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Characterisation and control of foot-and-mouth disease outbreaks in Zambia between 2015 and 2020

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Declaration

I declare that the thesis, which I hereby submit for the degree PhD (Veterinary Tropical Diseases) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required research ethics approval/exemption for the research described in this work. The author declares that they have observed the ethical standards required in terms of the University's Code of Ethics for scholarly activities.

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Dedication

Amama, this is for you… my biggest cheerleader of all times …I will always cherish you.

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List of Abbreviations

Ag-ELISA	Antigen detection ELISA
ARC-OVI	ARC-Onderstepoort Veterinary Research
BHK-21	Baby hamster kidney fibroblast cells-21
BIC	Bayesian information criterion
BSL	Biosecurity level
BTy	Bovine thyroid
BVI	Botswana Vaccine Institute
CFT	Complementary fixation test
CPE	Cytopathic effect
CVRI	Central Veterinary Research Institute
DIVA	Differentiate infected from vaccinated animals
DVS	Department of Veterinary Services
EA	East Africa
ELISA	Enzyme-linked immunosorbent assays
FAO	Food and Agriculture Organization
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus (renamed *Aphthovirus vesiculae*)
IB-RS-2	Instituto Biologico-Rim Suino-2
IgG	Immunoglobulin G
IZSLER	Istituto Zooprofilattico Sperimentale della Lombardia e Dell'Emilia Romagna
GFRA	Global Foot-and-Mouth Disease Research Alliance
KNP	Kruger National Park
LAMP	Loop-mediated isothermal amplification
LFD	Lateral flow device
LPBE	Liquid-phase blocking ELISA
MAbs	Monoclonal antibodies
MCF	Malignant catarrhal fever
MEM	Minimum essential medium
ML	Maximum likelihood
NGS	Next-generation sequencing
NSP	Non-structural proteins
NWZ	Northwest Zimbabwe
OIE	Office International des Epizooties (renamed WOAH)
PAbs	Polyclonal antibodies
PCP	Progressive control pathway
PCR	Polymerase chain reaction
PVM	Post-vaccination monitoring
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT-qPCR	Real-time reverse transcription-PCR

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RT	Reverse transcriptase
RT-iiPCR	Reverse transcription-insulated isothermal PCR
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcriptase polymerase chain reaction
SADC	Southern African Development Community
SAT	Southern African territories
SP	Structural protein
SPCE	Solid-phase competitive ELISA
SSA	Sub-Saharan Africa
SSARRL	Sub-Saharan Africa regional reference laboratory
SVDV	Swine vesicular disease virus (renamed *Enterovirus betacoxsackie*)
TAD	Transboundary animal diseases
TFCA	Transfrontier conservation areas
UK	United Kingdom
UTR	Untranslated
VCF	Veterinary cordon fence
VP	Viral protein
VI	Virus isolation
VN	Virus neutralisation
VNT	Virus neutralisation test
VSV	Vesicular stomatitis virus (renamed *Vesiculovirus indiana*)
WOAH	World Organisation for Animal Health
WRLFMD	World Reference Laboratory for Foot-and-Mouth Disease

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Abstract

Repeated outbreaks of transboundary animal diseases, particularly foot-and-mouth disease (FMD), have posed significant challenges to the development of the livestock industry in Zambia and the broader Southern African region. This sector is crucial to national food security and livelihoods and holds potential for greater economic contribution due to Zambia's favourable natural resources. This study explores the molecular epidemiology of the FMD virus (FMDV) in Zambia, focusing on the emergence and spread of serotypes O/EA-2, A, SAT-2, and SAT-3. A scoping review of diagnostic assays for SAT serotypes recommended the combined use of SAT-1, SAT-2, and SAT-3 lateral flow devices (LFDs) for effective viral detection, with the PrioCheck®-NSP kit proving highly effective for non-structural protein antibody identification. The immunogenicity of the O-Manisa vaccine was assessed in cattle challenged with the O/EA-2 outbreak strain, revealing significantly higher antibody titres in double-dosed animals at 56 days post-vaccination (dpv) compared to single-dosed groups. Furthermore, a strong positive correlation was observed between virus neutralisation tests and solid-phase competitive ELISA (SPCE), supporting the use of SPCE in immunogenicity assessments, particularly in resource-constrained settings. The findings underscore the complexity of FMD epidemiology in Zambia and highlight the urgent need for continuous monitoring of serotype O/EA-2 and a coordinated regional strategy for FMD control across Southern Africa.

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Chapter 1. Introduction

1.1. Introduction

Foot-and-mouth disease (FMD) is a contagious viral disease of livestock and wild cloven-hoofed animals. The most important epidemiological and economic hosts are cattle, pigs, sheep, goats and free-ranging African buffalo (*Syncerus caffer)*. The disease is characterised by acute fever and the development of vesicular lesions in the mouth and feet of infected animals. The diverse FMD strains (Maree et al., 2014) and the involvement of wildlife in the epidemiology of FMD pose a challenge to controlling this disease in sub-Saharan Africa (SSA) (Weaver et al., 2013).

The aetiology of the disease, *Aphthovirus vesiculae,* is a single-stranded, positive-sense RNA virus which exists as seven discrete serotypes, namely types O, A, C, Asia-1 and the South African territories (SAT) serotypes (SAT-1, SAT-2, SAT-3). These serotypes are divided into topotypes, genetic lineages, sub-lineages and strains (Mahapatra et al., 2015, Dill et al., 2017). Globally, there are seven FMD endemic pools of infection, which often require tailored diagnostics and vaccines (Freimanis et al., 2016, Paton et al., 2009), as each virus pool has more than one serotype, within which FMDV strains evolve and circulate (Knowles et al., 2016c, Knowles and Samuel, 2003). Greenland, Iceland, New Zealand, and the smaller islands of Oceania are generally recognised as FMD-free and have never experienced an outbreak due to their strict biosecurity measures and geographic isolation (WOAH, 2022b). Further, Antarctica's harsh climate and absence of domestic animals contribute to its status as FMD-free. The disease circulates in Asia, Africa and parts of South America, but it has a global distribution that mirrors poverty and livestock density (Knight-Jones et al., 2016). It is endemic in Africa, and its epidemiology here is more complicated than in other parts of the world as its home of six of the seven FMD virus serotypes, with multiple topotypes within these serotypes complicating vaccine development and disease control. In Southern African Development Community (SADC) countries, the FMDV SAT strains are reported commonly, with sporadic incursions of serotypes O and A reported from only Northern Zambia as a spill over from East Africa (pool 4) (Rweyemamu et al., 2008b, Overby and Zyambo, 1983, Kasanga et al., 2014b).

Since 1933, when the first reports of FMD in Zambia were recorded (Morris, 1934), sporadic disease outbreaks have been reported from three high-risk areas (Perry and Hedger, 1984, Chilonda et al., 1999), with FMDV serotypes O, A, SAT-1 and SAT-2 documented in Zambia. Since 2015, an unprecedented rise in the incidence of FMD outbreaks in livestock has been observed in the country, a scenario which is similar to the rest of Southern Africa (Thobokwe et al., 2010, Thomson et al., 2012, Fana et al., 2021). The reasons for this are multifaceted, but some probable factors are described in more detail in this study.

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1.2. Problem statement and justification

Foot-and-mouth disease (FMD) remains a significant global animal health issue, particularly impacting livestock farming in regions such as sub-Saharan Africa. Despite having low overall mortality, the highly contagious nature of FMD and the resulting productivity losses in animals pose substantial economic challenges for the livestock sector (Knight-Jones and Rushton, 2013). In Zambia, FMD is one of the key constraints limiting the economic potential of the livestock sector, severely affecting farmers' livelihoods, food security, and income generation (Sinkala et al., 2014b, WorldBank, 2011). FMD outbreaks disrupt local markets, leading to the forced sale of cattle at reduced prices (Mumba, 2019) and further exacerbating poverty in vulnerable communities, especially women and children (Scoones et al., 2010, Ferguson et al., 2013.).

