH_4Cl on BHK-21 cells treated with various concentrations of NH_4Cl at 6h and 24 h, in comparison to untreated cells. a) BHK-21 untreated cells (negative control). b) BHK-21 cells treated with 10 mM NH_4Cl. c) BHK-21 cells treated with 15 mM NH_4Cl. d) BHK-21 cells treated with 20 mM NH_4Cl. The cells were observed under an Olympus CK2 Inverted Phase Contrast inverted microscope (Thermo Fisher Scientific, USA) and the ZEISS AxioCam ERc5s microscope camera (ZEISS, Germany) was used to capture the image

4.2 The effect of ammonium chloride on BHK-21 SAT 2 virus-infected cell cultures

To determine the establishment of persistent infection in BHK-21 cells inoculated with the SAT 2 virus and exposed to various concentrations of NH_4Cl, morphological changes, resulting from the cytopathic effect (CPE) due to FMDV infection, were monitored and compared between the control, acute infection and persistent infection cultures, at 6, 24 and 48 hpi.

The control cells, i.e., BHK-21 cells neither inoculated with SAT 2 virus nor treated with NH_4Cl, showed some signs of increased cell density and overgrowth over time, with most cells remaining intact while there were some signs of detachment (Figure 4.2 a). In comparison to the control, notable morphological changes were observed in acutely infected cell cultures, with the CPE becoming more prominent with increasing time (Figure 4.2 b). Signs of cell detachment and formation of some syncytia were noticed from 6 hpi. On the contrary, signs of CPE due to viral infection were delayed in cells treated with NH_4Cl (persistent infection) (Figure 4.2 c, d, e) compared to untreated SAT-2-infected cells (acute infection) (Figure 4.2 b). Acutely infected cells also showed more severe CPE than persistently infected cells. However, at 48 hpi, there were no noticeable differences between acute and persistent infections, especially in 15 mM and 20 mM NH_4Cl-treated cells. All the cells showed rounding, detachment, and formation of syncytia, an indication of complete CPE.

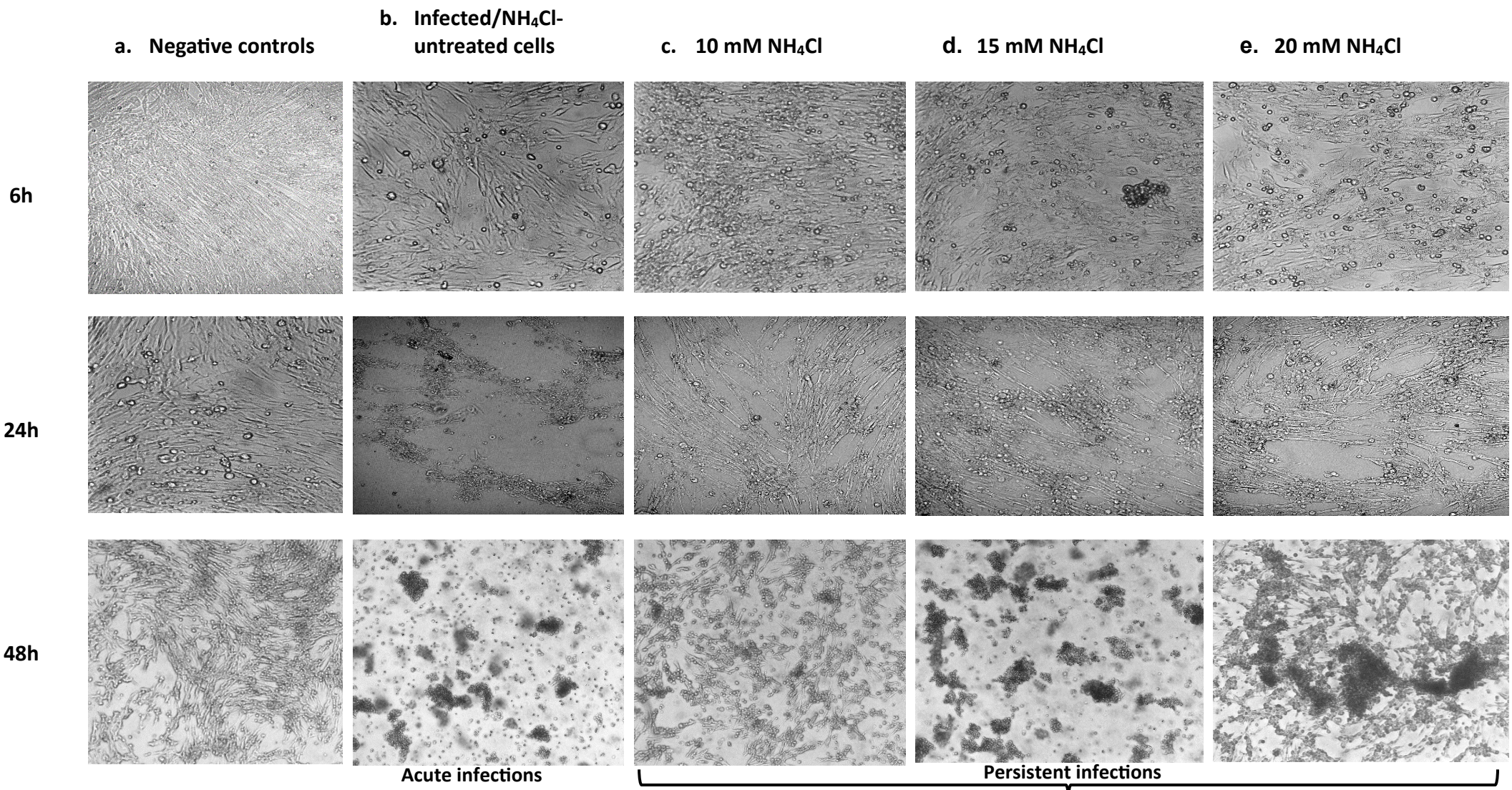


Figure 4.2: The cytopathic effect observed between acute and persistent infected BHK-21 cells with SAT 2 virus at 6, 24 and 48 hpi. a) BHK-21 control cells (neither infected with SAT 2 virus nor treated with NH_4Cl). b) BHK-21 cells infected with SAT 2 virus (acute infection), BHK-21 cells infected with FMDV SAT 2 and treated with either 10, 15 or 20 mM NH_4Cl respectively (persistent infection). The cells were observed under an Olympus CK2 Inverted Phase Contrast inverted microscope (Thermo Fisher Scientific, USA) and the ZEISS AxioCam ERc5s microscope camera (ZEISS, Germany) was used to capture the images.

4.3 Cell viability of BHK-21 SAT 2 infected cells in the presence of various concentrations of NH_4Cl

BHK-21 control cells and those acutely and persistently infected with FMDV SAT 2 were harvested and counted to determine and compare cell viability at different time points, i.e. 6, 24, and 48 hpi. Overall, the highest average % cell viability was observed at 6 hpi, on all the infected cultures (Figure 4.3). Notably, the BHK-21 control (uninfected and NH_4Cl-untreated), and 15 mM NH_4Cl-treated cell cultures had the highest cell viability at 6 hpi, up to ~ 65%. The cell viability for all the cultures decreased with the increase in time. However, in acutely infected cultures, the cell viability was maintained at the same levels between 6 and 24 hpi, followed by a major decrease at 48 hpi. On the contrary, the cell viability of the 10 and 20 mM NH_4Cl-treated cell cultures showed a drastic decrease as early as 24 hpi, as low as 28 and 35% respectively. The lowest cell viability was detected in acute infection and 10 mM NH_4Cl-treated persistent infection culture, at 48 hpi. Overall, the BHK-21 control, and the 15 mM persistently infected cultures had the highest % cell viability at all three time points.

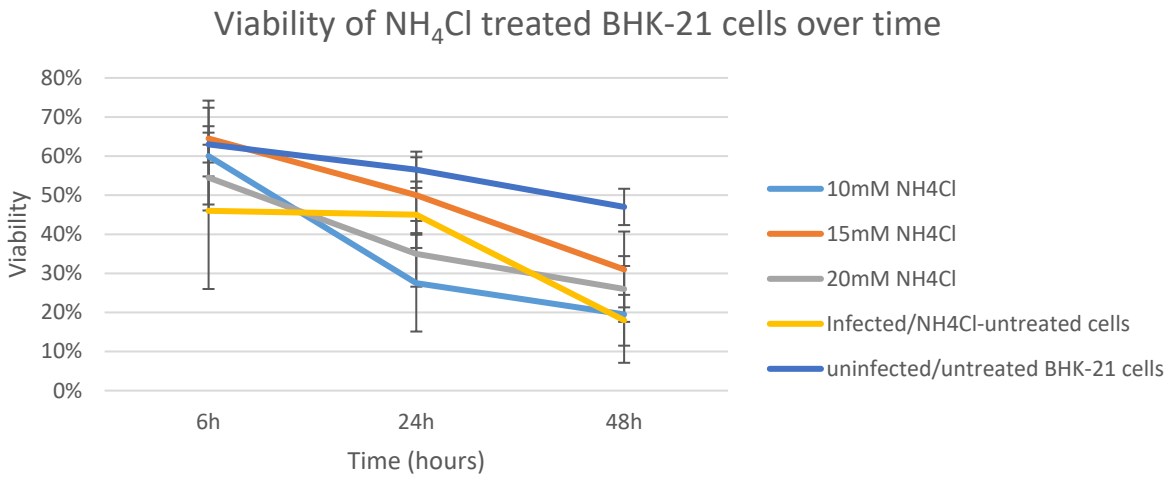


Figure 4.3: Average percentage cell viability on FMDV SAT2-infected BHK-21 cell cultures treated with various concentrations of NH_4Cl (persistent infections), in comparison with untreated cells (acute infections), measured at 6 h, 24 h, and 48 h post-infection.

4.4 Detecting viral genome replication on BHK-SAT 2 Cells treated with or without NH_4Cl

Quantitative real-time PCR (qPCR) targeting the FMDV 3D region was performed on acutely and persistently infected cell cultures at 6, 24 and 48 hpi. The 3D region was specifically targeted because it is highly conserved across FMDV serotypes, encoding the RNA-dependent RNA polymerase (RdRp), which plays a critical role in viral replication (Sarry et al., 2023, Du et al., 2011). The qPCR analysis resulted in positive quantification cycle (Cq) values ranging from 15-38. Samples with Cq values of ≤35 were considered positive for the presence of FMDV RNA and were used for analysis, whereas all samples with Cq values above 35 were eliminated from the analysis. Samples with a threshold value of above 35 were considered unreliable because they had a higher likelihood of nonspecific amplification or background noise, reducing the confidence in the accuracy of their detection.

The results shown in Figure 4.4 highlight differences in the detection of viral RNA in cells treated with NH_4Cl (persistent) and those without NH_4Cl treatment (acute). During the persistent infection, Cq values were higher (29-35) compared to acute infection at 6 h and 24 h time points, indicating lower amounts of the target RNA, especially at 20 mM NH_4Cl treatment. The results also showed a time- and dose-dependent effect of NH_4Cl on BHK-SAT 2 cells. Notably, the Cq values in the NH_4Cl-treated group were observed at 10 mM with Cq values ranging from 19 to 27 as time increased, followed by 15 mM with higher Cq values between 24 and 34, and the highest Cq values were recorded for 20 mM cultures (32-35). Notable increases in Cq values from persistent infection cultures were mainly observed between 6 and 24 h. During the acute phase (infected/untreated cells), the results showed consistently lower Cq values across all time points, in all samples within the group (15-25), indicating high viral RNA quantities. These observations also showed a time-dependent relationship with viral RNA replication (Figure 4.4).

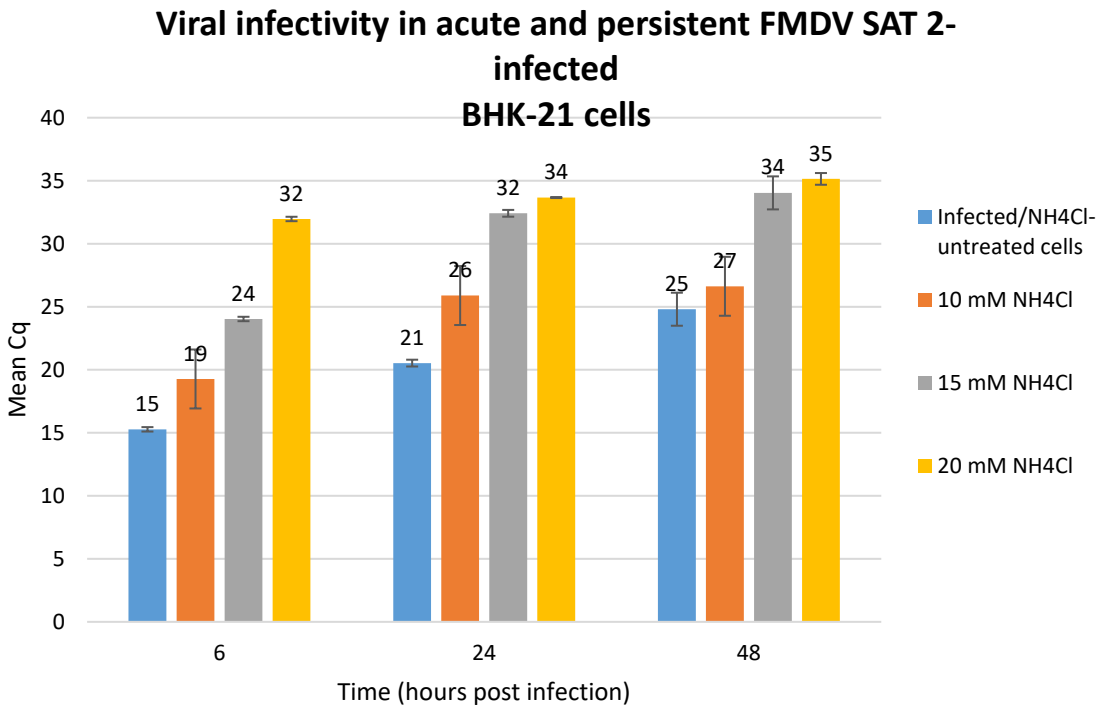


Figure 4.4*:* Cq values of obtained from the qPCR analysis of RNA samples from SAT 2 virus-infected BHK-21 cells treated with 10 mM, 15 mM and 20 mM NH_4Cl at different time points (6 h, 24 h and 48 h) to emulate persistent infection, and the 0mM negative control (virus-infected/NH_4Cl-untreated cells representing acute infection).

Change in Cq (ΔCq) was determined to understand the effect of NH_4Cl on FMDV replication. ΔCq was calculated by subtracting mean Cq values of all the acute infection (untreated cells) samples from the Cq value of the persistent infection samples (NH_4Cl treated cells).

$$\Delta Cq = Cq('persistent\ infection') - Cq('acute\ infection')$$

The ΔCq values were determined and compared to assess differences in the viral RNA levels under the experimental conditions. The ΔCq values revealed distinct patterns in RNA levels across different treatment groups at different time-points, Figure 4.5. The 10 mM persistent cultures had a slight fold increase (2 times) in ΔCq at 6 hpi, and the highest amounts of target RNA of the three treatment cultures at all three-time points (Figure 4.5). Meanwhile, the 20 mM persistent cultures had the highest fold increase in ΔCq values

and lowest reduction in RNA levels at 6 hpi, 4 times, and a similar increase in ΔCq values with the 15 mM cultures at 24 and 48 hpi, 4 and 3 times respectively.

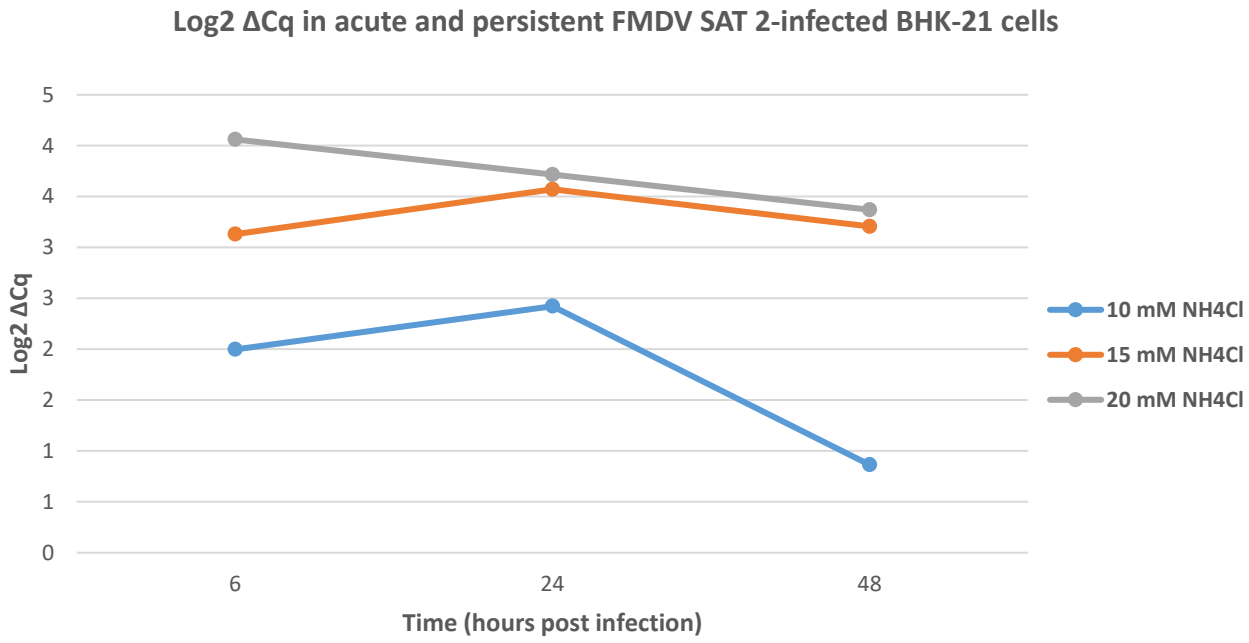


Figure 4.5: A $\log_2$ graph of ΔCq values obtained from BHK-21 cell cultures infected with FMDV SAT 2 serotype and NH_4Cl-treated to emulate persistent infection, showing the fold change differences in FMDV target RNA levels between the different NH_4Cl treatment concentrations.