The disease is endemic in Zambia's African buffalo population and has historically been confined to three high-risk areas (Perry and Hedger, 1984, Chilonda et al., 1999). However, since 2015, the number of outbreaks has increased, often occurring outside these traditional endemic zones. The underlying causes of changes in the epidemiology remain unclear, with some studies previously attributing the problem to inadequate epidemiological data and ineffective control measures (Chilonda et al., 1999), while others pointed to the diversity of circulating FMD virus (FMDV) strains as a potential contributing factor (Sinkala et al., 2014a). This study aims to investigate Zambia's specific FMDV genetic and antigenic characteristics, assess the suitability of current diagnostic tests, and evaluate the effectiveness of FMD vaccination programs.

The increasing frequency and spread of FMD outbreaks beyond traditionally high-risk areas underscore the urgent need for a deeper understanding of the virus's genetic diversity, improved diagnostic methods, and more effective vaccination strategies. By analysing the genetic and antigenic profiles of FMDV strains circulating in Zambia, this research will provide critical insights into the factors driving the disease's spread and persistence. Furthermore, assessing the current diagnostic tools and vaccine programs will enable the identification of gaps in disease management, leading to more targeted and efficient control strategies. The findings from this study are expected to enhance FMD control efforts, not only in Zambia but also in the broader region, ultimately improving livestock health, food security, and the livelihoods of farmers impacted by the disease.

1.3. Aim of the study

The study aimed to determine factors contributing to repeated outbreaks and the spread of FMD in Zambia, focusing on the 2015-2021 outbreaks.

The specific aims were to:

- Characterise FMDV causing recent cases in Zambia, to provide data to help understand the epidemiology of the disease in Southern Africa. Extensive research on FMD has been

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conducted in southern Africa, especially South Africa, Zimbabwe and Botswana (Gainaru et al., 1986, Thomson et al., 1992, Vosloo et al., 2001, Thomson, 1995, Sutmoller et al., 2000, Kasanga et al., 2014a). The situation differs in Zambia, where past FMD outbreaks have not been systematically studied (Sinkala et al., 2014a). Recent FMD outbreaks in Zambia were investigated to obtain an in-depth understanding of the epidemiology of the disease and its implications for the epidemiology of FMD in southern Africa. This study on FMDV diversity and circulation patterns provides insights into the factors driving the disease's spread and persistence.

- Among the probable factors leading to increased incidences of FMD in Zambia and the SADC region could be the performance of FMD diagnostic tests in use. The lack of appropriate diagnostic tools to identify carriers thus preventing movement of cattle from tainted areas to clean areas has been recorded as a hindrance to effective FMD control in endemic countries (Rodriguez and Grubman, 2009, Jamal and Belsham, 2013). The need to develop simple and affordable diagnostics, particularly for the FMDV SAT serotypes, for use in under-resourced laboratories was considered a priority at the Global Foot-and-Mouth Disease Research Alliance (GFRA) 2014 conference (Robinson and Knight-Jones, 2014). It's therefore important to review diagnostic techniques used in SAT serotype diagnosis to understand the accuracy, reliability and check on advancement in technology. This exercise assisted identify whether current diagnostic tests are contributing to the rising incidence of FMD in the region and provided support for the development of more effective strategies to prevent and manage future outbreaks.
- In endemic regions like SSA, vaccination is central to FMD control (Armstrong et al., 2000), and in Zambia, vaccination programmes have been conducted as a control measure (Perry and Hedger, 1984). With the increased reports of FMD outbreaks in southern Africa (Fana et al., 2021), new FMDV serotypes could likely be involved, raising concerns about the effectiveness of available vaccines. *Prima facie* evidence has previously pointed to the poor performance of prophylactic vaccination programmes as one of the reasons for the unprecedented rise in the incidence of FMD outbreaks in livestock in southern Africa (Thomson, 2020). Routine field evaluations of vaccination exercises were recommended as part of disease control policy and or standard practice for FMD-endemic countries by Lyons et al. (2015). The lack of independent evaluation of vaccine quality is a significant deficiency that holds back many control programmes (Robinson and Knight-Jones, 2014) as this is critical in validating the findings of manufacturers and meeting national regulatory standards. Post-vaccination monitoring is therefore required to ensure appropriate and effective vaccines are being utilised in a vaccination program (FAO-OIE, 2016). Therefore, a first documented study was conducted in Zambia to estimate the immunity of the population targeted for protection by vaccination. This exercise helped identify gaps in the current vaccination regime. It further determined whether or not protection against infection was likely using the available vaccines, ensuring a targeted and efficient disease control strategy, thereby preventing the disease's spread and/or reoccurrence.

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1.4. Study objectives

- Investigate the genetic diversity and phylogenetic relationships of FMDV from 2015-2021 FMD outbreaks in Zambia and determine their epidemiological implications.
- Conduct a systematic review and a meta-analysis of diagnostic techniques used in SAT FMDV diagnosis.
- Evaluate FMDV-specific antibody responses after a single dose and a second booster dose in vaccinated cattle.

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Chapter 1. Literature Review

1.1. Foot-and-mouth disease

Foot-and-mouth disease (FMD) is a highly contagious disease affecting *Artiodactylae*, primarily cattle, swine, sheep, goats and many species of wild ungulates (Brooksby, 1982). It is a major global animal health problem on the list of diseases notifiable to the World Organisation for Animal Health (WOAH). The infection may be subclinical in some species, e.g., African buffalo, but in most domestic animals, typical cases of FMD are characterised by vesicles of the feet, buccal mucosa, and in females, the mammary glands. The vesicles rupture and then heal, whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof (WOAH, 2022a). Clinical signs can vary from mild to severe and fatalities may occur, especially in young animals.

The capability of the causative agent to gain entry and initiate infection via different sites, the small infective dose, the short incubation period, and the release of FMDV before the onset of clinical signs make it a highly contagious disease (Abd El Wahed et al., 2013a, Charleston et al., 2011, Paton et al., 2018). The transmission of FMDV mostly occurs through direct contact, such as between infected and susceptible animals. However, indirect transmission, though less common, can occur when susceptible animals are exposed to the excretions and secretions of acutely infected animals or raw meat (WOAH, 2022a). Mechanical transmission, which involves the spread of the virus via contaminated environments, plays a role as well. Contaminated animal products, agricultural tools, people, vehicles, and airborne transmission have all been identified as contributors to the mechanical dissemination of FMDV (Longjam et al., 2011). The multiplication and spread of the virus are influenced by factors such as the host species, the host's nutritional and immunological status, population density, animal movements, and interactions between domestic and wild host species. Animals, for example, as they move from one place to another and objects capable of mechanical dissemination also contribute to the spread of the virus (Nishiura and Omori, 2010).

Foot-and-mouth disease virus has been recognised as a significant epidemic disease threatening the cattle industry since the sixteenth century (Longjam et al., 2011). Despite its unclear origins in SSA, the disease was first reported in southern Africa in 1892 (Bruckner et al., 2002). It causes substantial economic losses to the livestock industry in terms of high morbidity in adult animals, resulting in loss of draught power and milk production, reduced weight gain, and mortality in young stock. It’s one of the impediments on the continent of Africa that prevents access to lucrative export markets for animal products (Vosloo et al., 2007). The United Kingdom's 2001 FMD epidemic, which resulted in approximately 7 million animals slaughtered and £8 billion in losses, is one of the well-documented examples of the severe impacts of the disease in a previously FMD-free country (Knowles and Samuel, 2003, Thompson et al., 2002). At the country level, the effect on economic growth and overall poverty has been reported (Knight-Jones and Rushton, 2013, James and Rushton, 2002, Jibat et al., 2013). The following are the main hindrances in controlling FMD, thus rendering it the

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most dreaded viral disease. First, its high contagiousness, wide geographical distribution and broad host range. Further, this disease is able to establish carrier status, including antigenic diversity, leading to poor cross-immunity and relatively short duration of immunity (Longjam et al., 2011). Vaccination campaigns and stringent control measures were utilised to eradicate FMD in previously endemic areas of Europe and most of South America (Freimanis et al., 2016). Despite this, the disease continues to circulate on several continents due to complex epidemiological dynamics and maintenance patterns within wildlife and livestock (Knowles et al., 2007). It was suggested by Maree et al. (2014) that more effective control of FMD in Africa could be achieved if the primary endemic areas and factors that influence disease dissemination are known, to assist in vaccine design and design of appropriate control strategies, which may include; (1) more effective movement control, (2) improve decisions on appropriate vaccine strains, (3) improved vaccines and (4) point-of-disease diagnostic assays. It has also been documented that livestock marketing is inseparable from FMD control in Africa; hence, research on market structures in various localities may provide sustainable solutions to control FMD (Knight-Jones and Rushton, 2013).

1.2. Foot-and-mouth disease virus genome

Aphthovirus vesiculae, the causative agent for FMD belongs to the family *Picornaviridae* and genus Aphthovirus (Domingo et al., 2002, Brown, 2003). It is a small, non-enveloped virus with a sedimentation coefficient of 146S (Bachrach, 1968) and an icosahedral capsid composed of 60 copies each of four structural proteins and several non-structural proteins (NSP) (Grubman and Baxt, 2004, Porter, 1993). The genome is single-stranded, positive-sense and ~8.5 kb in size (Longjam et al., 2011, Grubman and Baxt, 2004). Functionally, the genome can be categorised into three main regions: (a) 5' noncoding regulatory region, (b) polyprotein coding region (subdivided into L, P1, P2, and P3), and (c) 3' noncoding regulatory region (Figure 1.1). The P1 region encodes the leader proteinase (Lpro) and structural proteins. The P2 region encodes NSP 2A,2B and 2C, while P3 region 3A, 3B (VPg), 3C protease and 3D polymerase (Carrillo et al., 2005, Lin et al., 2008).

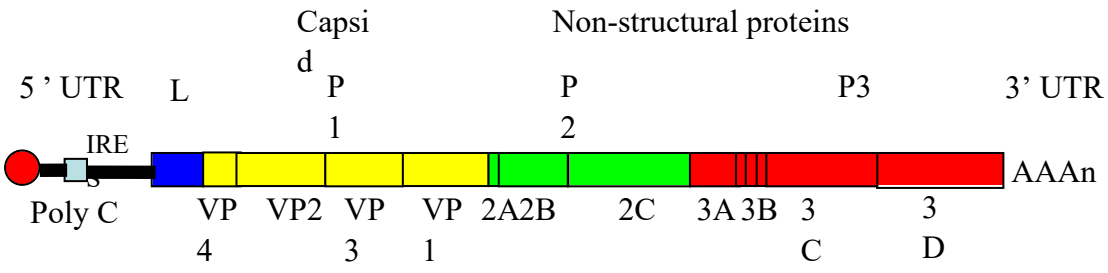


Figure 1.1. Foot-and-mouth disease virus genome orientation. Source: (Jamal and Belsham, 2013).

This virus is translated immediately into a polyprotein upon entry into a host cell (Chen et al., 2011). The four structural proteins VP1, VP2, VP3 and VP4, also known as 1D, 1B, 1C and 1A, respectively (Bachrach, 1968, Chen et al., 2011), form the viral capsid with VP4 internal and VP1 – VP3 exposed on the virion surface (Acharya et al., 1989). Except for 1A, the structural

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proteins are the first contact between the host cell and the virus (Chen et al., 2011). The structural proteins are more variable than the NSPs, which are highly conserved and not specific to any serotype; hence, their detection is not limited by serotype. As a consequence, the detection of the NSP is not serotype-restricted. Mutations or deletions in the genes of the structural proteins may help FMDV evade the host immune response (Carrillo et al., 2005). The genetic variation is most pronounced in the VP1 gene due to its significant roles in virus attachment, protective immunity and serotype specificity (Ma et al., 2011, El-Bagoury et al., 2022). As the most variable and immunogenic protein of FMDV (Cottam et al., 2008a, Logan et al., 1993), VP1 contains a serotype-specific amino acid sequence that can help differentiate various serotypes (Lin et al., 2008, El-Bagoury et al., 2022). This is why the coding sequence of VP1 has been extensively used for molecular epidemiological studies (Knowles et al., 2005, Baranowski et al., 2000, Jackson et al., 2002).

The high rate of nucleotide mutation and amino acid substitution in the FMDV genome is due to the lack of proofreading capabilities of viral RNA-dependent RNA polymerase, resulting in seven distinct serotypes with limited cross-protection between them (Jamal and Belsham, 2013, Domingo et al., 2002, Yang et al., 2022). As a result of this continuous generation of errors, the virus population is constantly evolving (Jamal and Belsham, 2018), resulting in numerous and continuously changing variants showing a spectrum of antigenic diversity (Ayelet et al., 2009, Klein, 2009). Scholars (Mwiine et al., 2019, Balinda et al., 2010, Kasanga et al., 2014b) found significant FMDV genetic diversity in East Africa, including intraserotypic diversity. The sequence differences can modify the virus's biology, but can also help identify the sources of viruses that cause disease outbreaks (Jamal and Belsham, 2018, Hall et al., 2013, Vosloo et al., 2001, Knowles and Samuel, 2003). Most of these substitutions are non-synonymous, so antigenic change is likely. Such high rates of change suggest tracing individual transmission events between animals may be possible using sequence data (Vosloo et al., 2007, Paton et al., 2018). It is important to recognise that improved knowledge of FMDV molecular epidemiology will enhance the development of vaccines and control strategies (Paton et al., 2018, Klein, 2009).

1.3. Epidemiology of foot-and-mouth disease

The FMD and global burden of FMDV infection have been maintained and are widely distributed in the developing world, in particular Africa, Asia, and South America (Alfouz et al., 2021, Tully and Fares, 2008, Maree et al., 2014, Brown, 2003). In these regions, livestock farming forms the backbone of rural economies that support approximately 70% of the world's poor. Europe, North and Central America, Greenland, Australia and Oceania remain free without vaccination (Figure 1.2).

Foot-and-mouth disease virus exists as seven different serotypes that are not uniformly distributed globally (Di Nardo et al., 2011, Liu et al., 2018). Although there are commonalities in the spread of FMD, the seven serotypes and even their variations (topotypes) exhibit distinct modes of transmission, clinical manifestations, and species tropism (Kitching et al., 2005). Six out of the seven serotypes have been recorded in Africa (O, A, C, SAT-1, SAT-2, SAT-

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3), while in the Middle East and Asia, only four serotypes (O, A, C, Asia-1) are usually present. However, there have been periodic incursions of exotic serotypes from Africa into the Middle East (Di Nardo et al., 2011, Liu et al., 2018). The SAT serotypes are generally restricted to SSA (Vosloo et al., 1996). Serotypes O and A are the most abundant in endemic regions worldwide (Chen et al., 2011, Knowles and Samuel, 2003, Yang et al., 2019) except for southern Africa. In this region, outbreaks due to serotypes A, O and C have occurred infrequently in the past (Rweyemamu et al., 2008b, Vosloo et al., 2002) and were shown to have been introduced from elsewhere (Sangare et al., 2001). Type C is restricted to the Indian sub-continent, and Asia-1 occurs typically only in southern Asia (Knowles and Samuel, 2003). However, serotype C type has not been reported since 2004 (Jamal and Belsham, 2013, Rweyemamu et al., 2008a, Paton et al., 2021) and is probably extinct. The SAT serotypes are prevalent in southern Africa (Maree et al., 2014), with serotypes SAT-1 and SAT-2 circulating throughout Africa, while SAT-3 is limited to southern and a small area in eastern Africa. Historically, it has been shown that SAT-2 has the highest incidence and SAT-3 the lowest (Bastos et al., 2003a, Vosloo et al., 2006).