4.5 Sequencing and quality control

The RNA samples from acute and persistent infection (represented by 15 mM NH4Cl-treatment) cell cultures were used for genome sequencing. The quality control results were combined and are displayed in Table 4.1 below. All samples had a library of 150 base pair fragments, the GC content was also consistent across all sequences, ranging from 57-61%. The total counts were high across all samples, indicating good coverage and there were no sequences flagged for poor quality.

Table 4.1: FastQC report for the quality analysis of fastq sequences from SAT 2 FMDV acute and persistent infection cultures, across three-time points.

Sample names	Sequence length	%GC	Total sequences	Sequences flagged as poor quality
Persistent 6 h	150	59	23413517	0
Acute 6 h	150	61	30286694	0
Persistent 24 h	150	59	27783144	0
Acute 24 h	150	60	28362210	0
Persistent 48 h	150	57	18785231	0
Acute 48 h	150	58	21040192	0

4.5.1 Genomic variations on SAT 2 FMD virus during persistent and Acute infection in BHK-21 cells

Variant calling was performed. The number of mutations detected were recorded for each viral genome region by displaying all the columns using the awk command and are displayed in Table 4.2. Out of the 12 samples sequence, the data from 10 mM and 20 mM NH_4Cl-treated cell culture were eliminated due to lower coverage at certain time points total (less than 10 million total sequences and GC content around 50-55%), leading to incomplete sequences, which are not suitable for the analysis. The acute cell culture sequence data at the 6 h time point served as a reference genome for variant calling and served as a baseline for comparison against persistent infection sequences, so it wasn't included on Table 4.2. The types of mutations detected from both the acute and persistent infection culture genome sequences included single nucleotide polymorphisms (SNPs), deletions, and insertions.

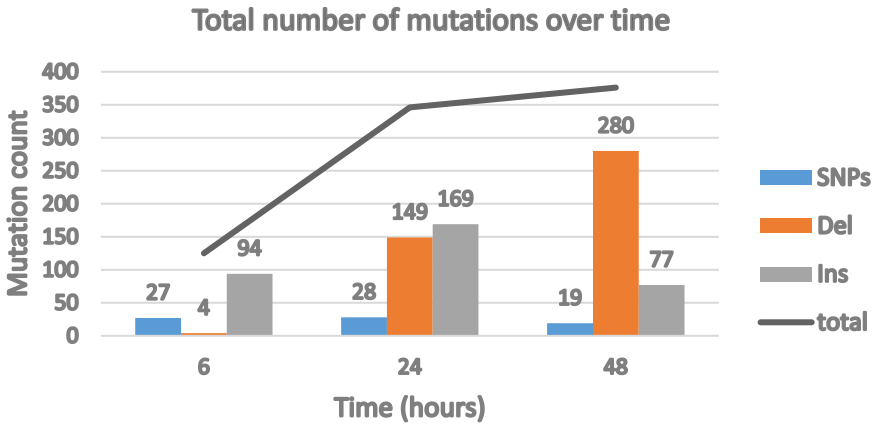
Table 4.2: The distribution of single nucleotide polymorphisms (SNPs), deletions (Del), and insertions (Ins) across different genomic regions of the foot-and-mouth disease virus (FMDV) in BHK-21 cells acutely and persistently infected with SAT 2 FMDV, at three different time points.

Region	Sequences	SNPs	Del	Ins
5' UTR (1-1100 bp)	Persistent phase 6h	6	0	21
	Persistent phase 24 h	2	1	25
	Persistent phase 48 h	0	22	13
	Acute phase 24 h	0	10	25
	Acute phase 48 h	0	38	7
L-protease (1101-1350 bp)	Persistent phase 6h	1	0	11
	Persistent phase 24 h	0	0	11
	Persistent phase 48 h	0	4	4
	Acute phase 24 h	0	4	9
	Acute phase 48 h	0	7	1
Structural proteins (1351-3750 bp)	Persistent phase 6h	4	1	27
	Persistent phase 24 h	10	8	21
	Persistent phase 48 h	2	26	9
	Acute phase 24 h	1	40	22
	Acute phase 48 h	5	55	14
Non-structural proteins & 3' UTR (3751-8300 bp)	Persistent phase 6h	16	3	35
	Persistent phase 24 h	12	18	30
	Persistent phase 48 h	6	60	6
	Acute phase 24 h	3	68	26
	Acute phase 48 h	6	68	23

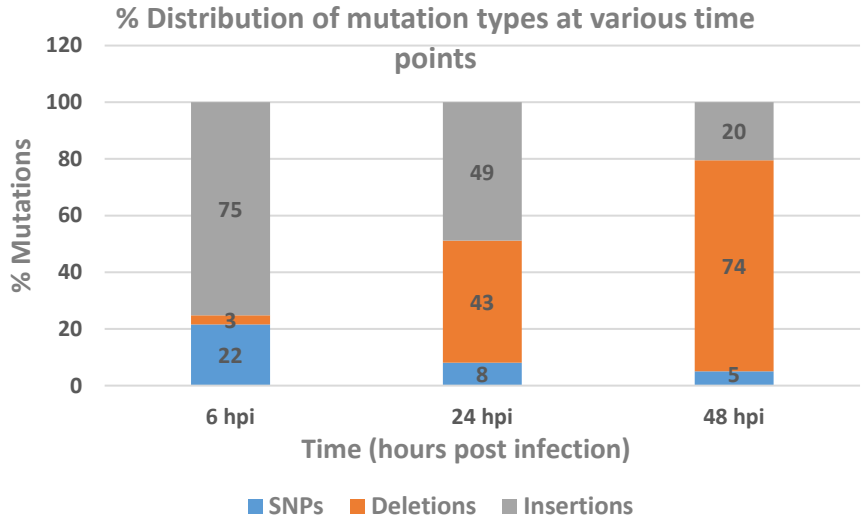
The increase in the total number of mutations was generally time-dependent (Figure 4.6). However, the number of specific types of mutations over time varied. The number of ‘deletions’ increased with time, while ‘insertions’ fluctuated, and ‘SNPs’ were generally similar at different time points. Notably, the majority of mutations at 6 hpi were ‘insertions’ (75%) and ‘deletions’ (74%) were the most occurring at 48 hpi (Figure 4.6: b). These two

mutation types were represented at similar proportions (49 and 43% respectively) at 24 hpi.

The comparison of the overall distribution of the different types of mutations between sequences from acute and persistent infection cultures showed a high proportion of 'deletions' (67%) and 'insertions' (51%), respectively (Figure 4.7; Figure 4.6:). The SNPs were the least represented, at 4% and 14% in acute and persistent infections, respectively.

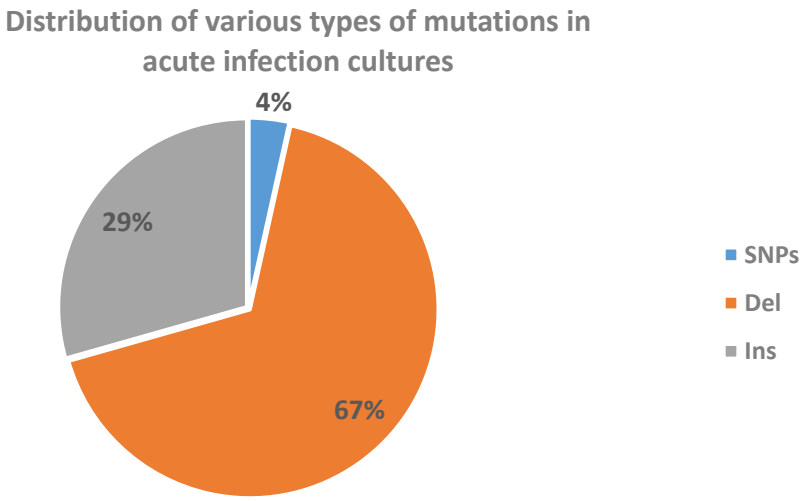


a.

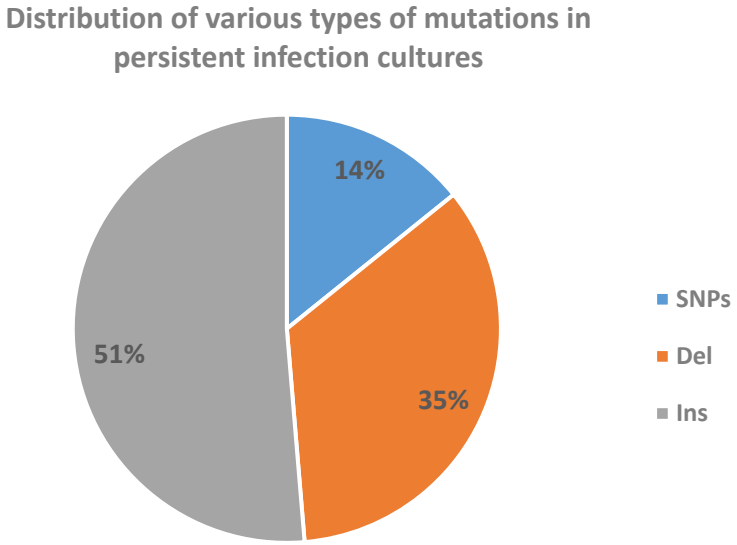


b.

Figure 4.6: Distribution of various types of mutations over time following *in vitro* infection of BHK-21 cells with FMDV SAT 2. SNPs = single nucleotide polymorphism. The total number of each mutation at specific time points is shown in (a) and the % distribution in (b).



a.

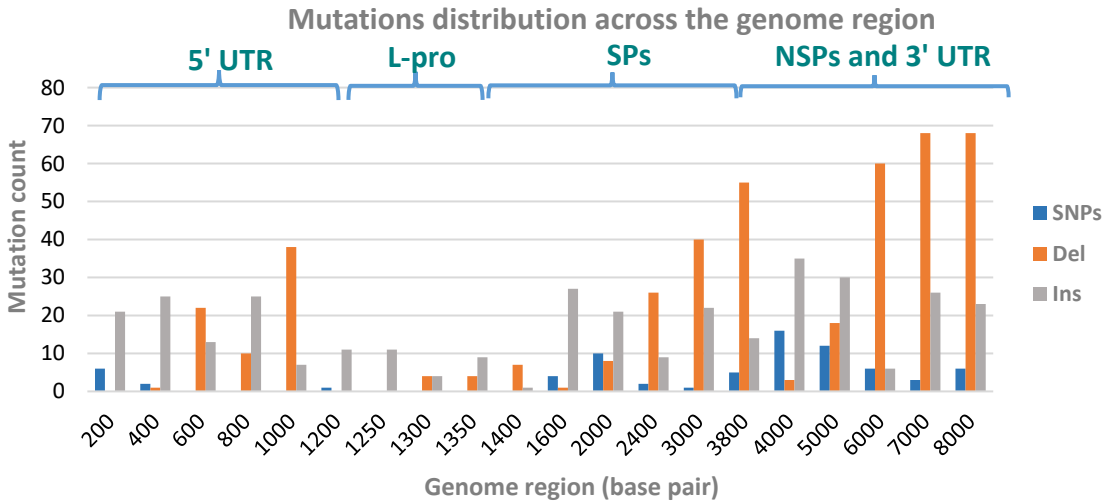


b.

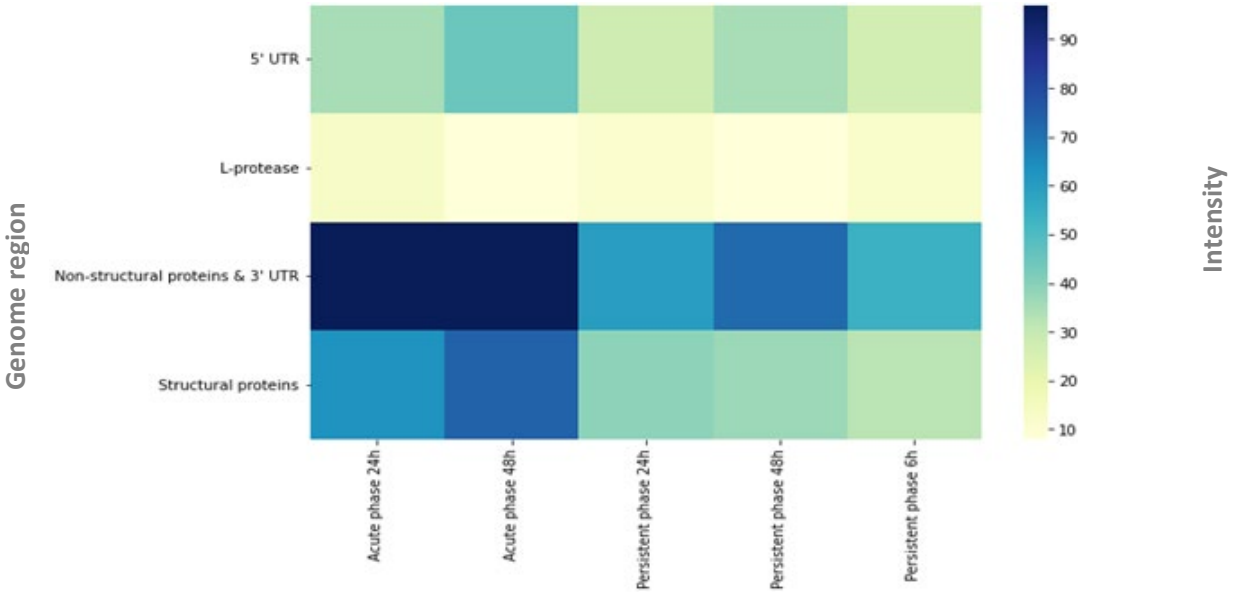
Figure 4.7: Comparison of the distribution of various types of mutations in BHK-21 (a) acute and (b) persistent infections with FMDV SAT 2. SNP = single nucleotide polymorphism, Del = deletions, Ins = insertions.

The mutations were distributed across the four FMDV genomic regions (Figure 4.8a; Figure 4.7). The most frequently occurring mutation type was ‘insertions’ which was detected from all FMDV genomic regions, followed by ‘deletions’ (Figure 4.8a). Notably, ‘deletions’ occurred at higher numbers compared to ‘insertions’, especially in the non-structural (NSP) and structural (SP) genomic regions. Fewer mutations were distributed

across the 5' UTR and L-protease regions compared to other regions (Figure 4.8 a). Consistently, the mutation intensity was low (below 50%) in these two regions (5' UTR and L-protease), compared to >50% in NSP and SP regions (Figure 4.8 b).



a



b.

Figure 4.8: (a) The distribution of different types of mutations across the four FMDV genomic regions (1= 170, 2= 52, 3= 245, 4= 380) in BHK-21 cells *in vitro* inoculated with FMDV SAT 2. SNPs = single nucleotide polymorphism, Del. = deletions, Ins. = insertions, and (b) a heatmap showing the intensity of mutations across genome regions in acute and persistent BHK-21 cell cultures inoculated with FMDV SAT 2, at various time points post-infection.

The 5' UTR region displayed a higher number of 'insertions' and 'deletions' compared to SNPs for both acute and persistent infections (Figure 4.9). Overall, the highest number of mutations were identified from the non-structural proteins (NSP) region, followed by the structural protein (SP) region, especially in sequences from acute infection cultures (Table 4.2; Figure 4.9). The least number of mutations were detected from the L-protease region. Notably, the highest number of mutations were detected from sequences from acute infection cultures.

Variations in sequences from persistent infection cultures, according to the mutation types described above, were detected as early as 6 hpi (Table 4.2; Figure 4.9). The comparison of mutations between sequences from acute and persistent cultures, at similar time points, revealed fewer SNPs and a higher number of 'deletions' in acute than in persistent cultures, irrespective of the time point or genome region. While the number of 'insertions' between the two culture groups (acute and persistent) varied based on the genome region and time point. However, at 48 hpi, the virus from acutely infected cells exhibited a higher number of insertions compared to the virus from persistently infected cells particularly in the 5' UTR, L-protease, SP, and NSP.

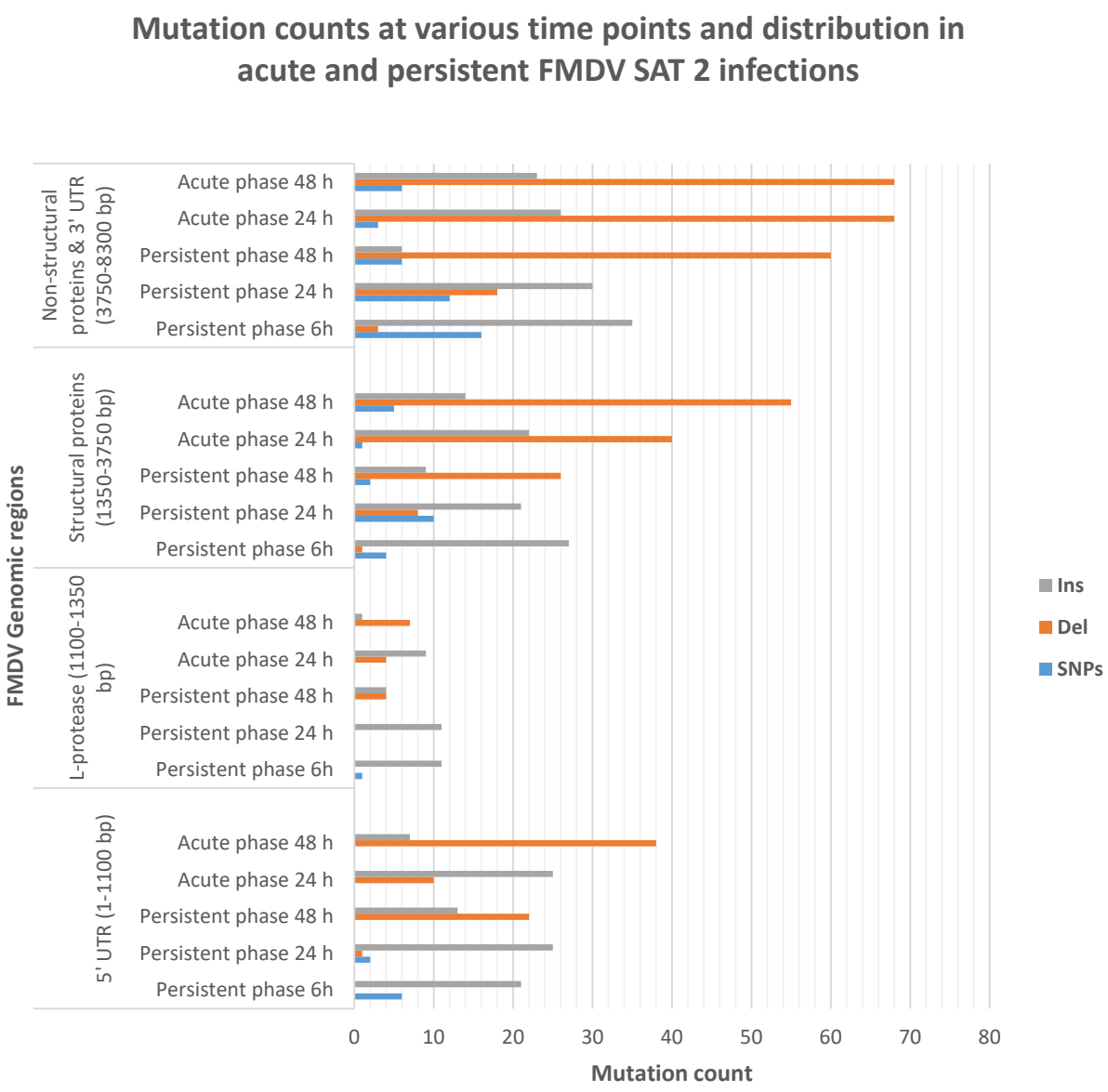


Figure 4.9: Comparison of the distribution of various types of mutations between acutely and persistently infected BHK-21 cells count across the FMDV genomic regions, at 6, 24 and 48 hours post-infection. Ins. = insertions, Del.= deletions, SNPs= single nucleotide polymorphism.