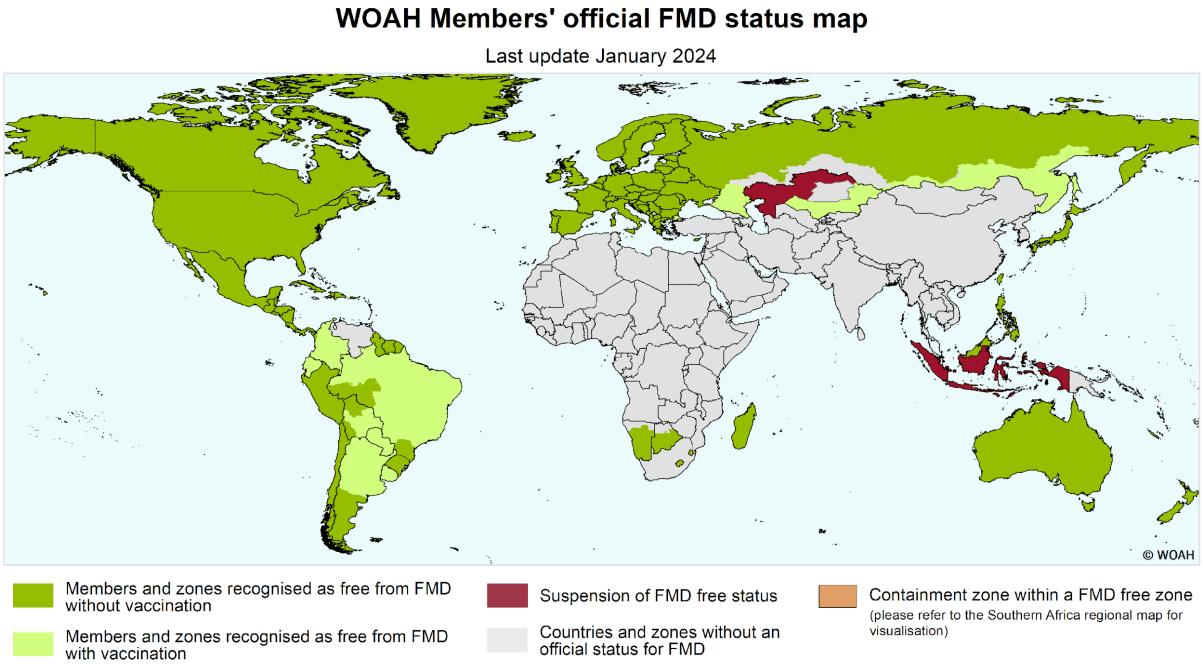


Figure 1.2 January 2024 WOAH global FMD status; map extracted 11th January 2024 from https://www.woah.org/app/uploads/2024/01/fmd-world-eng-2024.png

In Africa, FMD epidemiology is complicated by the widespread movement of animals, the broad host range of the virus involving wild and domestic animal reservoirs and the presence of multiple strains and sub-strains (Alexandersen and Mowat, 2005, Maree et al., 2014). However, in southern Africa, the epidemiology of FMD is said to be substantially different than in other regions. This is mainly due to specific circulating SAT serotype strains and African buffalo populations, which are reservoirs for the virus (Vosloo and Thomson, 2004, Vosloo et al., 2002) and can harbour the virus for 24 years (Condy et al., 1985). The role of wildlife species such as African buffalo and impala (*Aepyceros melampus*) in the epidemiology and

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maintenance of FMD SAT serotypes has been well documented in wildlife conservancy areas of Zimbabwe and South Africa (Hargreaves et al., 2004, Bastos et al., 2000, Vosloo et al., 2009) and their potential role in East Africa has also been assessed (Bronsvoort et al., 2008, Kivaria, 2003). However, the obtaining scenario on gaps in FMD epidemiology will continue to exist until it is adequately investigated in the rest of SSA countries (Vosloo and Thomson, 2004), as findings regarding FMD epidemiology in wildlife from one country should not be extrapolated to other countries because of ecological, host and viral variability differences (Vosloo et al., 2009). Wildlife reservoirs play a crucial role in controlling FMD. Therefore, vaccinating cattle with FMDV strains that are antigenically closely related to those found in buffalo is essential for effective FMD management in southern Africa (Bastos et al., 2003a).

Cattle are usually the main reservoir for FMDV elsewhere in the world, although, in some instances, the viruses involved appear to be specifically adapted to pigs. Small ruminants can play an important role in the spread of FMDV, but it is unclear whether the virus can be maintained in these species for long periods in the absence of infection in cattle. Livestock movement driven by trade has been identified as the main method of FMD transmission in Africa and other regions (Tekleghiorghis et al., 2014, Knight-Jones and Rushton, 2013, Rweyemamu et al., 2008b). In southern Africa, where several countries have controlled FMD by separating infected buffalo from livestock (Hunter, 1998) and by limited use of vaccination, disease-free areas are recognised (Thomson et al., 2003, Chitray et al., 2018).

The epidemiology of FMD in Africa has been reviewed by many scholars (Vosloo et al., 2002, Vosloo et al., 2004, Paton et al., 2009) and specifically for Zambia, by Sinkala (2015), Overby and Zyambo (1983), Perry and Hedger (1984). Four FMD serotypes, namely SAT-1, SAT-2, A and O, have been isolated from different areas in the country (Sinkala, 2015, Banda et al., 2014). An increase in FMD cases has been observed in Zambia, raising the possibility of a shift in the disease's epidemiology. This may have implications for the epidemiology of the disease in southern Africa, highlighting the need for further investigation to support the development of effective surveillance and control strategies for the region.

There is no clear understanding of the origins of FMD in Zambia. After the reports of FMD outbreaks in 1933 (Morris, 1934), the disease was re-recorded in Mazabuka, Mbala, and Sesheke districts in 1948, 1976 and 1995, respectively. Since then, repeated outbreaks have primarily occurred in three high-risk (Perry and Hedger, 1984, Chilonda et al., 1999, Overby and Zyambo, 1983), as earlier alluded to Figure 1.3.

The clinical disease of FMD in Zambia was recorded mainly in cattle, with few reports in small stock because of the subclinical manifestation of the disease in these species, the low numbers of goats and sheep and the lack of surveillance reports targeting small livestock (Akafekwa, 1980, Perry and Hedger, 1984). Like most of SSA, the majority of the FMD outbreaks in Zambia have been caused by the FMDV SAT-2, followed by SAT-1 (Mweene et al., 1996, Thomson and Bastos, 2004).