4.5.2 Phylogenetic relationship between genome FMDV sequences from acutely and persistently infected cells

The maximum likelihood phylogenetic tree analysis of FMDV SAT 2 genome sequences shows divergence based on time points post infection, for both acute and persistent infection cultures. As a result, sequences from acute and persistent cultures were distributed in two distinct groups, irrespective of the infection type. In the first group of sequences, the acute infections formed a cluster (6 h and 24 h) strongly supported by a bootstrap value of 95% (Figure 4.10). The sequences from persistent infections 6 h formed part of this group, though on a separate branch supported with a bootstrap value of 83%. The second group consisting of persistent infection sequences (24 h and 48 h) and acute infection 48 h formed a separate cluster from group 1. In this group, the 48 h acute infection sequence grouped closely with the sequence from the 24 h persistent infection culture, supported with a bootstrap value of 93%. On the other hand, persistent infection (48h) sequence shows the most divergence, forming a separate branch from the rest of the sequences analysed (Figure 4.10).

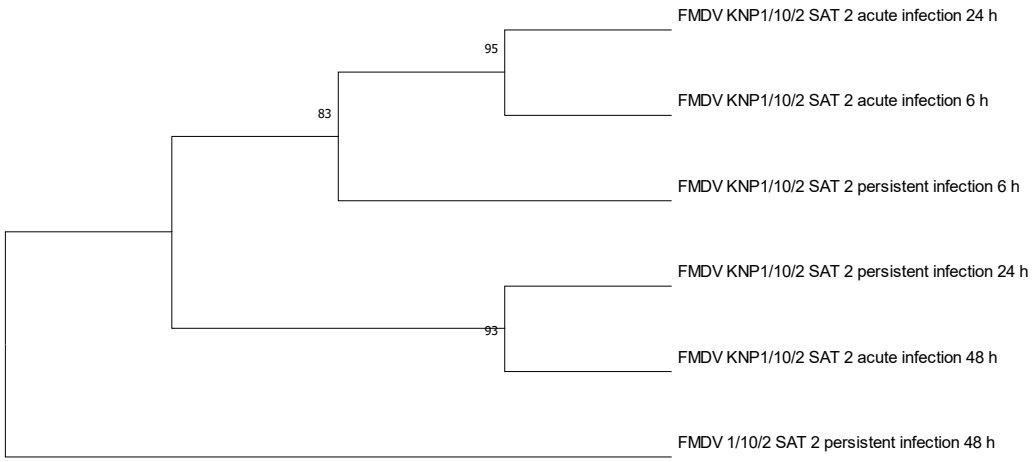


Figure 4.10: The evolutionary inference of FMDV SAT virus sequences by Maximum Likelihood phylogenetic tree constructed using the Jukes-Cantor model (Jukes, 1969). The consensus tree was derived from 500 bootstrap replicates with branches collapsed when showed less than 50% bootstrap replicates. The bootstrap values of the sequences where associated taxa grouped together and the bootstrap values are shown next to the branches (Felsenstein, 1985). The Neighbor-Join and BioNJ was used to generate the initial trees, followed by pairwise distance estimation using the Jukes-Cantor model. The final distance contained 7744 positions and the evolutionary analyses were performed in MEGA X software version 11.0.10 (Tamura et al., 2021).

CHAPTER 5

5 Discussion

To understand genomic mutations or variations in FMDV SAT 2 resulting from persistent infections, this study used NH_4Cl to mimic persistent infections *in vitro* due to its ability to inhibit lysosomal function by increasing intracellular pH, crucial for FMDV replication in host cells (Martín-Acebes et al., 2010). Once inside the cells, NH_4Cl dissociates into ammonium (NH_4+) and chloride (Cl-) ions, and NH_4+ can further dissociate into ammonia (NH_3) and hydrogen ions (H+) (Mohiuddin and Khattar, 2019). Ammonia diffuses into the lysosomes and reacts with H+ within the lysosomes, reducing the proton concentration, and increasing the pH (Carraro-Lacroix et al., 2011). At certain concentrations, NH_4Cl is known to induce cytotoxicity on various cell lines by impairing normal cellular degradation processes, leading to cellular stress, cytotoxicity, and sometimes cell death (Xu et al., 2013, Li et al., 2014).

When we investigated the cytotoxic effect of NH_4Cl on BHK-21 cells, the results indicated concentration- and time-dependent cytotoxicity. The 10 mM NH_4Cl treatment had minimal impact on BHK-21 cells, while higher concentrations (especially 20 mM) resulted in severe cell damage, with evident cell detachment. These results corroborate findings by Blancoa et al. (2013), which showed that swelling in neuroblastoma cells treated with NH_4Cl was dose-dependent. When neuroblastoma cells were exposed to varying concentrations of NH_4Cl (0.5 to 30 mM), significant cell swelling was particularly noted at higher concentrations, especially $\geq$20 mM (Blancoa et al., 2013). This swelling was associated to changes in intracellular pH and water balance, confirming that NH_4Cl disrupts the pH homeostasis (Li et al., 2014, Rooke et al., 2004). At high concentrations, NH_4Cl causes severe disruption of intracellular pH homeostasis, resulting in lysosomal enzyme dysfunction, thus affecting cellular structure (Suzuki et al., 2002). This pH disruption can inhibit some lysosomal enzymes that are responsible for the turnover of damaged organelles, triggering cellular stress responses (Repnik et al., 2012). The combination of cellular stress, intracellular alkalization, and lysosomal disruption can trigger cell death. At higher NH_4Cl concentrations, these stress signals are amplified,

leading to the activation of apoptosis-inducing factors (Li et al., 2024, Wang et al., 2018). As indicated earlier, this study also found that the cytotoxic effect of NH_4Cl on BHK-21 cell cultures was time-dependent, with prolonged exposure to NH_4Cl resulting in more prominent cytotoxic effects. Thus, BHK-21 cell cultures may have a time threshold for tolerance to NH_4Cl, hence they could withstand NH_4Cl exposure for a limited period before the cytotoxic effects became severe, and the threshold in this study was 24 h. The duration of NH_4Cl exposure has been linked to oxidative stress responses in various cell types, as well as cellular damage and apoptosis (Bento et al., 2007).

Subsequently, the effect of NH_4Cl treatment, at various concentrations, in BHK-21 cell cultures infected with the FMD SAT 2 virus, was monitored by cytopathic effects (CPE); the characteristic signs of viral infection and cell viability. In comparison to the negative control (BHK-21 cell cultures not infected with the virus nor treated with NH_4Cl), the acutely infected cell cultures (BHK-21 cell cultures infected with the same virus without treatment with NH_4Cl) showed notable morphological changes, as expected. The CPE observed in these cultures included cell detachment and formation of syncytia, which is typical of FMDV-infected cultures. This serves as evidence that the *in vitro* viral inoculation was achieved. Notably, the CPE severity gradually increased over time, from subtle changes at 6 hpi to complete CPE by 48 hpi, which is consistent with the typical progression of FMDV infection in susceptible cell lines (Arzt et al., 2011).

On the contrary, the onset of visible signs of CPE due to FMDV infection was delayed in BHK-21 cultures treated with NH_4Cl, compared to the negative control and acutely infected cultures. The increase in the pH within the endosomes causes the delay in the uncoating and release of FMDV's RNA, effectively suppressing the rate of FMDV replication (Sturzenbecker et al., 1987) and consequently its effect on the host cells. The delay of the signs of CPE in NH_4Cl-treated cultures suggests a successful establishment of FMDV SAT 2 persistent infection in BHK-21 cell cultures. Although the onset of CPE was delayed in persistently infected BHK-21 cultures, the formation of syncytia was observed in both acute and persistent cell cultures, which is a characteristic feature of FMDV infection mediated by viral proteins, particularly the VP1 protein (Ali et al., 2019). This demonstrates that FMDV SAT 2 infection was successful in both acute and

persistent infection cultures and NH_4Cl treatment does not necessarily prevent infection but delays viral entry to the host cells and consequently replication (Sturzenbecker et al., 1987). Accordingly, NH_4Cl has been reported to reduce the viral infectivity of equine infectious anaemia virus by over 90% at 30mM (Brindley and Maury, 2005). Furthermore, cells treated with NH_4Cl have been reported to have some resistance to FMD replication (Huang et al., 2011a).

Although we have observed morphological changes in NH_4Cl-treated BHK-21 cell cultures, a study by Huang and colleagues reported that NH_4Cl does not have an impact on BHK-21 cells' morphology and growth, when infected with FMD serotype O and treated with various concentrations of NH_4Cl (10 mM, 20 mM, 30 mM and 40 mM) (Huang et al., 2011a). This may suggest that the reaction to NH_4Cl treatment may be influenced primarily by the virus serotype. Nonetheless, in the current study, the morphological observations in acutely and persistently infected BHK-21 cell cultures revealed distinct cellular responses to viral infection under the respective conditions. The cellular responses were also influenced by time. Notably, the NH_4Cl treatment did not prevent complete CPE at time points longer than 24 h, which implies that the inhibitory effect of NH_4Cl on the virus replication is transient and cannot sustain long-term viability in the presence viral infection. Thus, NH_4Cl treatment seems to reduce the virus replication rather than completely prevent it. Its effect may influence cellular behaviour leading to changes in cell growth patterns consistent with results by Tesfaye and colleagues (Tesfaye et al., 2020).

Consistent with the time-dependent cellular response, the results in our study also showed an apparent time-dependent decline in cell viability across all infected cultures, acute and persistent, suggesting that as the FMDV SAT 2 infection progresses, the virus exerts increasing CPE in BHK-21 cells, which synergizes with NH_4Cl-induced cytotoxicity (Feng et al., 2021). Eventually, all FMDV SAT 2-infected BHK-21 cultures reached complete CPE and their lowest percent cell viability at 48 hpi. Infections with FMDV affect several biological processes in the host, including inhibiting host translation, obstructing protein secretion, and interfering with cellular signalling and the immune response, which may affect cell viability and even trigger cell death. (Gao et al., 2016). Notably, the

negative control and the 15 mM NH_4Cl persistently infected cultures had the highest % cell viability at all three time-points, suggesting that at 15 mM, NH_4Cl can induce persistent infection and retain high cell viability compared to other NH_4Cl concentrations investigated in this study. The data suggests that NH_4Cl, particularly at 20 mM, can significantly impair normal cellular degradation processes, leading to increased cellular stress and cytotoxicity. The elevated pH disrupts cellular processes such as autophagy and lysosomal activity (Lu et al., 2019), making the cells more vulnerable to damage and ultimately resulting in cell death. Additionally, the virus itself induces CPE in BHK-21 cells, and when combined with the cytotoxicity from NH_4Cl, this synergistic effect can be detrimental. The heightened cellular stress from both factors likely contributes to the severe reduction in cell viability at the 20 mM concentration. Meanwhile, at low concentrations such as 10 mM, the NH_4Cl alkalization effect does not seem to be enough to impair cellular degradation processes. However, the observed decrease in cell viability could be attributed to the fact that NH_4Cl at this concentration might not be sufficient to create a robust model of persistent infection, thus leaving the cells in a state similar to acute infection. Furthermore, NH_4Cl may compromise the cells' ability to handle the additional stress of viral infection, contributing to the overall decrease in viability observed at later time points.

Huang et al., reported that NH_4Cl-treated cells had fewer FMDV RNA copies when they were compared to cells without NH_4Cl treatment, demonstrating that NH_4Cl inhibits the viral replication (Huang et al., 2011b). In the current study, the qPCR analysis targeting the FMDV 3D region in infected BHK-21 cell cultures supports this finding as high Cq values were detected in persistent infection cell cultures compared to acute infection cultures. Consistent with the percent cell viability, the qPCR results also showed a dose-dependent effect of NH_4Cl across persistent infection cultures, with higher Cq values detected in cultures treated with high NH_4Cl concentrations, compared to lower concentrations. Accordingly, the highest fold change in Cq value was observed in cultures exposed to higher NH_4Cl concentrations, 4 times in 20 mM treated cultures, compared to 3 times at 10 mM NH_4Cl. Together, these results suggest there is a direct relationship between increasing NH_4Cl concentrations, and the decrease in FMDV RNA copy numbers and cell viability due to the virus-induced CPE and ultimately cell death.

A time-dependent relationship between Cq values and viral load was also evident in acute infections. As time progressed, the Cq values increased, indicating a decline in viral replication over time. This may reflect the natural cytopathic effects of the virus as the infection progresses, leading to cell death and, consequently the decrease in cell viability capable of supporting the viral replication. However, persistent infections showed a more stable rate of infection with a minor increase in Cq values, especially between 24 h and 48 h, consistent with a study by Han et al., which found that during persistent infection, host cells exhibit transcriptomic alterations that enhance their resistance to viral replication (Han et al., 2018). This suggests that the virus can induce changes in host cells while minimizing cytopathic effects.

The FMD virus is known for its rapid viral replication and a genome prone to mutations, contributing to its ability to persist in host populations (Domingo et al., 1996, Carrillo, 2012). In this study, mutations were detected in FMDV SAT 2 genome sequences from both acutely and persistently infected BHK-21 cells. Foot-and-mouth disease viruses have been previously reported to exhibit significant genomic variations during both acute and persistent infections, and have been targeted for understanding their pathogenicity and development of effective control measures (Klein, 2009). The FMDV's RNA polymerase lacks proofreading capabilities, resulting in errors during replication, and contributing to substantial genetic variability, including single nucleotide polymorphisms (SNPs), insertions, and deletions, which allows the virus to evolve as quasispecies (Palinski et al., 2022).

Comparative genome sequence analysis performed in this investigation showed that SAT 2 viruses from both acute and persistent infections exhibited SNPs, insertions, and deletions. Overall, the majority of mutations in virus from persistently infected cell culture at 6 hpi were insertions (75%) and deletions (74%) later at 48 hpi. In other viruses such as the African swine fever, indels can result to alterations in the protein coding sequences, resulting in changes in viral proteins, which are crucial for pathogenesis, immune evasion and replication (Elena, 2023). Accordingly in FMDV, deletions in certain genomic regions have been linked to viral attenuation (Azzinaro et al., 2022). Although, insertions have also been associated with viral attenuation (Arzt et al., 2017), it is possible that the

pathogenic effect of these mutations may depend on the genomic region affected. For instance, a higher number of deletions in the 3A protein-coding region of the FMDV serotype O has been linked to reduced virulence (Stenfeldt et al., 2018); while, an insertion mutation in the inter-AUG region of the leader proteinase is responsible for attenuation of a virulent field strain of FMDV (Arzt et al., 2017). Interestingly, insertions and deletions occurred at comparable proportions at 24 hpi, suggesting a possible transitional phase from pathogenicity to viral attenuation.

Notably, deletions were the most predominant mutations in acute infections, especially at 48 hpi (67%), compared to 35% in persistent infection. Also worth mentioning is that the accumulation of deletions in persistent infection was also predominant at 48 hpi, suggesting that deletions are more likely to occur during the latter stages of infection. Mutations in acute infections are generally driven by high replication rates (Carrillo et al., 2005). Our results suggest that, following rapid establishment of infection, characterised by high viral load, during the early phases in acute infection, the virus may exhibit reduced pathogenicity or attenuation in its virulence at later stages. This is consistent with the fact that, FMDV acute infections *in vivo* are characterized by rapid induction of clinical disease in cloven hoofed animals exposed to infection and the acute disease is equally rapidly resolved (Golde et al., 2008).

In contrast, insertion mutations were found to be predominant in persistent infection (51% compared to 29% in acute infection), particularly during the early phase of infection (6 hpi). It appears that the accumulation of insertion mutations in this case supports the early signs of viral adaptation, and a more stable rate of viral replication (Carrillo et al., 2005). The viral adaptation in persistent infection during early hours seems to occur without excessive disruption of viral proteins, suggesting that that while mutations accumulate, they may not substantially compromise the virus' ability to establish persistence (Yang et al., 2020). Similar to prior studies in cattle and buffalo, this gradual genetic variation is essential for the virus’ long-term persistence (Barros et al., 2007, Ramirez-Carvajal et al., 2018). The accumulation of deletions during the latter stages of persistent infection may be consistent with findings demonstrating that the pathogenicity of persistent FMDV strains is weaker than of the wild-type virus (Bao et al., 2011), considering that deletions

have been associated with reduced pathogenicity, ultimately attenuation (Azzinaro et al., 2022).

Regarding the distribution of mutations across the various genomic regions, insertions were the most frequently occurring mutations, detected in all regions. However, deletions occurred in high numbers although they were the second most frequent mutation, especially in the structural (SPs) and non-structural (NSPs)/3'-UTR protein regions. These findings highlight the complexity of viral evolution in response to different infection dynamics and the importance of developing targeted strategies for managing both acute and persistent FMDV infections. Since it has been established that deletion mutations could reduce the virus pathogenicity, it can be assumed that SPs and NSPs/3'-UTR regions, where most of these mutations occur, could comprise essential virulence factors. The structural proteins of FMDV include VP1, VP2, VP3, and VP4, which together form an icosahedral capsid that functions to protect the viral genome and are vital for viral stability, cell binding, and infection (Acharya et al., 1989, Domingo et al., 2005). The NSPs/3'-UTR region on the other hand plays an essential role in viral replication and immune evasion (Medina and Diaz San Segundo, 2024). Thus, the disruption of the functions of these proteins as a result of mutations will affect the propagation and infectivity of the virus.

The NSPs/3'-UTR region had the highest intensity of mutations, especially in acute infection cultures, dominated by deletions at all time points, compared to persistent infections dominated by insertions at two of the three time points (6 hpi and 24 hpi). There seems to be an early accumulation of deletion mutations in acute infections in this region, which is delayed in persistent infections, suggesting that the rapid viral replication during acute infection induces aggressive mutations such as deletions. The successful establishment of FMDV infections in host cells is largely due to virus' ability to interfere with the innate immune response with NSPs/3'-UTR region playing an essential role in immune evasion (Medina and Diaz San Segundo, 2024). The inhibition of cellular innate responses in FMDV infected cells has been demonstrated both *in vitro* and *in vivo* (Golde et al., 2008). Generally, viral pathogens mutate and evolve rapidly to counteract both the

non-specific and specific host immune responses, i.e. innate and adaptive immune response respectively.

Mutations in the NSP regions have been shown to affect the virus virulence, pathogenicity and host range, especially when they occur in the leader protease (Lpro) (Stenfeldt et al., 2018). FMDV leader protein (Lpro), and 3Cpro proteins are suggested to play a role in the inhibition of host proteins that lead to the suppression of cellular immune responses (Liu et al., 2015). Another three of the eight NSPs encoded by the open reading frame of the FMDV genome, 2A, 2B and 2C, are involved in viral replication, although their specific role in this process is well elucidated (Belsham, 1993, Belsham, 2005). Moreover, the 2B protein has been implicated in virus-induced CPE (van Kuppeveld et al., 1997, Ao et al., 2015). Considering the role of some of these NSPs, it could be that disruption of their function by mutations would affect the virus virulence and pathogenicity.

On the other hand, insertion mutations were predominant in NSPs/3'-UTR region in persistent infection cultures, particularly at 6 and 24 hpi. Furthermore, these mutations occurred in slightly higher numbers than in acute infections at the same time points. These results suggest that the rate at which the virus replicates have influence on the outcome of the types of mutation it effects to sustain adaptation and propagation, considering that insertions and deletions in the NSPs/3'-UTR region are linked to the virus' adaptation to different host environments (Ma et al., 2020).

A similar pattern of mutations observed in the NSPs/3'-UTR region was also observed in SPs in both acute and persistent infection cultures. Structural proteins are important in the maintenance of virion stability, host entry, and immune evasion (Gao et al., 2016). Similar to NSPs/3'-UTR, deletions in structural proteins have also been linked to reduced virulence in the Asia 1 serotype as time progresses (Li et al., 2010). Moreover, Li et al., also showed that mutations in structural proteins such as the VP3 protein are associated with attenuated virulence in cell cultures (Li et al., 2023a). Few deletions in this region, as observed in persistent infections in our study, have been linked to reduced viral replication (Alexandersen et al., 2002). Thus, the rate of viral replication plays a role in the determination of the type as well as the accumulation, of mutations in acute and persistent infections, as already discussed above. Critical in structural proteins is that

mutations can alter the capsid's antigenic sites and thus may affect the virus's ability to enter the host and/or evade host immune responses (Diez et al., 1990).

Notably, insertion mutations increased with decreasing deletions over time in persistent infections. Insertion mutations in FMDV can arise as a result of the virus adapting to persistently infected hosts (Hägglund et al., 2020). For instance, studies have shown that specific mutations can enhance the virus' ability to interact with host cell receptors, facilitating entry and replication in a manner less dependent on integrin receptors. This adaptation allows for continued viral replication despite changes in the host environment (Lawrence et al., 2013). Conversely, the frequency of deletions in the FMDV genome tends to decrease over time during persistent infections. The persistence of FMDV in host tissues has been associated with a stable viral population that exhibits reduced genetic diversity, suggesting that deleterious mutations are less likely to be maintained in the viral population (Zhu et al., 2020).

Sequences from both acute and persistent infection cultures had fewer mutations distributed within 5'-untranslated regions (UTR) and L-protease genomic regions, compared to the SPs and NSPs/3'-UTR regions. The 5'-UTR has a critical role in the regulation of viral replication and gene expression (Gao et al., 2016). Consistent with observations from SPs and NSPs/3'-UTR regions, deletions were detected in high numbers in acute infections although their accumulation was much less. Mutations such as deletions, are reported to have potential to interfere with regulatory signals in this region, leading to the reduction of viral replication efficiency over time (Kloc et al., 2017). In contrast, persistent infections maintained more conserved sequences, with insertions and SNPs dominating, particularly during the early infection phases (6 hpi, 24 hpi). This pattern may reflect the virus' need for stability in persistent infections, crucial for long term survival. (Carrillo et al., 2005, Ramirez-Carvajal et al., 2018).

Sequences in the L-protease genomic region were the most conserved in both acute and persistent infection cell cultures. This region is crucial for viral virulence, replication and pathogenesis (Brown et al., 1996, Mason et al., 1997, Gao et al., 2016). Thus, our results suggest that in both infections, acute and persistent, virulence is likely to be maintained, and persistent infections could be a risk of the spread of infection with virulent strains. In

this region, the less aggressive mutations, i.e. insertions, were the most occurring mutations, particularly in persistent infections. Deletions were prevalent in acute infections in this region, although in low numbers. Deletion mutations in the L-protease region can lead to viral attenuation, suggesting that sequence conservation in this region is essential for maintaining virulence (Azzinaro et al., 2022). Therefore, the fact that insertions are the most frequent mutations in this region could be an indication that FMDV, though highly mutable, regulates and preserves critical genomic regions necessary for its virulence, long-term persistence, and maintaining replication efficiency. Consistently, certain mutations in the L-protease coding region could contribute to the virus' capacity to evade host defences and establish a persistent infection (Medina et al., 2018). Although amino acid insertions and nucleotide deletions in L-protease have been associated with limited growth in bovine cells, which is typical of persistent infection, it has been reported that this may alter the pathogenicity of serotype O in cattle (Yang et al., 2020).

The phylogenetic analysis of FMDV SAT 2 sequences from persistent and acute cell cultures, revealed the divergence of these sequences into two distinct groups, which contained sequences from both infections. The tree branching patterns observed across all time-points suggest that the virus undergoes rapid genetic changes within a short time due to its rapid replication (Domingo and Holland, 1997). This evolution could be a key factor in the virus' ability to establish persistent infections. It also highlights the challenges in developing long-lasting vaccines, as the virus may quickly evolve (Kenubih, 2021). The sequences generally cluster according to the infection type and time points. The 6 h and 24 h sequences for acute infection cell cultures clustered together, suggesting the accumulated mutations between these time points were not enough to cause a genomic divergence between the two viruses. Notably, the sequence from persistent 6 h infection grouped closely to these two, supported by a bootstrap value of 83%. The same virus used for acute infection was used to establish persistent infection. Thus, this close relationship suggests limited genomic changes during the early phases of infection in both acute and persistent infection cultures. As viruses in these two infection cultures replicated at different rates, these results further suggests that the rate of replication is

not necessarily directly proportional to the rate of accumulation of mutations that can cause significant genomic changes.

The close relationship between the persistent infection at 6 h with acute infections (6 h and 24 h), may also indicate that during early hours acute infections and persistent infections may employ similar adaptive strategies. This is supported by the FMDV's ability to modulate host cellular pathways, crucial for both infection types. The virus can manipulate the signalling pathways to enhance replication while suppressing the host's immune activation, such as inhibiting the production of type I interferons, essential for antiviral defence (Ma et al., 2018, Peng et al., 2020). This suppression not only aids in establishing acute infections but also fosters long-term persistence by creating an immunosuppressive environment that hinders effective immune responses (Li et al., 2021).

Contrary to the acute infections, there were significant changes between 6 h and 24 h sequences from persistent infection cultures; enough for the two to group separate, and the latter closely with the acute infection 48 h sequence. This suggests that persistent infection is established by viruses that have undergone drastic genomic changes from the initial virus inoculum, and these changes can happen within a short period of time. Hence, the sequence from the 48 h persistent infection culture was very distant from all other sequences, taking a position similar to an outgroup on the tree, due to the extent to which the virus has changed. However, the above observations are contrary to the stable evolution of FMDV during persistence observed in African buffalo populations. Cortey et al. reported minimal genetic changes over time, suggesting that FMDV reaches a point of equilibrium within the host that allows for sustained replication (Cortey et al., 2019). Perhaps the stable evolution is reached over long periods of time, which then could explain the different results from the current study, as the experiments in this study were performed over a limited period (48 h).

Overall, the observed clusters for both acute and persistent infections indicate that each infection type follows a different evolutionary adaptation over time in response to the host cell environment (Biswal et al., 2019). The genomic variability observed among persistent infection samples may therefore represent an adaptive response that promotes viral

persistence (Macdonald et al., 2022). Since persistent infections may harbour more genetically diverse populations, they could serve as reservoirs of new, potentially vaccine-resistant strains (Alexandersen et al., 2002, Yang et al., 2020). The quasispecies nature of persistent infections implies that a single strain used in vaccines might not provide broad protection against all variants, particularly those emerging from prolonged infections.

5.1 Conclusion

This study successfully established an *in vitro* model of persistent infection for serotype SAT 2 of FMDV in BHK-21 cells, with 15 mM NH_4Cl. The cultures exhibited the hallmark characteristic of persistent infection, which is a reduced rate of viral replication. The viral RNA remained detectable but at much lower levels compared to acutely infected cells. Persistent infection was maintained for up to 24 h before severe signs of CPE were observed. Moreover, cell viability was maintained at high levels in 15 mM NH_4Cl treated cultures. At 15 mM, NH_4Cl successfully reduced replication without eliminating the virus.

Comparative genomic analysis of FMDV STA 2 sequences between acutely and persistently infected cells revealed distinct mutational changes. Mutations in persistent infections were predominantly insertions, while deletions were common in sequences from acute infection cultures. In both persistent and acute infection sequences, the most mutable genomic regions were NSPs/3'-UTR and SPs, while the 5'-UTR and L-protease regions were generally conserved, suggesting that the virus strategically mutate specific regions to ensure successful adaptation and survival in its host. The clustering patterns observed suggest that FMDV evolves differently under acute and persistent conditions, with limited divergence in the acute infections but notable genetic variability during persistent infections, as time progresses. The extreme diversity in sequences from persistent infection cultures reveals that the virus persistence depends on rapid evolution in order to rapidly acquire traits that will allow it to evade the host immune response and enable prolonged survival in its host.

The results from this study creates an opportunity to study FMDV SAT 2 serotype *in vitro.* They also enhance the understanding of FMDV persistence, and the genomic variations associated with different infection states. This knowledge is critical for improving strategies for the control and prevention of FMDV outbreaks, particularly in regions where the SAT 2 serotype is prevalent.

6 LIMITATIONS AND RECOMMENDATIONS

6.1 Limitations

The long-term effects of NH_4Cl on cells and viral behaviour were not thoroughly explored in this study. There was also a limited time frame of up to 48 h, which is relatively shorter compared to natural viral persistence in animals, which can last for several months or years. Due to budget limitations, the study could not include all three SAT serotypes of FMDV, which was the original intention. As a result, the findings from this study may not fully reflect the behaviour of other serotypes, which could exhibit different mechanisms of viral persistence and genomic variations. Moreover, the *in vitro* model lacks interaction with the host immune system, which is a contributing factor to viral persistence *in vivo*. This also limits the understanding of how the virus might undergo persistence and evolution within host animals.

6.2 Future studies

While this study successfully established persistent FMDV infection *in vitro*, the mechanisms driving viral persistence remain poorly understood. Future research should focus on identifying the molecular pathways involved in maintaining persistent infections and their interactions with host cells including host immune response during persistent infections. A more detailed study to understand genomic variations during persistent infections over extended time needs to be done to understand viral evolution and adaptation in host animals. This could help us understand how FMDV evolves inside the host during persistent infections. The effectiveness of NH_4Cl in inhibiting viral replication and inducing persistent infections would be beneficial to explore its use in drug discovery.

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8 APPENDICES

8.1 Ethics attachments

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

28 February 2024

LETTER OF APPROVAL

Ethics Reference No	**REC168-22**
Protocol Title	**Comparative genomic analysis of Foot-and-Mouth Disease Virus Serotypes SAT1/2/3 in BHK-21 cells**
Principal Investigator	**Miss L Nocaka**
Supervisors	**Prof KP Sibeko-Matjila**

Dear Miss L Nocaka,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC168-22) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Marna Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1(a)/3009(HP)

Linda Nocaka
ARC - OVR - TADP
100 Old Soutpan Road
Agricultural Research Council
Transboundary Animal Diseases Programme (TADP)
E-Mail: Nocakal@arc.agric.za

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

Dear Linda Nocaka

Your fax / memo / letter/ Email received 2023-05-02, 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 research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form received 2023-05-02 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study using the specified waste contractor. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
4. The study must be conducted in the TAD-ARC-OVR BSL3 facility.
5. Only samples from the TAD-biobank may be used.
6. Only extracted DNA samples may go to the Agricultural Research Council Biotechnology Platform for sequencing, subject to the TAD BSL3 SOPs.
7. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
8. This Section 20 approval is valid until 2026-05-31.

Title of research/study: Comparative genomic analysis of foot-and-mouth disease virus serotypes SAT1, SAT2, SAT3 in BHK cells in vitro
Researcher (s): Linda Nocaka
Institution: ARC - OVR - TADP
Your Ref./ Project Number: None
Our ref Number: 12/11/1/1(a)/3009(HP)

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date:
2023 -05- 05

SUBJECT: RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

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ONDERSTEPOORT VETERINARY INSTITUTE
ANIMAL ETHICS COMMITTEE

Application for clearance to use vertebrate animals (including their embryos and foetuses) for an experimental study or in a standard operating procedure for diagnostic purposes

Where clearance is sought for a standard operating procedure (SOP), a separate clearance form must be completed per procedure

NOTE:

- Please read the Animal Ethics Categories (AEC 01) form before you complete this application.
- This application must be typed.
- It must be signed by the Principal Investigator (the applicant) and other persons who are vouching for specialised aspects of the experimental design (i.e. statistician, safety officer, and persons responsible for supervising the use of scheduled medicinal substances).
- The application needs to be written simply but include all relevant detail.
- A score sheet must accompany all applications.
- If any animal during the experimental period gets sick/dies due to causes not related to the experimental work, a morbidity/mortality report must be submitted to the AEC accompanied by a full post mortem report.
- Once the experimental work starts a copy of the score sheet and a summary of the experimental work conducted must be visible at the experimental facilities together with the weekly animal health checks by the responsible veterinarian for inspections by AEC.
- **Submitting applications:** An electronic copy of the application should be emailed to Ms Thapelo Rametse (RametseT@arc.agric.za), Secretary of the Onderstepoort Veterinary Institute – Animal Ethics Committee (OVI-AEC), (Tel: 012-529-9545).
- **Deadline** for researchers to submit protocols will be the **1st of each month**. Late protocols go through to the next month.
- An AEC-reference no will be allocated to the submission and must be inserted as a Header on each page of the Application. It will be distributed electronically to all AEC members for review and comments. Comments from AEC members will be submitted to Ms Smit by the 10th of the month.
- Ms Smit will collate all the comments and send them back to the AEC members, and applicants after the monthly AEC meeting.
- Applicants must respond within 3 months to the comments. If now response is received the Application will be cancelled.
- **Approved applications:** the original document with all the relevant signatures must be submitted to the secretary to arrange that it be Stamped and signed by the AEC-Chairman. The original document will be filed on AEC-Submission file and a copy be sent to the applicant.
- Revised applications must be submitted by the 20th of the month.
- AEC meetings will be held on the third Wednesday of each month (except December) where all protocols and comments will be discussed, and final committee approval (or not) given, and communicated to applicants asap.
- Telephone enquiries on any animal ethics related matters may be directed to the Chairperson, Dr Paidamwoyo Mutowembwa (Tel: 012-529 9593, or MutowembwaP@arc.agric.za)
- **NB: No animals will be allowed at the ARC-OVI campus unless Section 20 approval is obtained from the Department of Agriculture, Forestry and Fisheries.**

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BIORISK MANAGEMENT STATEMENT

Does the project pose any hazards to other animals and staff, community and the environment from the use of GMO's, infective agents, toxic or carcinogenic agents or ionising radiation? If it does, state safety procedures to be adopted to contain these hazards. Provide a brief approval statement below from the Institutional/Departmental Safety Officer to provide assurance of safety for this project with this person's signature of ratification.

Please indicate the biosafety and biosecurity mitigation measures to be taken to minimize the risks:

According to the OIE standards (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals) foot and mouth disease virus (FMDV) is identified as a Containment Group 4 animal pathogen based on the severe consequences of spread from the laboratory and according to the risk it poses to animal health and the agricultural economy of a country. Therefore, the following measures will be adhered to.

1. Animal experiment,

The study design involve opening of stable and movement of animals after viral infection. This may pose a huge bio risk management to susceptible animals. For this reason, all work will be undertaken at the ARC-OVR (TAD) BSL-3 high containment animal facility and BSL-3 laboratory to minimise chances of viral escape. As mitigation measures against possible viral exposure, the use of Standard Operating Procedures (SOP) and Personnel Protective Equipment will be in place. This study design involves opening of stables and movement of animals after viral infection. This may pause a risk of contamination of animal rooms with naïve animals. This practice is a deviation from normal operations of TAD-Stable facility. To mitigate Bio risk, all transfers of naïve cattle from clean stables to stables with infected animals will only be through the central corridor. The cattle will be move out of a clean stable into the central corridor, then the door to the clean stable will be closed immediately before opening the door of the stable with infected cattle (dirty stable). Soon after the animals are moved into the "dirty" stable the door is closed immediately followed by decontamination of the central corridor as described in TAD-BSF 08 Biosafety Manual section 9. The Animal experiment will use one viral isolate at a time to avoid cross contamination.