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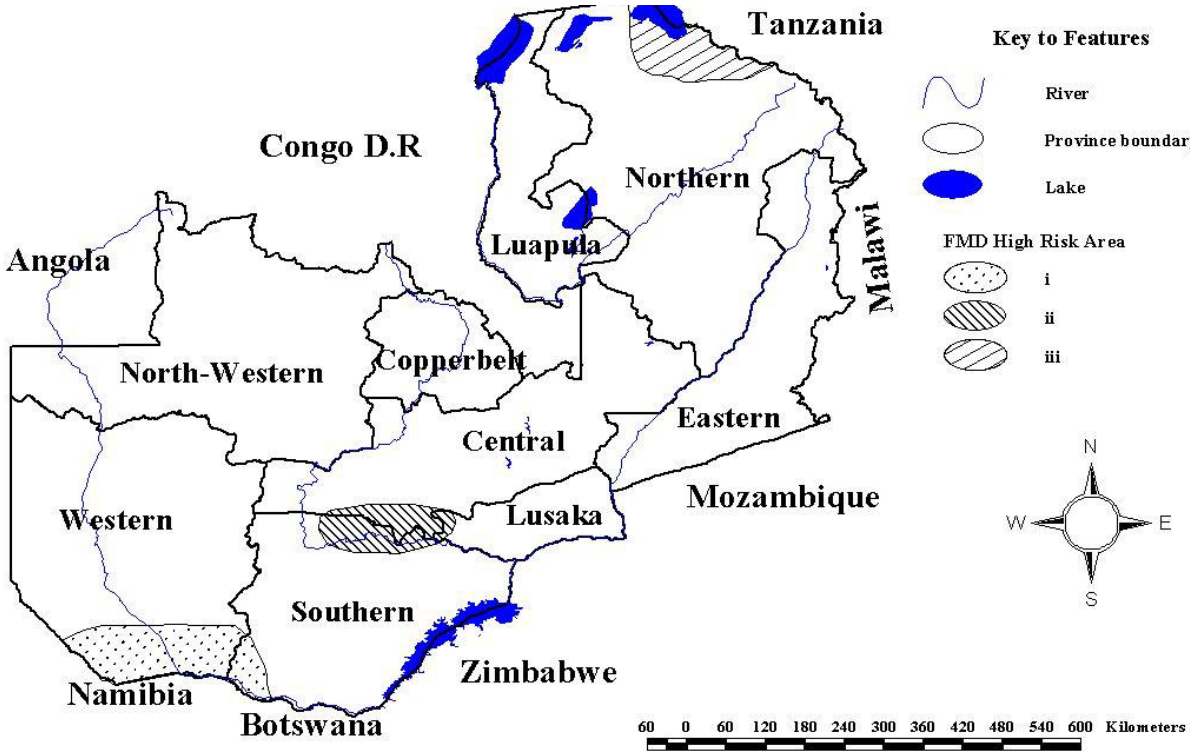


Figure 1.3 Map of Zambia showing the provinces and the areas at risk of foot and mouth disease. Source: Mweene et al., 1996

Serotypes O and A were reported mainly in northern Zambia as incursions from Tanzania (Banda et al., 2014, Rweyemamu et al., 2008b, Overby and Zyambo, 1983, Kasanga et al., 2012). The World Reference Laboratory for FMD (WRLFMD) reported that FMDV topotypes that have been isolated from Zambia were topotype I (NWZ), topotype II and III for SAT-1; topotype III and IV for SAT-2; topotype EA-2 for serotype O and topotype AFRICA G-1 for serotype A (FAO-WRLFMD, 2018).

The role of wildlife in the epidemiology of FMD in Southern and East Africa has been explained, with the two probable scenarios of maintenance and transmission patterns of SAT type viruses highlighted (Vosloo et al., 2002). In the first pattern, wildlife, particularly the African buffalo plays a role in maintaining and spreading the disease to other susceptible domestic animals and wild ungulates (Vosloo and Thomson, 2004, Vosloo et al., 2002). The second being one where domestic animals possibly maintain the infections independently of the agency of wildlife (Vosloo et al., 1992, Vosloo et al., 2002). In Southern Africa, the former cycle predominates due to the presence of the African buffalo (Syncerus caffer), the only wildlife species for which long-term maintenance of FMDV has been described (Hedger et al., 1973, Vosloo et al., 1996). The role these patterns play in Zambian FMD epidemiology was discussed by Sinkala (2015) whilst elaborating on the challenge these two patterns cause in selecting sufficiently cross-protective FMD vaccines. The FMD outbreaks in Zambia have often been associated with areas where livestock and wildlife interact, such as the Kafue flats and lower Zambezi, with the movement of buffalo, along migratory routes, probably facilitating the spread of the virus to new areas (Sinkala, 2015).

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Several socioeconomic factors also contribute to the outbreaks of FMD in Zambia by assisting the spread of the disease. These may include livestock movement, especially for trade, grazing and cultural practices such as mafisa (Muuka et al., 2012). Transhumant pastoralism, where herders move their animals seasonally, increases the risk of disease transmission (Mwacalimba et al., 2013, Muma J.B., 2006). And to access the market for their livestock, farmers often move livestock to central trading areas, for instance Munanda in northern Zambia (Banda et al., 2014). Central trading areas where animals gather can become hotspots for disease transmission due to inadequate biosecurity measures, further exacerbating the issue. Economic constraints in resource-limited countries like Zambia restrict funding for effective disease control measures such as vaccination and surveillance. Additionally, poor infrastructure—particularly limited veterinary services and facilities—hinders timely disease detection and response. Low levels of awareness and education about FMD among farmers further contribute to inadequate disease management practices.

The methods used to control FMD in Zambia have been summarised (Perry and Hedger, 1984), and currently, surveillance, quarantine, movement control, and biannual vaccination of cattle in the outbreak and high-risk areas are implemented. However, the fundamental components of FMD control, such as vaccination, biosecurity and livestock movement controls, are often inadequately implemented in smallholder systems in developing countries (Knight-Jones et al., 2016). The scenario is similar in Zambia where biannual vaccination of cattle has been central in the effort to control FMD, but the practice is not followed conscientiously. Uncontrolled animal movement is also a significant factor in disease spread in the country (Mumba et al., 2018, Muuka et al., 2012), with restrictions on movement and animal quarantine being applied during disease outbreaks only. Zambia also lacks veterinary infrastructure, human resources and appropriate vaccines, which has been previously reported to expose many developing countries to the spread of FMD (Perry and Rich, 2007, Sutmoller et al., 2003). Lastly, in Zambia, as a disease control measure, animal disease surveillance is mostly done passively, with active surveillance being conducted during FMD outbreaks.

Northern Zambia hosts an intersection of virus pools 4 and 6 (Knowles, 2013). Freimanis et al. (2016) previously reported that, periodically, viruses are spread between pools and free regions, and countries at the interfaces between pools have been reported to often experience FMD outbreaks from different regional sources. This threat is imminent for Zambia and the rest of southern Africa, which do not harbour viruses found in pool 4. On the other hand, evolutionary studies on SAT-1 topotypes isolated in Kenya and Tanzania highlight the potential for cross-border exchange of FMDV through transboundary livestock and wildlife movements. Southern Africa was ascribed as a likely source of this virus introduction into Eastern Africa (Sangula et al., 2010). The challenge with transboundary transmission of FMD is that surveillance systems are not usually synchronised between countries, and control strategies are not carried out simultaneously during outbreaks. The lack of synchronised control and surveillance strategies raises the risk of back-and-forth transmission of FMD across borders. Therefore, the need to comprehensively evaluate FMD viruses circulating in

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Zambia, which links southern Africa with the rest of SSA, cannot be over-emphasized. Detection of the viral genome, serotyping and eventual characterising circulating FMDV will lead to a sound epidemiological understanding of FMD endemicity, hence the development of effective, acceptable and rational control methods for FMD in SSA.

1.4. Molecular epidemiology of foot-and-mouth disease virus

In recent years, considerable progress has been made in understanding FMD epidemiology (Knowles and Samuel, 2003, Rweyemamu et al., 2008b, Di Nardo et al., 2011). However, a clear global picture of the distributions and movements of FMDV strains within and between regions and countries is still lacking. This is due mainly to limited diagnostic capacity and sampling in many endemic countries (Namatovu et al., 2013). Scholars have elaborated that the integral part of any viral disease control strategy is the epidemiological tracing of virus transmission together with conventional field investigations (Logan et al., 2014). Applying molecular techniques such as polymerase chain reaction (PCR) amplification and nucleotide sequencing is recommended for studies of RNA viruses with high evolutionary rates like FMDV (Wright et al., 2011, Cottam et al., 2008b). The data from such studies can be used to classify FMDV field isolates into topotypes and define the genetic relationships between the viruses causing outbreaks (Knowles et al., 2016c).