2. Sample collection,

Sampling will be done by well-trained veterinarian (Dr Mutowembwa P SAVC reg: D08/9606) as well as animal technician (Thapelo Rametse SAVC reg: H09/10185). This official will be assisted by animal attendance in the BSL3 facility.

I. Ante mortem sample collection

Rompun (2% Xylazine) will be used for analgesia and sedation during all procedures other than taking of temperature. Ante-mortem sampling will consist of the blood, serum, nasal swabs, clinical blisters, saliva and the palatine tonsil swab. Blood sample collection

Blood will be collected as follows:

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i. Two BD Vacutainer-EDTA tubes (Becton Dickson) (~20 ml) for innate and adaptive immune responses and Elispot assays
ii. One VacuLab® serum tube per animal (~10 ml) for serological assays.

Palatine tonsil swab

The palatine tonsil swab will be collected as previously described by Casteleyn et al 2011. Briefly the tonsil sinuses will be swabbed individually with a nylon brush. A laryngoscope will be will be used to depress the tongue and provide lighting while swabbing the tonsillar crypts. In between the sampling, the Laryngoscope will be disinfected with citric acid and rinsed with PBS thereafter. The nylon brushes will be transferred to a container with 0.5 ml PBS and snap frozen. These samples will be used for RT-qPCR or virus isolation.

II. Post-mortem sample collection

Necropsies will be performed immediately subsequent to euthanasia at predetermined time points as described in figure 1. The following tissues will be collected: larynx, nasopharynx, palatine and lingual tonsils, spleen, retropharyngeal lymph nodes, submandibular lymph nodes, popliteal lymph nodes and prescapular lymph nodes. For each defined specimen, two 30 mg tissue samples will be aliquoted into separate screw-cap 1.5 ml cryovials and within 2h to a -80°C freezer where they will be stored until the time of processing. These samples will be subjected to virus isolation, RNA isolation followed by RT-qPCR, transcriptome sequencing and genome sequencing.

Sample handling, and Sample processing,

Considering the potential threat of FMDV to other cloven-hoofed livestock, especially in an FMD free Zone of the country. All experiments will be done in the BSL3 facility ARC-TAD-OVR to minimize chances of viral and contacts between staff and other cloven-hoofed species. All cattle groups (32 per group) will be challenged at this facility to minimise virus outbreak. Work with viral samples will be in biosafety cabinets. Infectious material will be stored in cryovials for long-term storage. As mitigation measure, the use of standard operating procedure (SOP) and personal protective equipment (PPE) will be in place. Access control in and out of the facility will be strictly controlled throughout the study period according to the facility access control system. Personnel showers and gown/degown, deep gumboot baths when entering and exiting. All blood, tissues and PBMC samples will be handled according to Good Laboratory Practice and necessary PPE will be used. All waste from the trial will be will be incinerated according to the laboratory SOP. PBMC will be isolated in a Biosafety Cabinet within the diagnostic lab inside TAD BSL3 facility. All other experimental assays will be performed within BSL3 facility and ARC- Biotechnology Platform.

Biological material storage:

Due to sampling nature of the project, at the end of trial all the animals would have been sacrificed. For each defined specimen, two 30 mg tissue samples will be aliquoted into separate screw-cap 1.5 ml cryovials

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and frozen immediately in liquid nitrogen for transfer within 2 h to a -70°C freezer in which they were stored until the time of processing or needed within the BSL3 facility.

4. Waste management and discarding of biological specimens and cultures: (disinfection protocol) Incinerator commissioning letter is attached.

All the biological/infections wastes will be disposed as indicated in the Laboratory Biosafety Manual. Biological waste requires special handling to protect human health and environment. It includes solid waste, which if improperly treated and handled may transmit diseases. Solid non-sharp biohazard waste such as paper, towel, cotton wool, gloves, specimen containers, blood tubes, pippete tips and 96 well plates from the laboratories is securely within autoclave waste bags inside biohazard containers until collected. Used tissue culture plates, specimen containers, blood tubes, and pipette tips are soaked in 2% citric acid solution for the minimum effective contact time as per manufactures instruction. After complete disinfection, all the used consumables are stored in autoclave waste bags positioned in biohazard bins. The laboratory assistant closes the opening of the waste bag securely to avoid spillage and load them on the push cart and takes them to the washroom where the autoclave is located. The bags are autoclaved then transported to the small incinerator where they will be disposed of by incineration. Organ samples within the animal facility will be put in double biohazard waste bags bearing a biohazard signage. The double bagged package is then sent to the basement incinerator for disposal by incineration. Liquid waste will also be disinfectanted by heat treatment in the effluent plant at 140 °C for 60 minutes before release into the municipal system.

6. Incidence report procedure

Incidence will be reported to HR through line managers by research principal investigator (Dr T Nefefe).

7. Exposure and Emergencies

It is important to understand that, FMDV is not readily transmissible to humans and is not a public health risk. Only a few benign cases of human infections have been documented, none requiring hospitalization
In case of emergency the institute first aid will be called and assist while the matter is escalated to the occupational health officer of the Institution. The general laboratory procedures for treating any emergencies will be carried out according to the laboratory safety manual.

8. Material Control and Accountability plan.

Dr Nefefe the project PI will be responsible of material control. There is a guideline on handling materials that will emanate from the research and the plan is fully described in the protocol. The project will adhere to OIE guidelines for the containment level for Group 4 pathogens and this include the following precautions:

- Access to the building through a system of air lock's
- Maintaining the building under negative air pressure
- Filtering incoming air through a single HEPA filter
- Filtering outgoing air through double HEPA filters

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- Conducting all work with infective materials in specialized cabinets

9. Transport Security: Once animals are confirmed FMDV negative, they will be transported from Kaalplaas using a secured truck to ARC-OVR-TAD South Africa Biosafety level 3 laboratory that is less than 3KM away from the farm mitigating transit risk factors for FMD transmission. Mr Ntshangase/Pule will responsible for animal welfare during their transportation to TAD stables. Within the BSL3 samples will be moved from the stables to other sections of the laboratory, which does not transport security measures.

10. Information Security: The ARC-TAD is secured facility with 24 hours on guard; armed security beefed up by camera surveillance.

11. Attached DAFF BSL3 compliance certificate (Need to be renewed and the process undergoing with management)
The BSL3 laboratory have all the SOPs for handling group 4 risk pathogens and this SOPs will be adhered to throughout the research project.

APPROVAL STATEMENT:

The revised application as requested by the Facility Manager was re-evaluated by the IBBC and approved on 29 August 2023, based on the risk mitigation plan for animal movement within the BSL-3 stables.
NAME: Faith Peta SIGNATURE: Faith Pera DATE: 05/10/23

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Submission Date		For Administrative Purposes	AEC REF.	
AEC approval Date		Signature (only on approval)		

A. PROJECT NO:

B. PROJECT TITLE

Genomic analysis of Foot and mouth Disease Virus and Global Transcriptome profiling of host immune response: Defining mechanisms of persistent infection and activation of immune response.

C. PROJECT LEADER/RESEARCHER (PRINCIPAL INVESTIGATOR)

Name	**Contact Number**	**e-mail address**	**Contact Address**
Tshifhiwa Nefefe	0125299575	NefefeT@arc.agric.za	ARC-OVR,TAD
Division		Vaccine and Diagnostic Development department (VDD)	
Qualifications		PhD Veterinary Tropical Diseases	
Appropriate experience in animal research		10 years in Veterinary Research Experience in animal experimentation involving FMD vaccine efficacy trials. Experience in immunology, processing animal blood for various immunological assays using PBMC, Lymph cells processed by ELISPOT, Flow cytometry and RNA-Sequencing and RT-PCR.	
Details of involvement		Study leader Day to day supervision and evaluation of samples and reagents for good laboratory practice. In addition, assist students in performing all the immunological, molecular and serological experiments.	

D. RESEARCH TEAM MANAGER (RTM)

Name	**Contact Number**	**e-mail address**	**Contact Address**
Arshad Mather	0125299236	MatherA@arc.agric.za	ARC-OVR
Division	Vaccine and Diagnostic Development department (VDD)		
Qualifications	PhD Biochemistry		
Appropriate experience in animal research	Over 16 years of research experience at the ARC-OVR. Currently is a Research Team Manager, head of the Vaccine and Diagnostic Development unit. The unit conducts research focused on new generation vaccine development for veterinary diseases of economic importance. Dr Mather's research interests include the		

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	development of vector-based vaccines for viral diseases and has been involved a vast number of projects and animal experimental work.
Signature:	Date: 05/10/23 .....

E. RESPONSIBLE VETERINARIAN

Name	Contact Number	e-mail address	Contact Address
Paidamwoyo Mutowembwa	012 529 9593	MutowembwaP@arc.agric.za	ARC-OVR, TAD
Division	Transboundary Animal Diseases- Vaccine Production		
Qualifications	BVSc, MSc		
Appropriate experience in animal research	He the Veterinarian involved in the management of clinical animal studies in the TADs High Containment Animal Facility. He is registered with the South African Veterinary Council. REG NO: D08/9606		
Details of involvement	Clinical veterinary procedures: vaccinations, challenging and treatment if required.		

Name	Contact Number	e-mail address	Contact Address
Dr M Mparamoto	012 529 9493	MparamotoM@arc.agric.za	ARC-TAD
Division	Public Health and Zoonoses		
Qualifications	BVSc		
Appropriate experience in animal research	More than 20 years' experience as a veterinarian		
Details of involvement	Back up veterinarian		

F: Co-WORKERS (involved directly with procedures on Animals)

Name	Contact Number	e-mail address	Contact Address
Thapelo Rametse	012 529 9545	RametseT@arc.agric.za	ARC-OVR, TAD
Division	Transboundary Animal Diseases- Vaccine Production		
Qualifications	B Tech Animal Health		
Appropriate experience in animal research	13 years' experience as an AHT of SA Vet Council H09/10185.		
Details of involvement	Animal monitoring and administrative activities involving animals at the TADP BSL-3 stables, Animal care, sample collection and processing.		

F. OTHER Co-WORKERS

Name	Contact Number	e-mail address	Contact Address
Dr Livio Heath	012 529 9501	HeathL@arc.agric.za	ARC-OVR, TAD

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Division	Transboundary Animal Diseases- Vaccine Production		
Qualifications	PhD		
Appropriate experience in animal research	Over 15 years' experience doing research involving animals.		
Details of involvement	Project design, reagents preparations, activities scheduling and overall management.		
Name	**Contact Number**	**e-mail address**	**Contact Address**
Prof. Kgomotso P. Sibeko-Matjila	012 529 8402	kgomotso.sibeko@up.ac.za	Faculty of Veterinary Science Room 2-20.3, Level 1,Paraclinical Building University of Pretoria, Private Bag X04 Onderstepoort 0110, South Africa
Division	Department of Veterinary Tropical Diseases		
Qualifications	PhD		
Appropriate experience in animal research	My experience in animal research is in protozoan infectious diseases of cattle, specifically Theileria parva. I am a principal investigator in research projects involving in vivo, in vitro, in silico and molecular studies, with the primary aim of characterizing pathogens and identifying targets or candidates for disease control.		
Details of involvement	Assist with execution of animal work		
Name	**Contact Number**	**e-mail address**	**Contact Address**
Dr Selaelo I. Tshilwane	012 529 8566	selaelo.tshilwane@up.ac.za	Faculty of Veterinary Science Room 2-12, Level 1, Paraclinical Building University of Pretoria, Private Bag X04 Onderstepoort 0110, South Africa
Division	Department of Veterinary Tropical Diseases		
Qualifications	PhD		
Appropriate experience in animal research	Experience in animal research on vector-borne diseases of veterinary importance including heartwater and African horse sickness. Performing vaccine efficiency trials as well as immune response evaluation following infection and/or vaccination in small ruminants and horses. Experience in immunology, including preparation of; PBMC from whole blood, immune cells from lymph nodes and spleen as well as immune monitoring using various immunological assays including ELISPOT, Flow cytometry, ELISA, RT-PCR and RNA-Sequencing.		

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Details of involvement	Assist with the design of animal experiments and laboratory experiments on immune response evaluation		
Name	**Contact Number**	**e-mail address**	**Contact Address**
Dr Jonathan Artz	(631) 323-4421	jonathan.arzt@usda.gov	Animal Disease Research Unit, Plum Island Animal Disease Center. USA.
Division	Foreign Animal Disease Research Unit, Plum Island Animal Disease Center.		
Qualifications	DVM, MPVM, PhD, DACVP.		
Appropriate experience in animal research	Experience in Pathology, Animal Virology, Microscopy, Foot-and-Mouth Disease Virus, Fluorescence Imaging, Veterinary, Virology, Immunohistochemistry, Virus-Host Interactions, Vaccinology, Veterinary Medicine, Immunology, Virus Infection, Virology, Histology, Pathogenesis, Cattle Epistemology, Epidemiology, PCR Vaccines, Interferon, Vaccination		
Details of involvement	Establishment of INP inoculation using SAT viruses		
Name	**Contact Number**	**e-mail address**	**Contact Address**
Dr Caroline Stenfeldt	(631) 323-3008	carolina.stenfeldt@usda.gov	Plum Island Animal Disease Center, Agricultural Research Service (USDA) Orient Point, United States
Division	Foreign Animal Disease Research Unit, Plum Island Animal Disease Center.		
Qualifications	PhD Veterinary Virology.		
Appropriate experience in animal research	I have substantial experience in design, coordination, and practical performance of in vivo experiments investigating the pathogenesis and transmission of high-impact viral infections of livestock, such as foot-and-mouth disease and African swine fever. I have specialized in elucidating the tissue- and cellular tropism of viral infections in the natural host species using simulated-natural experimental systems. I care deeply about the intricacies and appropriateness of experimental design, as well as the welfare of study animals. I have experience of performing complex experimental animal studies in high containment (BSL-3+Ag) research facilities		
Details of involvement	Establishment of INP inoculation using SAT viruses		
Name	**Contact Number**	**e-mail address**	**Contact Address**
Prof. Melvyn Quan	+27 12 529 8142	melvyn.quan@up.ac.za	Room 2-18

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			Paraclinical Building
Division	Department of Veterinary Tropical Diseases		
Qualifications	PhD Veterinary Virology.		
Appropriate experience in animal research	Modelling in vivo dynamics of foot-and-mouth disease virus in pigs 4 years experience as veterinarian 14 years experience as researcher		
Details of involvement	Assist with the design of animal experiments and laboratory experiments.		

H. DECLARATION

1. Moral Philosophy
   The ethical review of proposed animal experiments is predicated upon the acceptance by the OVI that vertebrate animals are organisms of moral concern and as such, must be accorded rights in their exploitation for the advancement of biological knowledge and for the promotion of the health and welfare of animals and man.
2. Animal Rights
   In the research use of laboratory animals these comprise the right:
   - Not to be used for research and/or to be killed for trivial, irrational, unjustified or inappropriate reasons
   - To live, reproduce and grow under conditions that are comfortable and reasonably natural to their species
   - To be kept free from disease, parasitism, injury and pain by prevention, rapid diagnosis and treatment
   - To be able to express normal behaviour through providing sufficient space, proper facilities in which to live and in the company of the animal's own kind
   - To be free from fear and distress by ensuring that the animal's living conditions, handling and treatment will either minimise or eliminate the infliction of these states upon the animal.

   A. Humaneness
   The principles of Humane Experimental Technique must be followed in the planning and conduct of animal experiments. These comprise:
   - Replacement of animals with non-sentient research systems i.e. researchers should strive to avoid using laboratory animals if alternative methods can yield the data they need
   - Reduction of the numbers of animals which are to be used to a minimum by design in order to achieve only sufficient statistical power to allow the objectives of the experiment to be achieved
   - Refinement of the experimental methodology to be adopted by the implementation and if necessary improvisation of procedures which will have the least distressing or harmful effects on the animals and when this is not avoidable to counter those effects with the use of anaesthesia, analgesia and other effective strategies.

   B. Relevance
   The research must address an important question relevant to the OVI's objectives in advancing education, science and human and animal welfare through research.
   C. Responsibility

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Everyone using animals, whether for experimentation, testing, diagnosis, teaching or sourcing of tissues or body fluids is responsible in their personal capacity for assuring that the animals which they use are afforded the highest levels of welfare and protection from abuse, and violation of the rights accorded to them.

D. Declaration

6.1 I, **Dr Tshifhiwa Nefefe**, as Principal Investigator in this application hereby declare that I am familiar with the precepts, policies, and responsibilities outlined under Section D and will personally undertake to see that these are upheld in the conduct of this study, should it be approved.

6.2 I also undertake not to deviate from the approved protocol without clearance for changes which may become desirable or necessary when the experiment is in progress without submitting a request for a deviation and having these changes cleared by the OVI-AEC.

6.3 Should the OVI-AEC request a report, at the conclusion of the study I undertake to report on its outcome to the OVI-AEC and if it has not been completed within six months of it being cleared by the Animal Ethics Committee, to submit progress reports at six monthly intervals until the study has been completed.

07/11/2022

.....

Signature of Applicant **Date**

6.4 In my opinion, all persons named and working under my supervision have the appropriate training and skills needed to carry out their responsibilities for experimental procedures, care and handling of the species being used.

Signed by:ARSHAD SALEH MATHER
Signed at:2023-10-05 11:41:13 +02:00
Reason:Witnessing ARSHAD SALEH MA

ARC • LNR

05/10/23

....

Signature of Supervisor (When applicable) **Date**

I. PEER REVIEW STATEMENT

I Dr Melanie Chitray (Reviewer's Name) declare that this research protocol has been peer reviewed by myself in my capacity as (tick answers):

Director	
Research Team Manager	
External review	

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Other (specify)	Senior Researcher - VDD (TADs)

Of the (Institute/Unit) on VDD-TADs and has been judged by myself to be relevant, designed in accordance with accepted scientific practices and norms and is in my opinion likely to be successful in achieving its aims.

03 October 2023

Signature (Reviewer) Date

J. PROTOCOL

Nature of Project (tick applicable answer)

A.

New Project	Extension to Approved Project	Amendment to Approved project
X		

B.

Research	Training	Animal products	SOP	Other (specify)
X				

C.