Sub-Saharan Africa encounters a significant challenge in controlling and eliminating FMD due to the enduring presence of specific genetic subtypes (topotypes) over the last 40 years (Knowles and Samuel, 2003). Each African topotype leads to distinct epidemiological patterns (Maree et al., 2014). There are significant gaps in knowledge, particularly about how viruses persist, spread, evolve and how to stimulate a good immune response through vaccination (Paton et al., 2009). This adds to the complexity of the disease's epidemiology in this region. Africa has been remarked as having the greatest diversity of FMD serotypes (Rweyemamu et al., 2008b). Molecular epidemiology studies are critical to understanding the disease's natural history and relationships among various serotypes and to developing more effective prevention and control methods (Sobhy et al., 2018). Further, molecular epidemiology, with its power to resolve transmission pathways, has contributed enormously to our understanding of the spatial and evolutionary traits underlying FMD outbreaks. Understanding the geographical distribution and genetic diversity of FMDV is essential for enhancing control measures and developing effective vaccines (Bastos et al., 2003a). This genetic variation can partially explain the persistent occurrence of outbreaks even with obligatory governmental vaccination programmes in Zambia and Egypt (Sinkala, 2015, Sobhy et al., 2018). Genomics can ultimately drive the reconstruction of FMDV movements on multiple scales, ranging from intra-host to farm, region to country, and a more comprehensive global picture (Orton et al., 2013). This may provide a more detailed breakdown of the virus transmission chain (i.e., 'who infected whom') and support decisions and policymakers in outbreak management (Freimanis et al., 2016).

Traditionally, FMDV molecular epidemiology is based on analyses of the partial or full-length sequence of the gene encoding the capsid protein VP1 (Knowles et al., 2016c, Xu et al., 2013,

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Knowles and Samuel, 2003). Studies in which genetic and antigenic variation have been compared indicate that viruses of different VP1 gene topotypes tend to be antigenically poorly related. This observation holds for European and SAT serotypes (Bastos et al., 2003a). Genetic characterisation of strains is important to determine if variants exist in the field, especially for the SAT serotypes, in which it has been shown that more significant genetic variation occurs compared to other serotypes (Bastos, 1998, Wright et al., 2011). Sometimes, there is a lack of efficient cross-protection between different subtypes within a single serotype. Thus, the characterisation of subtypes becomes necessary for selecting appropriate vaccines to control an outbreak in countries where vaccination is employed (Jamal and Belsham, 2018). Assessment of the genetic variation of the viruses in the field also helps determine the origin of outbreak (Bastos et al., 2003a). It is speculated that some viruses circulating in Zambia are shared with neighbouring countries (Sallu, 2016, Banda et al., 2014, Kasanga et al., 2012). Given the lack of detailed information on specific serotypes and topotypes circulating in various parts of Zambia, it is crucial to conduct surveillance and characterisation of the virus. The geographical position of Zambia makes it an excellent resource for ensuring disease filtration between Southern Africa and the rest of Southern Africa, hence the need for continuous monitoring. Determination of the factors influencing the maintenance and spread of the disease, as well as knowledge of circulating subtypes of FMDV in Zambia, is therefore essential for effective control of the FMD in the region. Such data provide science-based support for designing vaccination control programmes/policies to ensure the most appropriate vaccine-candidate strains are used. The knowledge of circulating subtypes would also provide information on the efficiency of diagnostic tests currently in use and more information on the development of tailored diagnostic tests for the region.

1.5. Phylogeny of foot-and-mouth disease virus

Phylogenetic classification of the Foot-and-mouth disease virus (FMDV) using reverse transcriptase PCR (RT-PCR) amplification of FMDV genomic RNA isolates and VP 1 nucleotide sequencing has supplanted serological methods (Sobrino et al., 2001, Domingo et al., 2002). These genetic and antigenic analyses show that FMDV worldwide have been subdivided into seven regional pools, also referred to as topotypes, based on their geographic origin

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(ecological isolation or adaptation)(Di Nardo et al., 2011, Samuel and Knowles, 2001a, Foglia et al., 2021) (Figure 1.4).

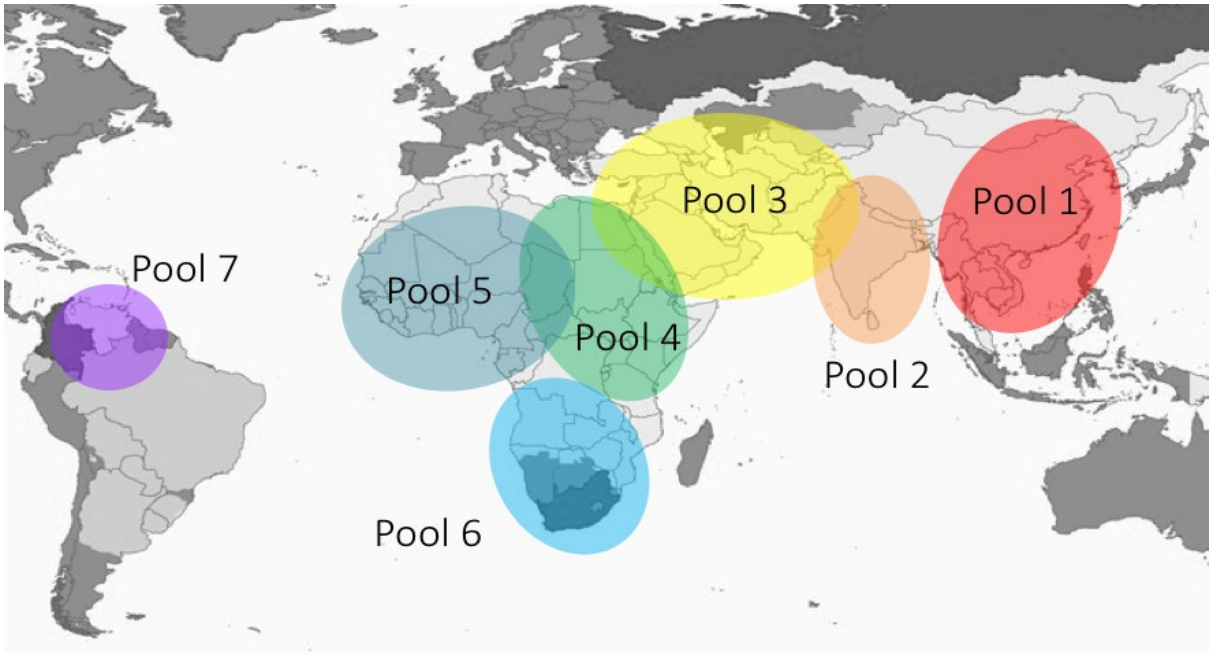


Figure 1.4 Distribution of the seven endemic pools of FMD: Source https://www.pirbright.ac.uk/.

A nucleotide difference of up to 15% in the VP1-coding sequence is required to define a topotype within the serotypes A, C and Asia-1 (Dill et al., 2017). Due to higher variability within the FMDV SAT serotypes, a nucleotide difference of up to 20% is required (Samuel and Knowles, 2001b, Carrillo et al., 2007). There are currently 50 FMDV topotypes: serotype O (11); serotype A (3); serotype C (3), serotype Asia-1 (1); serotype SAT-1 (13); serotype SAT-2 (14); and serotype SAT-3 (5) (WOAH/FAO, Knowles and Samuel, 2003).

Out of the seven world's FMD regional pools (Foglia et al., 2021, Brown, 2003, Di Nardo et al., 2011), Africa has been divided into three, with pool four encompassing East and North Africa, where serotypes A, O, SAT-1, SAT-2 and, SAT-3 are predominant (Maree et al., 2014). Pool five is limited to West and northern Africa, where serotypes O, A, SAT-1 and SAT-2 are found, whilst pool six is primarily confined to southern Africa with SAT-1, SAT-2 and SAT-3 serotypes (Figure 2.3). Some countries share viruses belonging to two different pools, for instance, Egypt and Libya have viruses from pools 4 and 5, and Tanzania and Zambia (northern part) have viruses from pools 6 and 4. This scenario complicates disease control because of marked regional differences in the distribution and prevalence of various serotypes and intratypic variants. The viral and antigenic diversity demonstrated by the two intersecting virus pools in northern Zambia may need to be studied to effectively control FMD in Zambia and the SADC region (Sinkala et al., 2014b). This could be achieved through tailored diagnostics, appropriate vaccine selection and scientifically informed control strategies (Di Nardo et al., 2011, Jamal and Belsham, 2013).