Start Date	End Date
01 August 2023	30 August 2026

1. Classification of project according to Categories A-E

In order to comply with the Institutes requirements for animal experimentation it is essential that you read the attached list for the explanation of categories carefully before marking the relevant block with an **X**

Category	
A	
B	
C	
D	X

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2. Background Information

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hooved animals caused by FMD virus (FMDV), a member of the Aphthovirus genus in the Picornaviridae family (Grubman and Baxt, 2004). FMDV is a small (approximately 30 nm) positive-sense single-stranded RNA virus with a non-enveloped icosahedral capsid. There are four structural proteins (VP1-4) found in the capsid of FMDV (Jackson et al 2003). It has been reviewed by Mason et al 2003, that the genome of FMDV is approximately 8.3 kb long and composed by a 50 untranslated region (UTR, approximately 1.3 kb), a single open reading frame (ORF, approximately 7 kb) and a 30 UTR (approximately 90 nucleotides long). FMDV consist of seven serotypes (A, O, C, Asia1, SAT1, SAT2, and SAT3) as well as multiple subtypes (Grubman and Baxt, 2004). In Southern Africa, the Southern territories SAT 1, 2 and 3 are prevalent and occur differently based on their incidental occurrence. Most of the outbreaks reported in South Africa for both livestock and wildlife are due to SAT 2, followed by SAT1 and then SAT3 (Bastos et al 2003; Hall et al 2013 and Dyason, 2010). These serotypes are maintained in domesticated livestock (aldazo-Gonzalez et al 2012; Ahmed et al 2012) and in buffalos (Maree et al 2016).

FMD poses major constraints to international trade in animal products owing this to its high contagiousness and broad spectrum of host species that includes both wild and domesticated ruminants and suids (Alexandersen et al. 2002). When FMD virus is not completely cleared during acute infection, it results in persistent asymptomatic infection, which limits the control and eradication of FMD, in addition to the number of serotypes and subtypes that exists and the transmissibility by air (Eschbaumer et al. 2016). Thus, asymptomatic FMDV carrier animals are a major threat and the existence of the carrier state complicates regaining FMD-free status. The carrier state can last for up to 5 years in buffalos, but the proportions of carriers under field and experimental conditions start declining after 3 years. In cattle, it is known that the virus can survive up to 30 days and remain infectious to susceptible cattle via direct inoculation with oropharyngeal fluid (Arzt et al., 2018). There is still knowledge gap in how the carrier animals regularly transmit infection to susceptible animals in livestock species due to several failures in attempt to prove if carriers can infect naïve animals (Salt, 1993; Alexandersen et al., 2002; Sutmoller and Casas, 2002; Hayer et al., 2018; Bertram et al., 2018). Previously Li et al 2019, reported on differentially expressed immune-related genes during persistent infection and during acute infection in BHK cells infected with FMDV strain serotype O (Akesu/58/2002). This finding provide the foundation to further understand the mechanisms of persistent FMDV infection including the genes involved in FMDV replication specifically using in vitro experiments. To date there has been no study done whether in vivo/in vitro on genome analysis and immune related genes on SATs viruses' serotypes using next generation sequencing technologies.

Foot-and-mouth disease virus infection have several effects on host response. When the host is infected, fever, skin vesicular lesion, lameness, and salivation are common clinical signs in cloven-hoofed animals during the acute stage of FMDV infection (Meyer and Knudsen 2001). Following acute infection by FMDV, the innate immune system of the host will be activated to establish an antiviral state (Guo et al 2015). Innate immunity can be defined as a semi-specific and widely distributed form of immunity, which represents the first line of protection against pathogens (Kumagai et al., 2008; Riera Romo et al., 2016). Its main elements are physical and chemical barriers, humoral and

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cell-mediated components. In order to prevent onset of the disease the innate immune response play a pivotal role as first line defence (Cole et al 2017).

Transcriptomics has improved the understanding of complex and interconnecting mechanisms of the host immune response to diseases (Mutz et al., 2013). Transcriptome analysis using RNA sequencing technology has been used in several viral and parasite studies that sought to understand host response to infection, including FMDV (Zhao et al 2017, Saravanan et al 2021) African horse sickness virus serotype 4 (AHSV4) (Faber et al., 2022), bluetongue virus (Singh et al., 2017) and Toxoplasma gondii (Zhou et al., 2016). Recently, Zhao et al. (2017) analysed the transcriptome of porcine peripheral blood mononuclear cells (PBMCs) to establish the differential regulation in FMDV infected and non-infected cells.

The relationship between host gene expression and the persistent infection is not well understood. Understanding the mechanism of persistence in the host, can be enhanced through understanding of the molecular mechanism that regulates viral infection, cell killing capacity and immune system evasion during persistent infection in the host. Herein, we propose to use a combination of transcriptome sequencing, genome sequencing and immunology to understand and define persistent infection at the selected anatomical sites that have been proven to maintain persistent infection in vivo; nasopharynx, larynx and lymph nodes in cattle as well as in baby hamster kidney (BHK) cells. Complementary, whole genome sequencing will be used to track any significant FMDV genetic variation at the specified anatomic sites that may occur during a persistent infection. This will in turn help us understand how and where the reservoir site of FMD in the host cells is. The Southern African Territories 1,2 and 3 (SAT 1,2&3) serotypes will be used to experimentally infect 32 cattle (between 6 and 12 months of age mixed genders) in vivo. Both Antemortem and Post-mortem samples will be collected as indicated in figure 1. Time points from 2-14 will assist in defining innate immunity, viremia and sequence variations during acute infection whiles from 28-49 will be for persistence infection and adaptive immunity. Two PhD and one MSc students have already been identified and have recently finalized their registration. The first PhD student will focus on global genomics analysis of FMDV whiles the second student will focus on global transcriptome profiling of host immune responses after infection with FMDV. Lastly, the MSc student will perform FMDV genomic in vitro studies on BHK cells infected with SAT 1, 2 and 3.

3. Aim/s of the Proposed Study/SOP

Study 1 aim: **To determine genomic variation of SAT 1, 2 and 3 in BHK cells.**
Objectives:
- Initiate conventional FMDV SAT 2 infection in BHK-21 cells
- Establish persistent infection
- Perform next generation sequencing analysis

Aim/s of the Proposed Study/SOP

Study 2 aim: To compare genomic variation of FMDV in acute and persistent infection.
Objectives:
- Infect cattle with SAT viruses using intra-nasopharyngeal inoculation (INP) method

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- Confirm FMDV infection in animals infected with newly INP inoculation system
- Perform next generation sequencing analysis to determine genomic variation during acute and persistent infection.

Study 3 aim: To determine global transcriptome of host immune response after infection with FMDV.
Objectives:
- To characterise mucosal immunity to FMD SAT infection.
- To characterise B and T cells phenotype and proliferation.
- Perform transcriptomics analysis to determine innate and adaptive immune response.

4. Potential Benefits of the Research Findings

Foot-and-mouth disease is a major viral disease in animals worldwide, affecting both national and international trade of livestock and animal products and leading to high economic losses and social consequences. Control and eradication of FMD is often limited by the number of serotypes and subtypes that exists and the transmissibility by air and the occurrence of prolonged asymptomatic carrier state in a large population of infected animals. Thus, there is a need for better understanding of factors that contributes to the prolonged persistent infection. Knowledge will be available to policy/regulation makers and scientist for assisting in managing and controlling the disease. Understanding of transmission mechanism underlining persistent FMDV infection and activation of innate and adaptive immune response in livestock will educate policy makers. Furthermore, the knowledge generated will also assist in development of next-generation vaccines and antiviral products against FMDV. The project will provide an opportunity to previously disadvantaged students (black female) to be trained on trending cutting edge technologies which are a scarce skill in our country, especially in veterinary science Two PhD and one MSc students.

At least four peer-reviewed scientific articles are envisaged:

1. Pathogenesis and clinical manifestation of FMD Virus SAT2 Infections In acute and persistent experimentally infected cattle.
2. Genomic variation analysis of FMDV infection between acute and persistent infection in tissue samples.
3. Global transcriptome of host immune response during acute and persistent FMDV SAT 2 infection.
4. Genomic variation analysis of FMDV infection between acute and persistent infection in BHK cells.

Presentation of research work at conferences/scientific meetings.

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5. Hypothesis

We hypothesise that the anatomical site will present minimal or no genetic variation of FMDV during persistent infection in cattle and may possibly be the reservoir of the virus. Analysis of the genome sequencing will aid in identification of possible sites that may act as the reservoirs of the virus during persistent infection. Similarly, both innate and adaptive immunity will be evaluated from the acute to the carrier state. This will give us a better understanding of the host immune evasion during both acute and persistent FMDV infection.

6. Experimental Design and Procedure/s

Describe briefly in layperson's terms in a sequence of short annotated sentences (a), (b), etc. all the steps to be performed in conducting the experiment including operative procedures, collection of samples (give blood volumes and routes of collection if applicable), other measurements to be done, duration of study, data processing and comparisons. Include a description of how the animals will be allocated to experimental and control groups and the treatment assigned to each group (where applicable).

Material and methods

Study 1 aim: To determine genomic variation of SAT 1, 2 and 3 in BHK cells.

6.1.1 Viruses

SAT1 (KNP/196/91/1), SAT2 (KNP/19/89/2) and SAT 3 (KNP/1/08/3) FMD viruses will be obtained from the ARC-OVR-TAD biobank. Briefly SAT1/2/3 viruses will be propagated on BHK cells, clone 13 as previously described (Chitray, et al. 2020). Briefly, the cells will be infected with $1x10^5$ $TCID_{50}$ at 37°C in 5% $C0_2$. The viruses will be harvested when 70-80% of cells are infected and titrations will be performed using the standard plaque assay method as previously described (Chitray, et al. 2020).

6.1.2 Establishment of persistently and acutely infected cells

Establishment of persistently and acutely infected cells (in vitro) will be done to compare with in vivo experiments. This objective will form part of MSc student project. The same isolates used to infect the cells in vitro will be used in the in vivo experiments. Establishment of persistently infected cells will be performed as previously described by Li et al 2019. Briefly, BHK-21 cells will be propagated in 6-well plates and will be infected with FMDV (SAT1/SAT2/SAT3) at a concentration of 10^4 $TCID_{50}$. After 1 h of absorption at 37°C, cells will be washed for 1 min with 0.1 M phosphate buffer (pH 6.0) to inactivate unabsorbed virions and washed again extensively with GMEM. The infection will be allowed to proceed in GMEM (5% FBS) supplemented without or with 10, 15, 20, 30, 40 mM ammonium chloride for 48 h at 37°C without CO2, respectively.

Acutely infected cells will be developed by incubating BHK-21 cells with wild-type FMDV at a concentration of 10^4 $TCID_{50}$ (SAT1/SAT2/SAT3) according to a previously published protocol by Huang et al. 2011. Cells will be collected 24-48h post infection. Persistently

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infected BHK-21 cells will be collected, and wild-type BHK-21 cells will be used as a negative control. Three independent replicates will be conducted for each cell group.

RNA extraction

Total RNA will be isolated from SAT 1-3 acutely and persistently infected cells according to the TRI® Reagent protocol (Sigma Aldrich®). Contaminating genomic DNA will be removed by using a DNA-free kit (Ambion) according to the instructions of the manufacturer. The total RNA will be quantified using a spectrophotometer ND-1000 Nanodrop® (Thermo Scientific) where samples with an absorbance ratio value (A260/A280) of 1,8–2,0 will be considered for further analysis and assessed by agarose gel electrophoresis (Li et al., 2020).

Quantitative real-time RT-PCR

Real-time RT-PCR will be performed on acutely and persistently infected cells. Real-time RT-PCR will be done using GoTaq® Probe 1-Step RT-qPCR System (Anatech) according to the manufacturer's instructions. The sense and antisense primers targeting the FMDV 3D region will be used, with the 5' end labeled with 6-FAM, and the 3' end with TAMRA (Callahan et al., 2002). The CFX96™ Real-Time PCR Detection system (Bio-Rad, CA, USA) will be used for detection of viral nucleic acid material. Specimens with a cycle threshold value ≤35 will be considered positive (Lazarus et al., 2019)

Illumina library preparation and genome sequencing

The cDNA library will be constructed within the high facility contaminant area following protocols provided by Illumina (NEB Next Ultra RNA Library Prep Kit, Illumina, USA). The cDNA will be subjected to all requirements for transferring materials from the BSL3 facility to Biotechnology platform for sequencing.

One nanogram of each dsDNA sample will be used to prepare sequencing libraries using the Nextera XT DNA Sample Preparation Kit (Illumina) according to manufacturer's instructions. Libraries will be sequenced on MiSeq at the Agricultural Research Council Biotechnology Platform. The 300 cycle version 2 reagent cartridges (Illumina) will be used to produce paired end reads of approximately 150 bp each (Logan et al., 2014).

6.1.4 Genome Sequence data analysis.

Genome data will be visualised and analysed using the CLC Genomics Workbench 21.4. (https://www.qiagenbioinformatics.com/). Consensus sequences will be obtained using a complete published sequence as the reference genome, or, where a closely related reference will not be available, a de novo assembly will be used. Sequence read quality will be monitored with FastQC prior to Sickle trimming all bases with a score of <30. For de novo trimmed Fastq files will be processed using Velvet v1.2.10 with an optimum Kmer length determined by Velvet-Optimiser. A minimum contig length of 1000 will be included in L fragment analysis. A BLAST search with the contigs will be used to confirm viral origin. Final contig assemblies will be completed manually in BioEdit. All genome sequences produced in this study will be submitted to NCBI GenBank.

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Study 2 aim: To compare genomic variation of FMDV in acute and persistent infection in vivo

6.2.1 Animals

A total of 108 cattle, which will be divided into three phases of studies representing each serotype (P1 SAT 1 N=32, P2 SAT2 N=32, P3 SAT 3 N=32). There will be 4 additional animals for standardizing Intranasopharyngeal infection method in each serotype or study phase. These animals will be obtained from the ARC-OVR farm (Kaalplaas) and will be between 6 and 12 months of age mixed genders. In case the animals are unavailable from Kaalplaas or tested positive for FMDV antibodies, animals will be purchased by the University of Pretoria in Makapanstad farm (University of Pretoria large animal supplier). Prior transportation of animals to TAD laboratories blood samples will be collected and the animals will be confirmed to be free from SAT antibodies by the OIE reference laboratory, Transboundary Animal Diseases (TAD) (Agricultural Research Council). Once animals are confirmed FMDV negative, they will be transported using a secured truck to ARC-OVR-TAD South Africa Biosafety level 3 laboratory. Mr Ntshangase/Pule, in conjunction with the TADs Veterinarian will be responsible for animal welfare during their transportation to TAD stables. Daily rectal temperatures and general health observations will be monitored daily. Prior to the experimental challenge, all the animals will be allowed to acclimatize to the environment for at least 7 days. One serotype will be used at a time. At the end of each experiment, the stables will be disinfected, using the OIE-recommended disinfectant for FMD. The commencement of the second experimental group and third experimental group will be determined by the availability of funds. Currently, we have funding approved to run SAT2 experimental challenge. Upon availability of funds to run all the three groups within one project funding, experimental challenges of the three different groups will be done minimum of four weeks apart from each other to avoid cross contamination of serotypes.

6.2.2 In vivo experiments

Confirmation of Intranasopharyngeal (INP) inoculation with SATs viruses.

Because the INP inoculation system has never been used in TAD facilities, as well as in SAT viruses, we will need to first confirm its effectiveness in establishing FMD clinical signs using two naïve cattle. These procedure will be repeated in all three phases of the project. If the initial dose does not result in clinical FMD in these cattle, we will need to adjust the dose to a higher level and inoculate in two further naïve cattle. In brief, 2ml of inoculum containing 10^6 $TCID_{50}$ of FMDV will be deposited within the nasopharynx of sedated animals using a 3ml syringe and a flexible plastic catheter measuring 30cm in length and 2mm in diameter. And these animals will monitored for FMDV clinical signs for 7 dpi (1 week). Upon successful development of clinical FMDV signs and accompanying virological parameters using 10^6 $TCID_{50}$, the dose will be adopted for the study. If the dose does not work, we will adjust the dose to 10^7 $TCID_{50}$ of FMDV using the next set of naïve animals.

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Experimental Procedure

All experimental cattle between 6 and 12 months of age mixed genders (N=36) weighing 400–500 kg and will be repeated in all phases of the study (SAT 1 and SAT3 study)

Animals will be divided into three groups

Group 1 (N=14, Intranasopharyngeal inoculation=INP)

Group 2 (N=14, Direct Contact inoculation=DCI)

Group 3 (N=4, Persistent Contact inoculation=PCI)

After Group 1 and group 2 transported to the facility and allocated separated stables (Group 1, N=14 will be divided in to 4 stables. Stable 1 and 2 will contain 3 animals each and stable 3 and 4 will contain 4 animals each. Group two will be housed in two separate stables each containing seven animals. All animals groups will be allowed to acclimatize for one week prior experiment commencement.

Group 1 will be inoculated with 10^6 or 10^7 $TCID_{50}$ of FMDV SAT 1, 2 and 3 using direct Intranasopharyngeal (INP) inoculation. Infectious dose will depend on the outcomes of initial test inoculation on section 7.2.2 paragraph 1. In brief, 2ml of inoculum will be deposited within the nasopharynx of sedated animals using a 3ml syringe and a 30cm length of 2mm diameter flexible plastic catheter. The challenge will be done as previously described by Pacheco et al 2016. After 24hrs post infection Group 2 will be introduced to INP infected cattle stable to allow direct contact, in the following arrangement; 3 naive animals will be introduced to 3 INP infected (Stable 1 & 2) and 4 naïve animals will be introduced to 4 INP infected (Stable 3 & 4). Now each stable will contain equal number of INP and DCI animals.

Sampling.

Day 0 samples (Antemortem samples) will be collected before animal experiment commence. The samples will be collected at three main stages of FMDV infection 1. Acute stage; Day 2, 4, 6, and 7. However, on day 4, 6 and 8 only Antemortem samples will be collected. 2. Transition stage; Day 14 and 21. 3. Persistent stage; Day 28, 35, and 49 as indicated in figure 1. In addition Antemortem samples will be collected from New naïve cattle introduced on D28 pi on days 2, 4, 6 and 8. At each time point four animals (2 INP and 2 DCI) will be sacrificed, and post-mortem samples will be collected. During sampling at a predetermined time point, no animal will be left as singlet. Figure 2 designate the sequence at which samples will be collect from stable 1-4. i.e., on Day 2 samples will be collected from stable 3 & 4. After day 28, four new FMD free animals will be introduced to the remaining 12 persistently infected cattle (to stable 3 and 4). After day 35 sampling point, remaining 8 animals ($4_{INP/DCI}$ and 4_{PCI}) will be housed together in one stable until day 49, to study the infection rate of persistently infected cattle to new FMDV free cattle.