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1.6. Diagnosis of foot-and-mouth disease

A presumptive diagnosis of FMD can be normally based on clinical signs but this can be easily confused with other vesicular diseases, such as swine vesicular stomatitis, swine vesicular disease and malignant catarrhal fever (Remond et al., 2002, Ferris et al., 2009, WOAH, 2022a). Therefore, confirming the diagnosis of suspected FMD in the laboratory is essential (Lazarus et al., 2010). Every strategy for controlling and preventing of FMD relies on diagnostic tests that vary in their convenience of application (Namatovu et al., 2013, Thomson et al., 2003). Given the rapid spread of FMD and the significant economic impact of the outbreaks, it is crucial to have prompt, sensitive and specific laboratory diagnosis which includes serotyping of the outbreak virus. This is essential for designing an effective disease control programme (Kasanga et al., 2014c, Jamal and Belsham, 2013). However, developing countries of SSA face many challenges in diagnosing FMD, notably the limited availability of on-the-spot field diagnostic tools (Namatovu et al., 2013, Jamal and Belsham, 2013). Rapid diagnosis and identification of FMDV serotypes are essential for controlling epidemics using an appropriate emergency vaccine and tracking the origin of outbreaks (Vosloo et al., 2006, Callens and De Clercq, 1997). In Zambia, FMD diagnosis typically begins with clinical suspicion, followed by laboratory serological testing at the Central Veterinary Research Institute (CVRI). Confirmatory diagnosis is conducted at regional reference laboratories, including Botswana Vaccine Institute (BVI) in Botswana and the WRLFMD in the United Kingdom (UK). As in other FMD-endemic countries, in Zambia, controlling FMD is challenging due to lack of tools to identify carriers and prevent the movement of cattle from infected areas to clean areas (Sammin et al., 2007). Poor surveillance and diagnostic facilities, including optimisation of the current serological tests, were previously highlighted by Sinkala (2015) as hindering FMD control in Zambia. This, coupled with ineffective FMD control programmes that are not monitored, enhances the repeated and increased disease outbreaks observed in Zambia.

1.6.1. Clinical diagnosis

The clinical outcome of the disease may vary between the host species and the infecting virus strain (Longjam et al., 2011). *Aphthovirus vesiculae* is found in virtually all organs during the acute phase of FMD as a consequence of the viraemia (Yang et al., 2019), and in general, viraemia occurs before the onset of clinical signs (Quan et al., 2004). In cattle and pigs, fever and viremia usually start within 24 – 48 hours after epithelium infection, leading to viral spreading into different organs and tissues and the production of secondary vesicles on the mouth and feet (Longjam et al., 2011). Lesions can also be observed in and around the oral cavity and on the mammary glands of females. In swine populations, typical FMDV clinical signs and lesions are indistinguishable from those caused by *Enterovirus betacoxsackie* (previously swine vesicular disease virus) or *Vesiculovirus indiana* (previously vesicular stomatitis virus) (Fernández et al., 2008). The pain from the lesions leads to lameness, loss of appetite, and excessive drooling which is usually indicative of the condition (Remond et al., 2002, Alexandersen et al., 2003a, Gorna et al., 2016). The vesicles rupture and then heal, whilst coronary band lesions may give rise to growth arrest lines that grow down the side of

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the hoof. The age of lesions can be estimated from these changes as they indicate the time since infection occurred. The severity of clinical signs varies with strain of virus, exposure dose, age, breed of animal, host species and the animal's immunity. Foot-and-mouth disease induces high morbidity but low mortality, and most reported deaths occur in young animals due to myocarditis (Gorna et al., 2016). The morbidity and mortality in FMD depend upon the breed and age of the animal, where morbidity can reach 100% in susceptible populations, with mortality being generally low in adult animals (1 – 5%) but higher in young calves, lambs and piglets (20% or higher) (WOAH, 2022a).

1.6.2. Laboratory diagnosis

The accurate diagnosis of infection with FMDV is of prime importance for control and eradication campaigns in FMD-endemic areas and as a supportive measure to the stamping out policy in FMD-free areas (Remond et al., 2002). Longjam et al. (2011) extensively elaborated on the history of research and diagnosis in FMD. Techniques for laboratory diagnosis of FMDV are available in the World Organisation for Animal Health (WOAH) Terrestrial Manual and have been utilised by various laboratories based on their national priorities and technological advancement levels. The laboratory diagnosis of FMD involves detecting and identifying viral agents or antibodies against FMDV.

The antigen ELISA and VI have been recommended laboratory procedures and have been traditionally used for FMD diagnosis for nearly twenty years based on their suitability to detect the presence of FMDV antigen in tissue samples (Shaw et al., 2004, Ferris and Dawson, 1988, Ferris et al., 1988, Hamblin et al., 1984, Roeder and Le Blanc Smith, 1987). The complement fixation and molecular diagnostic tests are also used for this. If the sample is inadequate or the diagnosis remains uncertain, sample materials can be tested by RT-PCR and/or VI. Demonstrating FMD viral antigen or nucleic acid is sufficient for a positive diagnosis (Colling et al., 2014, El-Shehawy et al., 2012, WOAH, 2022a).To detect antibodies against either the structural or NSP of FMDV, serological assays are utilised, and these include the NSP ELISA, virus neutralisation test (VNT), liquid-phase blocking ELISA (LPBE), and solid-phase competitive ELISA (SPCE) (WOAH, 2022a). The detection of antibodies to viral NSP is considered one of the most important indicators of infection, regardless of vaccination status (Barnett et al., 2015, Colling et al., 2014, WOAH, 2022a). This is particularly useful in countries like Zambia, where vaccination is used and in mild cases or where epithelial tissue cannot be collected (Colling et al., 2014). The gold standard for diagnostic samples, however, has been the collection of epithelial samples from lesions followed by tissue culture, antigen ELISA for serotyping and sequencing (Mark Bronsvoort, 2023).

The high level of intra-serotype nucleotide variability, especially in the VP1 coding sequence, makes it challenging to design broadly reacting molecular tests that can detect all representatives of a single FMDV serotype that are circulating in different geographical regions (Reid et al., 2014). Therefore, most successfully developed molecular assays have been tailored to a specific lineage found in more restricted geographical locations (Bachanek-Bankowska et al., 2016, Reid et al., 2014, Ahmed et al., 2012). However, low sensitivity and

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specificity have been noted in most developed molecular detection methods targeting FMDV SATs, which are prevalent in SSA due to the propensity for mutations of FMDV SATs, especially in SAT-2 (Ahmed et al., 2012, Bastos et al., 2003b, Liu et al., 2018). This was confirmed by Valérie Mioulet (2023), who stated that SAT-2 remains the more challenging serotype for detection in all test formats. The challenges of diagnostic tests currently in use especially for SAT serotypes, have also been previously highlighted (Thomson, 2020, Maree et al., 2014, Sinkala, 2015). Therefore, the need for a scoping review of FMDV SAT serotypes diagnostic assays to determine the most sensitive and specific diagnostic assays for detecting these serotypes cannot be overemphasised.