The experiment will be terminated on day 49 and all samples will also be collected as in all other time points. The animals will be sedated by IM injection of xylaxine (0.22 mg/kg) and placed in sternal recumbency. Sedation in steers will be reversed by intravenous injection of Tolazoline (2 mg/kg) during sample collection. The samples for genome

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sequencing will be stored in minimum essential medium supplemented with hepes at -80°C until further processing. Whereas immunology samples will be processed for immune assay within 12 hrs.

After day 28, four new FMD free animals will be introduced to the remaining 8 persistently infected cattle (to stable 3 and 4). After day 35 sampling point, remaining 8 animals ($4_{persistent}$ and 4_{naive}) will be housed together in one stable until day 49, to study the infection rate of persistently infected cattle to new FMDV free cattle.

The experiment will be terminated on day 49 and all samples will also be collected as in all other time points.

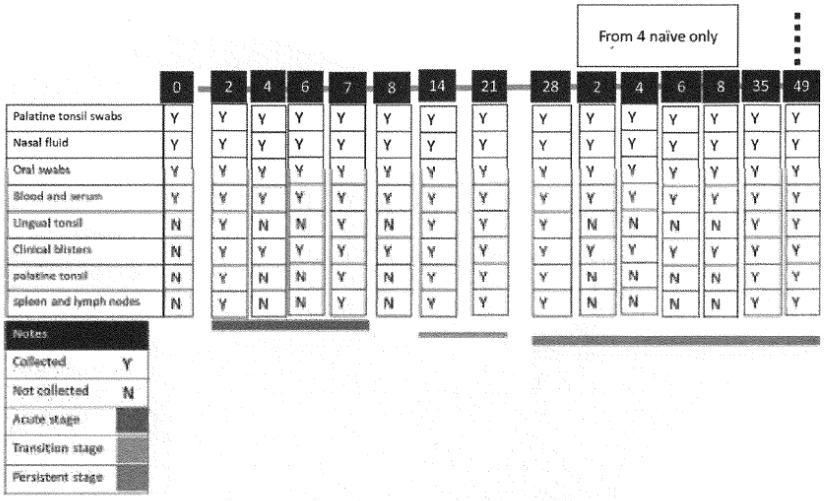

Figure 1. Sample collection guideline with types of samples collected from day 0 to day 49 in FMDV SAT 2 cattle infected using Intranasopharyngeal inoculation method (INP) and direct contact inoculation (DCI).

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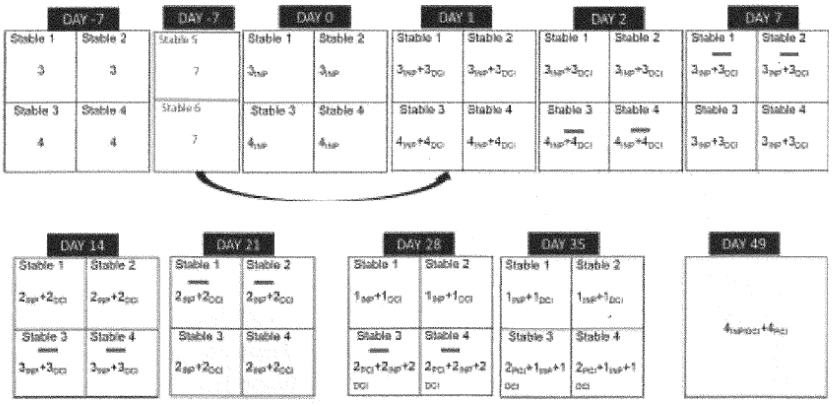

Figure 2. Animal sampling guideline in stables; Number of animals remaining are shown after each time point of collection. Red dash indicate stables to be sampled at time point. INP= Intranasopharyngeal inoculation; DCI= Direct Contact inoculation and PCI= Persistent Contact inoculation=PCI.

6.2.3 Sample collection

6.2.3.1 Ante mortem sample collection

Rompun (2% Xylazine) will be used for analgesia and sedation during all procedures other than taking of temperature. Ante-mortem sampling will consist of the blood, serum, nasal swabs, clinical blisters, saliva and the palatine tonsil swab. Blood sample collection

Blood will be collected as follows:

iii. Two BD Vacutainer-EDTA tubes (Becton Dickson) (~20 ml) for innate and adaptive immune responses and Elispot assays

iv. One VacuLab® serum tube per animal (~10 ml) for serological assays.

Palatine tonsil swab

The palatine tonsil swab will be collected as previously described by Casteleyn et al 2011. Briefly the tonsil sinuses will be swabbed individually with a nylon brush. A laryngoscope will be will be used to depress the tongue and provide lighting while swabbing the tonsillar crypts. In between the sampling, the Laryngoscope will be disinfected with citric acid and rinsed with PBS thereafter. The nylon brushes will be transferred to a container with 0.5 ml PBS and snap frozen. These samples will be used for RT-qPCR or virus isolation.

6.2.3.2. Post-mortem sample collection

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Necropsies will be performed immediately subsequent to euthanasia at predetermined time points as described in figure 1. The following tissues will be collected: larynx, nasopharynx, palatine and lingual tonsils, spleen, retropharyngeal lymph nodes, submandibular lymph nodes, popliteal lymph nodes and prescapular lymph nodes. For each defined specimen, two 30 mg tissue samples will be aliquoted into separate screw-cap 1.5 ml cryovials and within 2h to a -80°C freezer where they will be stored until the time of processing. These samples will be subjected to virus isolation, RNA isolation followed by RT-qPCR, transcriptome sequencing and genome sequencing.

6.2.4 RT-qPCR and virus isolation

Real-time RT-PCR will be conducted using GoTaq® Probe 1-Step RT-qPCR System, according to the manufacturer's instructions. Primers targeting the FMDV 3D region will be sense 5'-ACT GGG TTT TAC AAA CCT GTG A-3' and antisense 5'-GCG AGT CCT GCC ACG GA-3'. The probe will be 5'-TCC TTT GCA CGC CGT GGG AC-3'; its 5' end will be labeled with 6-FAM (Callahan et al., 2002). The CFX96™ Real-Time PCR Detection system (Bio-Rad, CA, USA) will be used for virus detection. Specimens with a cycle threshold value ≤35 were considered positive.

Virus Isolation

Virus will be isolated from tonsil swabs in RS cells and from tissue samples in ZZ-R 127 cells as described previously (Brehm et al 2009 and Charleston et al 2011). Samples with no cell death after three passages will be considered negative. Culture supernatants will be analyzed by qRT-PCR as described in this section.

6.2.5 Serology

6.2.5.1 Virus neutralization assays (VNT)

Neutralizing antibodies against SAT1/2/3 in serum samples collected from day 0, 2, 7, 14, 21, 28, 35, 42 and 49 from cattle will be measured with a VNT using the method previously described in the OIE Manual using IB-RS-2 cells in 96-well tissue culture plates (Maree, et al. 2015). The 50% end-point serum titres will be calculated using previously described methods (Chitray et al. 2020; Maree, et al. 2015). The antibody titres will be calculated as log10 of the reciprocal of the final serum dilution that neutralized 100 TCID50 of virus in 50% of the well.

6.2.5.2 Solid phase competitive ELISA (SPCE)

The antibodies to FMDV will be determined at 0, 2, 7, 14, 21, 28, 35, 42 and 49 using the SPCE as described previously. Briefly, 96-well microplates will be pre-coated with rabbit anti FMDV serotype-specific serum. Then 50 µl of the FMDV antigen (diluted in blocking buffer: 0.05% [w/v] Tween 20, 10% [v/v] NBS, 5% [v/v] normal rabbit serum.) will be added to each well of the ELISA plates and incubated on an orbital shaker at 37°C for 1 hour. Following washing three times with PBS, 50 µl of serotype-specific guinea-pig anti-FMDV antiserum will be added and samples diluted in clocking buffer. The plates will be diluted in a two-fold dilution series and incubated on an orbital shaker at 37°C for. Following

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washing four times with PBS, 50µl of the FMD conjugate reagent in casein will be added to each well and will be incubated at 37ºC+ 2ºC on an orbital shaker for 1 hour. The wash step will be repeated and 100 µl of the substrate will be added and the reaction will be stopped after 5-10 minutes incubation with the 50 µl stop solution. The plates will be read on Thermo Multiskan EX at 450nm. The results will be analyses as follows; 0% competition control (ODCa) the median will be calculated as the mean of two mid-values of the reaction control wells. For test serum a percentage of inhibition will be calculated for each well, using the following calculation: (100 – [optical density of each test or control value/median ty of the ODCa] × 100%), representing the competition between the test sera and the guinea-pig anti-FMDV antisera for the FMDV antigen on the ELISA plate. For the control sera the serum titre will be calculated as that dilution where a 50% reduction of the colour reaction is recorded. Antibody titres will be expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density of the reaction (antigen) control wells.

6.2.5.3 RNA extraction from blood and tissues.

Total RNA will be extracted by using QIAmp® viral RNA kit according to the manufacturer's instructions. The genomic DNAse contaminants will be removed by DNA-freeTm kit using the manufacturer's protocol. RNA quality and quantity will be determined by agarose gel electrophoresis, NanoDrop 2000 and Qubit RNA High Sensitivity (HS) Assay Kit.

6.2.6 Genome sequencing

Complementary DNA (cDNA) will be depleted from extracted total RNA samples through the activity of rDNase1 using the DNA-free DNAse kit. Briefly, 10 µg of extracted RNA in a 50 µl volume will be combined with 5 µl of DNase Buffer and 1 µl of rRDNase1 (2U) and incubated at 37°C for 30 min as recommended by the manufacturer's protocol and the sample will be incubated for a further 2 min at room temperature with periodic mixing. The samples will be centrifuged at 17,000 xg for 2 min and the DNAse-treated supernatant will be retained for subsequent processing.

6.2.7 Genome Sequence data analysis

Genome data will be analysed as described in section 6.1.4.

Study 3 aim: To determine global transcriptome of host immune response after infection with FMDV.

6.3.1 Animals will be infected as described in study aim 2 and samples will be collected as such.

6.3.2 Peripheral blood mononuclear cells (PBMCs) cells isolation

PBMC will be purified by using the Ficoll purification method as described previously (Nefefe et al 2017). Briefly, 20 ml of blood will be collected in BD Vacutainer-EDTA tubes (Becton Dickson) and will be diluted in 2:1 ratio with Hanks's Balanced salt solution (HBSS), containing 0.2% EDTA). The mixture will be under-layered with 15 ml of Ficoll-Histopaque and centrifuged at 900g for 40 min. The PBMC/Ficoll interface will be collected and diluted three times with Alsever's solution and centrifuged at 600 g for 10 min. The contaminating red blood cells in the pellets will be removed by lysis with 1 ml BD Pharm

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lysis solution. The pellets will be washed twice with the Alsever's solution followed by centrifugation at 300 g for 10 min. The pellets will be resuspended in 1 ml complete RPMI-1640 medium (RPMI-1640 medium containing 2 mM L-glutamine, 10% FBS, 5E-05 mercapto-ethanol, 0.1 mg/ml sodium benzyl-penicillin and 0.2 mg/ml streptomycin sulphate). An aliquot of the cells (1 µl) will be diluted in 90 µl complete media (1:100), and the diluted cells will be counted using TC10™ automated cell counter. These samples will be subjected to RNA isolation and transcriptome sequencing. Furthermore, a concentration of $1x10^7$ PBMCs samples will be shipped according to IATA guidelines and worked on in a Class II BSC inside the SAPO4 (equivalent to ABSL3) facilities at The Pirbright Institute. The package will be prepared within BSL3 TAD facilities according to laboratory shipping protocols.

6.3.3 RNA-sequencing and data analysis

The cDNA library will be constructed within the high facility contaminant area following protocols provided by Illumina (NEB Next Ultra RNA Library Prep Kit, Illumina, USA). The CDNA will be then heat inactivated and be subjects to all requirements for transferring materials from the BSL3 facility. RNA sequencing will be performed at the ARC Biotechnology platform from the prepared cDNA library. The methods for RNA sequencing of the host cells have been published previously (Nefefe, et al 2017; Tjale et al. 2018). RNA seq data will be visualised and analysed using the CLC Genomics Workbench 21.4. (https://www.qiagenbioinformatics.com/). The reference genome (bovine/ovine/FMDV) and gene annotation files will be downloaded from ENSEMBL. HISAT will be used to build the index of the reference genome. The expression levels of genes will be normalised by considering both the library size and gene length effects with respect to the RPKM values (reads per kilo base of gene model per million mapped reads). The average expression values will be normalised in quantiles prior to statistical test analysis. Differential gene expression will be defined as described previously (Tjale et al. 2018). Briefly, genes will be considered to be differentially expressed if the fold change (FC) ≥2, biological repeats>2 and p-value ≤0.05. Differentially expressed genes will be subjected to gene ontology databases to study the gene functions. Briefly, to study metabolic and signalling pathways KOBAS will be used based on Kyro Enclyocpedia of genes and Genomes (KEGG) (Xie et al. 2011). On the other hand, to study innate and adaptive immune responses pathway signalling, Amigo (http://amigo.gen eontology.org/amigo/landing) will be used. Genes that are up-regulated in experimental animals when compared to control will be determined. RNA seq data will be validated by RT-qPCR.

6.3.5 Flow cytometry
Lymphatic B-cells ($B_{Naïve}$), (2) regulatory B cells (B_{Reg}), (3) memory B cells (B_{Mem}), (4) plasmablasts (PB), and (5) plasma cells (PC) and T-cells $T_{Naïve}$, T_{AV}, T_{CM}, T_{EM}, T_{TE} and γδ-TCR cells will be stained from the PBMCs and tissues collected from infected animals using monoclonal antibodies (B-cells=S-IgL, CD21, CD40, CD71 and CD138 and T cells=CD8, CD4, CD45RO, CD25,CD62L and CD3+γδ) according to manufacturer's protocol. The Flow cytometry will be run using MACSQuant Analyzer 10 flow cytometer analyzer and data will be analysed by Kaluza version 1.2 software.

6.3.6 Cytokines ELISpot Assay

The ELISpot assay will be used to measure the amount of innate immune response cytokines produced upon stimulation of immune PBMC and tissues. The 96 well

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ELISpotPLUS IFN- γ kit and ELISpotPLUS IL-4 kit will be used and the assay will be carried out according to the manufacturer's instructions. Plates will be then read using the CTL-Immunospot S6 Universal Analyzer (ELISPOT) within TAD laboratory.

6.3.7 Movement of PBMCs procedure

Approvals

A material transfer agreement (MTA) will be compiled between the ARC and The Pirbright Institute to ensure that all legal, IP and commercialisation aspects are covered. A DALRRD Section 20 application has been submitted for approval for this work. No export will be conducted until the Section 20 is approved. Prior sending the samples to Pirbright, we will seek the master import permit issued by the Veterinary Import and Export office of Gauteng Department of Agriculture and Rural Development (GDARD) and the Pirbright. The Animal Disease regulations will also be followed when sending biological materials to the Pirbright from ARC-OVR: TAD. We will also seek approval from the Council for Non-Proliferation of Weapons of Mass Distraction as required under the Non-Proliferation of Weapons of Mass Destruction Act, 1993 (Act No.87 of 1993).

Packaging

Peripheral blood mononuclear cells (PBMC) will be transferred to Screw capped plastic Vials with 0-ring fitted top. The tubes will be completely closed to avoid any leakage of the frozen cells Each tube will contain $1x10^7$ concentration of cells. All the tubes will first be frozen in liquid nitrogen prior transportation. Then the frozen cells will be transferred in to a primary container with dry ice and then be wrapped in sufficient dry adsorbent material to adsorb the material in case of breakage. After the primary containers have been sealed, their exterior surfaces will be decontaminated with an appropriate decontaminating agent. The wrapped container will be placed in a small waterproof plastic bag that is sealed to prevent leakage if the primary container breaks. These sealed bags will be then be placed in a secondary container (metal construction). The bags will be cushioned with additional adsorbent cotton to prevent jarring of the contents. A sufficient quantity of dry sodium carbonate will be placed inside the secondary container to ensure inactivation of the virus should the primary container and the plastic bag break. The secondary container will also be sealed and the lids will be taped closed. Appropriate labels must be placed on the outer transport container (UN Recommendations on the Transport of Dangerous Goods – Model Regulations, 12th edition or updated requirements). The Biosafety Office will be requested to record in an appropriate register, the date when biological materials will be removed from The ARC-OVR: TAD. This Office will also maintain an appropriate logbook indicating the nature of the material (based on label information), the date it was moved, who sent it, and who will received it. Form BSF 12 for Authorisation to remove items from contaminated area will be filled.

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Transportation

The Biosafety personnel will be responsible for coordinating transportation for the PBMCs leaving the ARC-OVR: TAD. Necessary permits and approval form the Research Team Manager: Vaccine Production (RTM: VP), arranging for shipment of the PBMCs will be organised prior transportation. Personnel who have been trained in specific procedures for the transportation of biological materials will be assigned as couriers to transport the cells to airport for Pirbright Delivery. In case of an emergency, the Head of Division will be must be notified immediately; and ARC-OVR: TAD and Pirbright telephone number and appropriate extension numbers will be displayed for ease of contact.

7. Justify the use of selected species as an appropriate animal model

FMD is a globally important livestock disease affecting domestic cloven-hoofed animals such as goats, sheep, pigs and cattle. Cattle will be used as an animal model.

8. Animal Requirements	
Animal Species:	Cattle
Strain / Gender / Body mass / Age	6-12 months
Total Number Required:	108
Date Required:	1 August 2023
Microbial Status:	FMDV free
Source of Animals:	Kaalplaas/ Makapanstad
Large farm animals: Will Animal Health Technician be able to supply the animals?	YES Signed by:Jerome Nkosiyakhe Ntshangas Signed at:2023-10-03 16:25:02 +02:00 Reason:Witnessing Jerome Nkosiyakhe Signature: DATE 03/10/23
Large farm animals: Are there sufficient stables/pens to house the animals?	YES Signature: DATE 10/10/23
Stable/pen number/name where animals will be housed	TAD-Facility large stable 1-4.

9. Reduction of number of animals to a minimum to achieve scientific objective

The reduction of the number of animals to be used in the study was determined by both validated existing protocol and statistical design.

10. Animal Caging and Care

In the bio-containment stables, each group of animals will be housed together in a stable with a minimum floor surface of 25.23 m^2. The cattle will be able to move freely within

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the confines of the stable. Experienced animal attendants will conduct daily contact including grooming. Animals will be fed pellets (hay not suitable due to its impact on the facility's effluent sanitation system).

The stables and food troughs will be inspected daily to ensure cleanliness and adequate supply of feed. Water will be provided ad lib by automatic watering system. The ambient temperature between (18°C-26 °C) and relative humidity (30-70%) in the animal facility will be kept. Well experienced animal health technician, animal handlers will provide proper animal welfare during trial.

The floor of the stable is designed not to be slippery and disease security regulations for the agents used in the TADs stables dictate that no material which makes disinfecting stables problematic can be allowed. For this reason we avoid materials which could be difficult to disinfect so hay as fodder and shavings for bedding cannot be used since they block the effluent plant, which in turn could lead to biosafety problems.

11. Restraint of the Animals

Animals will be physically restrained in a holding pen and sedated for all procedures other than taking of temperatures.

Rompun (2% Xylazine) at 0.67ml/100kg will be used for sedation during all procedures other than taking of temperature.

All procedures will be performed by experienced personnel

12. Invasiveness

Describe the level of invasiveness of the procedure/s (nil, minor, moderate, major), the manipulation/s to be performed on the animals and the expected degrees of distress and pain which these may cause. State how these will be dealt with to ensure the welfare of the animals.

Blood collection: invasiveness is minor, animals will be physically restrained and sedated using (Rompun; 2% Xylazine) at 0.67ml/100kg.

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Nasal and Oral swabs collection are minor invasive procedures and the animals will be physically restrained and sedated using (Rompun; 2%Xylazine) at 0.67ml/100kg.

Intranasopharyngeal inoculation: invasiveness is moderate and animals will be sedated using (Rompun; 2% Xylazine) at 0.67ml/100kg for this procedure.

Tubing of animals for feeding will not be done as the risk of injuring the infected tongue mucosa is too high.

13. Refinement

Describe the specific steps that have been taken to refine the experimental procedures to make them as humane as possible.

Rompun (2% Xylazine) at 0.67ml/100kg will be used for sedation during all procedures other than taking of temperature.

When lesions are severe, animals will be treated with antibiotics (Duplocillin LA; 4.0 ml/100 kg) and/ or anti-inflammatory drugs (Flunixin Meglumine1.1 to 2.2 mg/kg). This will be done with the discretion of the veterinarian and with severity guidelines.

Using the guidelines in appendix B, substantial conditions is regarded as the severity limit for a challenged experiment.

All procedures are performed by experienced personnel.

14. End Point for Experiments that Induce Illness in Animals

Give the endpoints of data collection in experiments or procedures that may cause animals to become ill, lose weight, become distressed and justify these in terms of the needs of the experiment in order to attain its objectives.

Possible adverse effects from the infectious agent: Animals that are exposed to FMD viruses will show clinical signs that include a rise in body temperature, vesicles, erosions and ulceration on the feet or tongue, lethargy and inappetence. Any animal exhibiting exceptionally severe signs of disease such as secondary bacterial infection leading to severe lameness or hoof shedding, extreme difficulties in eating and drinking will be evaluated according to a humane score sheet and for a score exceeding 10, will be euthanised humanely by a competent person (veterinarian).

The welfare score sheet shown in appendix A. The score sheet will be used to define the severity levels in the cattle. The severity levels will determine when humane endpoint will be executed. International humane endpoints in FMD research are summarised in appendix B and the researcher would like to implement this in combination with the welfare score sheet. Using the guidelines in appendix B, substantial conditions is regarded as the severity limit for a potency experiment. The table with the endpoint and the welfare score sheet will be available at the stables where the animals are housed.

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15. Euthanasia

Animals will be sedated and euthanized using a penetrating captive bolt pistol followed by decapitation and exsanguination.

YES	**X**
NO	

Method of disposal of carcass:
Carcasses will be disposed of by incineration at the TADs facility. Animals will be sedated and euthanized using a penetrating captive bolt pistol followed by decapitation and exsanguination. Carcasses will be disposed of by incineration at the TADs bio-security level 3 facility.

16. Administration of Scheduled Medicinal Substances (Medicines Control Act)

Describe all substance administration to the animals and give routes of administration, dosages per body mass including anaesthetics, analgesics and agents used for euthanasia and state who is legally responsible for prescribing and administering the controlled Scheduled 3 – 6 Medicinal substances.

Xylazine 2%	0.67 ml/100 kg, Intramuscularly
Duplocillin LA	4.0 ml/100 kg, Intramuscularly
Flunixin Meglumine	2.2 mg/kg, Intramuscularly
FMD virus	0.2ml of FMDV at $TCID_{50}$ of 10^5/ml intranasopharengeally

Responsible Veterinarian (Print name): Dr Paidamwoyo Mutowembwa

Qualification: BVSc, MSc (SAVC Reg No: D08/9606)

Acceptance Signature:

Date: 03/10/2023

17. Statistical Analysis

Experimental data will be presented as mean ± SE. Statistical significance between groups will be determined by equal variance two-tailed Student's t tests. P values less than 0.05 will be considered to be statistically significant. P values less than 0.01 will be considered to be extremely significant. The Project supervisors (Profs Sibeko-

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Mathila and Melvin Quan and Drs K, Tshilwane, I and Nefefe T) will assist the students in performing statistical analysis.

18. Biohazard Statement

Live FMDV virus will be used as infection material and is neither infectious nor harmful to human beings. The proposed study involves the use of foot-and-mouth disease virus infected samples which pose a threat to cloven hoofed animals. All the work will be undertaken at the ARC-OVR (TAD) BSL-3 high containment animal facility and BSL-3 laboratory to minimise chances of viral escape. As mitigation measures against possible viral exposure, the use of Standard Operating Procedures (SOP) and Personnel Protective Equipment will be in place. The study design involves opening of stables and movement of animals after viral infection. This may pause a risk of contamination of animal rooms with naïve animals. This practice is a deviation from normal operations of TAD-Stable facility. To mitigate Bio risk, all transfers of naïve cattle to infected animal rooms will only be through the central corridor. The cattle will be move out of a clean stable into the central corridor, then the door to the clean stable will be closed immediately before opening the door of the stable with infected cattle (dirty stable). Soon after the animals are moved into the "dirty" stable the door is closed immediately followed by decontamination of the central corridor as described in TAD-BSF 08 Biosafety Manual section 9. The Animal experiment will use one viral isolate at a time to avoid cross contamination.

Safety Officer Name: Dr Paidamwoyo Mutowembwa

Signature: **Date:** 03/10/2023

19. Facilities Requirements

Are the proposed facilities where the animals will be housed in working condition and suitable for the housing of the animals for the duration of the entire experiment

Animal facilities are in functional order and are suitable for housing the cattle for the duration of the entire experiment. Biosafety Regulations and Instructions for the facility are directed to prevent spread of disease from the laboratory to animals that are in the immediate environment. These regulations are in line with national regulations administered by the department of Agriculture, Land Reform and Rural Development.

Facility Manager – Dr Livio Heath

Signature: Signed by:Livio Edward Heath
Signed at:2023-10-10 22:30:52 +02:00
Reason:Witnessing Livio Edward Heath

Date: 10/10/23

20.Score sheet included

YES	X
NO	

21. Has this application been submitted to any other Animal Ethics Committee?

YES	
NO	X

If yes, include the name of Institution/Department responsible and the approval number.

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Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

TRIAL PERIOD
STARTING DATE: 01/08/2023 ENDING DATE: 30/08/2026

PROJECT NUMBER:				
PROJECT TITLE:	Genomic analysis of Foot and mouth Disease Virus and Global Transcriptome of host immune response: Defining mechanism of persistent infection in cattle and activation of immune response.			
PROJECT LEADER:	Dr Tshifhiwa Nefefe			
DIVISION:	Vaccine and Diagnostic Development department (VDD)			
CATEGORY:				
SPECIES OF ANIMAL:	Cattle			
NUMBER OF ANIMALS:	108			

RECOMMENDATIONS BY ANIMAL ETHICS COMMITTEE

Date of AEC meeting for consideration:	Action Taken:	SIGNATURE: AEC-Chairperson Dr P. Mutowembwa
	APPROVED	pp deputy chairperson of the AEC-OVR

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

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Appendix A: Welfare score sheet

Date: _____

Animal number				
Clinical signs* feet-FR				
Clinical signs* feet-FL				
Clinical signs* feet-BR				
Clinical signs* feet-BL				
Temperature score <39,5 =0 39.5≤ to < 40=1 ≥ 40=2				
Nasal discharge score 1= serous 2= sero necrotic 3= necrotic				
Clinical signs* oral/snout/mouth				
Clinical signs tongue*				
Clinical signs teats/udder*				
Lameness = 1 Recumbent = 2 Neither = N/A				
Total score				
Off food/water				
Behaviour changes				
Secondary infection				
Summary				

*Clinical signs

None = 0. Elevated temperature/congestion or healing vesicle = 1.
Vesicle = 2. Severe lesions = 3 (up to detachment of heel or equivalent).

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Appendix B: International Humane Endpoint guidance

This table specifies the clinical findings and conditions for large animals (Cattle, Sheep, Pigs, Mini Pigs, Goats, and horses) used to define the Severity levels for individual animals. For every severity level the conditions described in the column to the right constitute humane endpoints, i.e. moderate conditions for a mild protocol, substantial conditions for a moderate protocol and Substantial Protocol Humane Endpoints for substantial Protocols. Any finding/condition observed during the routine inspections, which exceeds mild is recorded at least daily and the records of these observations are held in the isolation unit accessible for the NSPCA for the duration of the experiment.

MILD	MODERATE	SUBSTANTIAL	SUBSTANTIAL PROTOCOL HUMANE END-POINTS
General			
• Separation from others, • Delayed response to stimuli, slower movements, lethargic	• Abnormal posture e.g. head hung, back arched, lethagic	• Ignores human approach • rises and/or moves with great reluctance,	• Prolonged substantial behaviour of more than 3 days • signs of severe pain e.g. vocalization, grinding of teeth, trembling, oblivious to stimuli, • unwilling to rise for more than 4 hours, • unconscious
• Lack of enthusiasm for food	• Anorexia of up to 3 days duration	• Anorexia of more than 3 days duration.	• Anorexia of more than 4 days duration substantial weight loss
• Roughened coat	• Early signs of diarrhoea or other discharges	• Watery or bloody diarrhoea of more than 3 days duration, • heavy discharges	• Watery or bloody diarrhoea of 4 days duration, • severe dehydration

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		from eyes, nose or mouth, • sunken eyes, • staring coat, • evident dehydration of skin, • appearance of pressure sores	of 4 days duration, • multiple or large pressure sores unresponsive to therapy
• Body temperature increase	• Fever above 40°C	• Fever above 40°C for 4 or more days	
Infectious Agents: *Foot-and-Mouth Disease*			
• Local surface temperature increase just above hoof • Evidence of vesicular lesion development such as blanching on sites such as limbs, face, mouth, teats or vulva. • Some nasal discharge and salivation	• Lameness • Small vesicles or ulcers at no more then three sites. • Erosive lesions on feet & tongue/snout.	• Unable to bear weight on more than one foot • Large tongue erosions leading to hypersalivation and lip smacking, painful erosions on feet leading to loss of weight bearing, blisters or erosions on teats.	• Persistence of substantial signs beyond 3 days • Severely superinfected lesion that does not respond to therapy within 24 hours and prevents eating or standing, severe under-running or shedding of a hoof.
Interventions			
Normal blood collection Anxiety and resistance during blood collection.	• Severe inflammation or infection at site of venepuncture (Moderate)	• Haemorrhage leading to anaemia, dehydration, tachycardia or tachypnoea. 'Shock syndrome'	• Weakness or inertia due to blood loss. 'Shock syndrome' in pigs from which the pig does not

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Need to administer sedation. (Mild)		in pigs leading to cyanosis, dyspnoea	recover when left quietly for 1-2 hours
Physical administration of infectious agent	• Anxiety & resistance during administration. Possible need to administer sedation • Severe inflammation or infection at site of administration		
Probang sampling	• Blood flecks in probing material. Discomfort on sampling (especially small ruminants) • Regurgitation of rumen content with risk of inhalation. • Pronounced haemorrhage into oral cavity/pharynx (extremely rare). Insertion of probing cup into trachea with consequent transient asphyxia.	• Inhalation pneumonia due to rumen contents. Delayed recovery of normal breathing following obstructive asphyxia (unlikely)	• Non-recovery of normal breathing following removal of probing. Pneumonia not responsive to treatment.
Collection of lachrymal fluid, nasal, oral or rectal swabs and/or milk			
Mild discomfort. Flecks of blood on/in the sampled material.	• Pronounced discomfort, haemorrhage or secondary infection at site of sampling.	• Pronounced discomfort, haemorrhage or secondary infection at site of sampling.	• Severe signs that do not respond to treatment within 2 days.
Drug or reagent e.g corticosteroid, anti-inflammatory, vaccine, other infectious agent combinations, low dose general anaesthesia			

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Adverse drug reactions that are mild and are included on the drug datasheet as recognised side effects of the drug or its administration	• Severe drug reactions, whether or not they are included on the drug data sheet.	• Severe drug reactions that compromise animal welfare and that do not respond to palliative treatment within 2 days.	• Severe drug reactions that compromise animal welfare and that persist for more than 2 days despite palliative treatment
Actions			
No action required maintain monitoring	• No direct action required maintain monitoring, discuss signs with named veterinary surgeon. Vet to perform full clinical examination if signs are unexpected in nature or severity.	• Direct action required increase monitoring, inform veterinary surgeon. Vet to perform full clinical examination. • Animal to be segregated where possible. • Placed on straw bedding • Consider oral rehydration treatment. • Ameliorative treatment course initiated if appropriate	• Direct action required inform named veterinary surgeon &/or project licence holder. • Humane termination of affected animal/s by schedule 1 method

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Weekly animal health checks by veterinarian

1. Visually evaluate each group of animals for:
    a. Locomotion
    b. Appetite (feed and water intake)
    c. Discharge/excretions (faeces, urine, nasal and ocular discharge)
    d. Habitus (bright, alert, responsive)
2. Check the week's health records and record any abnormalities noted
3. Perform a full clinical examination on any animals found to have abnormalities and begin treatment if required
4. Discuss the health of the herd with the animal handlers and project managers
5. Check treatment records for the week to evaluate correctness of treatment
6. Evaluate general welfare of animals and suggest improvements where necessary

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Project: _____ Herd/Animal group: _____

G. PROJECT TITLE

1. Animals with visually detected abnormalities

Animal ID	Date	Abnormality

2. Abnormalities detected in week's health records (that have not already been tended to)

Animal ID	Date	Abnormality

3. Abnormalities detected in clinical examination

Animal ID	Date	Abnormality

4. Issues raised in discussion with animals handlers and/or project manager

Animal ID	Date	Abnormality

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General issues (please record date)

5. Problems noted in treatment records (please record date)

6. Welfare concerns and suggestions for improvement

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7. Responsible Veterinarian

Name	Contact Number	e-mail address	Contact Address

Division	
Qualifications	
Appropriate experience in animal research	
Details of involvement	

Signature: _____ Date: _____

8.2 List of codes used

#Perform Quality Control

```
fastqc -o QC_results myfile_1.fq.gz myfile_2.fq.gz
```

#Trim using trimmomatic

```
java -jar Trimmomatic-0.39/trimmomatic-0.39.jar PE -phred33 \
myfile_1.fq.gz myfile_2.fq.gz \
myfile_1_paired.fq.gz myfile_2_paired.fq.gz \
ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15
MINLEN:36
```

#de novo assembly

```
# Define input files
forward_reads="myfile_1.fq.gz"  #Forward reads
reverse_reads="myfile_2.fq.gz"  #Reverse reads
output_dir="spades_output"       #Output directory for assembly
```

#Run SPAdes for paired-end reads

```
spades.py \
  -1 $forward_reads \
  -2 $reverse_reads \
  -o $output_dir \
  --careful # Option to reduce mismatches and indels in the final contigs
```

#Align reads to reference genome

```
bwa mem ref_genome.fasta myfile_1_paired.fq.gz myfile_2_paired.fq.gz > myfile.sam
```

#Convert SAM to BAM file

```
samtools view -Sb myfile.sam > myfile.bam
```

#Sort BAM file

```
samtools sort myfile.bam -o myfile.sorted.bam
```

#Index the BAM file

samtools index myfile.sorted.bam

#Index the reference genome

samtools faidx ref_genome.fasta

#Variant calling

bcftools mpileup -Ou -f ref_genome.fasta myfile.sorted.bam | bcftools call -mv -Ob -o myfile.bcf

---

#Convert BCF to VCF

bcftools view myfile.bcf > myfile.vcf

#Compress and index the VCF file

bgzip myfile.vcf

tabix -p vcf myfile.vcf.gz

#Normalize and filter variants

bcftools norm -f ref_genome.fasta myfile.vcf.gz -o myfile_norm.vcf

---

#Compress and index the normalized VCF file

bgzip myfile_norm.vcf

tabix -p vcf myfile_norm.vcf.gz

#To list the positions of the variants

bcftools query -f '%CHROM\t%POS\n' myfile_norm.vcf.gz

#Manually inspect variants

bcftools view -v snps myfile_norm.vcf.gz

bcftools view -v indels myfile_norm.vcf.gz

bcftools view -v mnps myfile_norm.vcf.gz

#List all variants

```
echo "SNPs: $(bcftools view -v snps myfile_norm.vcf.gz | grep -v "^#" | wc -l)"
echo "Insertions: $(bcftools view -v indels myfile_norm.vcf.gz | grep -v "^#" | awk 'length($4) < length($5)' | wc -l)"
echo "Deletions: $(bcftools view -v indels myfile_norm.vcf.gz | grep -v "^#" | awk 'length($4) > length($5)' | wc -l)"
echo "MNPs: $(bcftools view -v mnps myfile_norm.vcf.gz | grep -v "^#" | wc -l)"
```

```
#Annotate variants using snpEff
snpEff ann hg19 myfile_norm.vcf.gz > annotated_myfile_norm.vcf.gz
```

```
#Verify and summarize annotation
grep -e "^##SnpEff" annotated_myfile_norm.vcf.gz > annotation_summary.txt
```