1.6.2.1. Identification of the virus

1.6.2.1.1. Virus isolation

The method used to determine whether any infectious virus is present or to identify viral activity is to attempt to isolate the virus by inoculating and passaging the original tissue suspension through susceptible cell cultures and looking for a cytopathic effect (CPE) (Hearps et al., 2002, WOAH, 2022a). Sensitive cell culture systems include primary bovine (calf) thyroid (BTy) cells and primary pig, calf or lamb kidney cells with established cell lines, such as baby hamster kidney fibroblast cells-21 (BHK-21) and Instituto Biologico-Rim Suino-2 (IB-RS-2) cells being used. The BHK-21 and IB-RS-2 cells are generally less sensitive than primary cells for detecting low amounts of infectivity (WOAH, 2022a). Virus isolation may take up to four days to demonstrate CPE, especially with low levels of viremia, and FMDV may fail to grow in a specific cell type (Yang et al., 2019, WOAH, 2022a, Jamal and Belsham, 2013). The cell cultures are examined for CPE for 48 hours. If no CPE is detected, the cells are frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. However, CPE is not specific to FMDV alone. The foregoing makes cell culture laborious and time-consuming (Kasanga et al., 2014c, Yang et al., 2019). Virus isolation also requires an infectious positive control, thus restricting the application of this method to high-security laboratories (Moss and Haas, 1999, Asfor et al., 2020) which are limited.

Biswal et al. (2021) reported that virus isolation is as sensitive as RT-PCR during the early phase of infection. The success of VI depends on the quality of the sample (Jamal and Belsham, 2013) coupled with special transporting conditions from point of sampling to the laboratory (Alexandersen et al., 2003b, Jamal and Belsham, 2013). This poses challenges for many developing countries, particularly in regions with high environmental temperatures, long distances between sample collection points and national laboratories, inadequate laboratory infrastructure and a lack of trained personnel (Belsham et al., 2011, Reid et al., 2001). Furthermore, as VI takes time to confirm the disease and inform us of what vaccine to use, the FMDV infection would have spread, thus increasing the cost and time needed for control (Sinkala, 2015). There is no biosecurity level three (BSL3) laboratory in Zambia required for VI; therefore, tissue and/or probang samples from infected animals are routinely sent to reference laboratories. Depending on foreign laboratories for disease control programs may not be sustainable, as the limited number of samples analysed does not

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provide sufficient detailed knowledge of the circulating FMDV strains needed for effective control measures (Rweyemamu et al., 2008a). Therefore, more rapid, on-the-spot, user-friendly diagnostic assays are necessary to address some of these challenges.

1.6.2.1.2. *Immunological methods*

1.6.2.1.2.1. Enzyme-linked immunosorbent assays

The ELISAs, whether blocking- or competition-based assays that use serotype-specific polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs), are quicker to perform and do not rely on tissue culture systems and the use of live viruses (WOAH, 2022a). The detection of specific antibodies to structural proteins in non-vaccinated animals signifies a previous infection with FMDV (Hamblin et al., 1986a, Crowther and Abuelzein, 1979, Paiba et al., 2004). This approach is particularly beneficial in mild cases or when epithelial tissue samples are unavailable. Tests for antibodies to NSPs of FMDV can indicate past or current infection, as they reflect viral replication in the host regardless of vaccination status. While methods based on VI or the demonstration of FMD viral antigen or nucleic acid in samples can confirm a positive diagnosis, the ELISA using type-specific serological reagents (Ferris et al., 1988, Roeder and Le Blanc Smith, 1987) is generally preferred for the detecting FMD viral antigen and identifying the viral serotype in the early research stages (Ferris and Dawson, 1988, Ferris et al., 2011). Serotype information derived from antigen-detection ELISA can provide valuable supporting data to aid the selection of primers for RT-PCR (Knowles et al., 2016c). Antigen ELISA, however, has a limited sensitivity and yields positive results only with vesicular material but not with saliva, nasal swabs, milk or serum (Longjam et al., 2011, Reid et al., 2006).

In Africa, the diagnosis of FMD primarily depends on antibody ELISAs, with the NSP ELISA being widely utilised (Namatovu et al., 2013). This NSP ELISA has two main advantages: i) only one antigen is necessary and sufficient to detect the seven different FMDV serotypes (it is a serotype-independent assay), and ii) the ability to differentiate infected from vaccinated animals (DIVA) (Ferris and Dawson, 1988, Brocchi et al., 2006, Bergmann et al., 2000). The latter makes it usable as a generic screening tool to support national programmes to ascertain the WOAH status of FMD freedom with or without vaccination (Brocchi et al., 2006, Paton et al., 2006). However, the use of non-purified vaccines in Africa complicates DIVA, particularly in regions where repeated vaccinations are conducted (Jamal and Belsham, 2013, Namatovu et al., 2013). Additionally, tests for detecting NSP antibodies cannot distinguish between recovered and carrier animals (Jamal and Belsham, 2013, Armstrong et al., 2005, Sørensen et al., 1998), which requires the use of virological tests.

1.6.2.1.2.2. Lateral flow devices

Rapid detection and containment of FMD outbreaks are critical to the efficient control of FMD. Unfortunately, most test assays, including VI, RT-PCR, ELISA and genomic sequencing, require a laboratory, well-trained personnel and specialised equipment. For FMD-endemic countries like Zambia, which have to ship samples to reference laboratories for confirmation,

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the problems of the high costs associated with shipment on dry ice, high biosafety levels and long transportation time (> 20 hr) make an onsite, field-deployable test for the rapid and accurate detection of FMDV highly desirable. Lateral flow devices (LFDs) have been designed to detect FMDV antigens rapidly (Valérie Mioulet, 2023) and have been described as being relatively quick to use (< 10 min), cheap and user-friendly (Ferris et al., 2009, Morioka et al., 2015). The LFDs have been developed to be either serotype-specific (Ferris et al., 2010, Yang et al., 2013, Yang et al., 2021) or pan serotypic, i.e able to detect all seven FMDV serotypes (Morioka et al., 2015, Ferris et al., 2009). The first reported SAT-1 and SAT-3 strip tests, when combined with the previously developed SAT-2 strip test, have been shown to be useful for quick diagnosis of FMD in endemic countries of Africa (Yang et al., 2021). The method uses the capture and detection of MAbs or specific polyclonal antisera on a strip test. Directly applying vesicular fluids and epithelial suspensions from infected farm animals to detect FMDV antigens can minimise diagnostic errors caused by nonspecific reactions, which may result from laboratory contamination. However, the LFDs will only work using certain materials, i.e., vesicular fluids and epithelial suspensions, but not saliva, nasal swabs, or serum (Ferris et al., 2009). Furthermore, the sensitivity of the strip test may differ for the various FMDV (Oem et al., 2009) and has been described as being less sensitive (~80%) compared with molecular assays (Morioka et al., 2015, Yang et al., 2015, Ferris et al., 2010), meaning a negative result still requires laboratory confirmation before any control measures can be implemented (Hole and Nfon, 2019).

1.6.2.1.2.3. Complement fixation test

The complement fixation test (CFT) was first used in 1929 to type antiserum and FMDV of guinea pig origin (Bachrach, 1968). It is the earliest laboratory test for diagnosing FMD and its still recognised by WOAH (WOAH, 2022a). The CFT has been used extensively to distinguish different strains of FMDV (Ferris et al., 1984, Rweyemamu et al., 1978). A comparison of CFT and other diagnostic methods like ELISA and VI has been done (Westbury et al., 1988) and several disadvantages have been found. To this effect, the CFT was later replaced (El-Shehawy et al., 2012, Ferris and Dawson, 1988).

1.6.2.2. Serological tests

Serological tests for FMD are of two types - those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral NSP. Antibodies against SP of FMDV can be detected by VNT, LPBE and SPCE (Jain et al., 2018, Hamblin et al., 1986b, Alexandersen et al., 2003b), while several ELISA tests have been described to detect FMDV NSP-specific antibodies using recombinant 3A, 3B, 3C, 3AB and 3ABC (Jaworski et al., 2011).

1.6.2.2.1. Virus neutralisation test

This test is considered the “gold standard” for detecting antibodies against vesicular viruses, including FMDV, *E. betacoxsackie* and *V. indiana* antibodies. It is a mandatory test for certifying the import and export of animals and animal products. The quantitative VNT for

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FMDV antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtitre plates (WOAH, 2022a). *In vivo*, serum antibodies are induced against the outer capsid structural proteins following vaccinations against and infection with FMDV. The assay measures the ability of a serum sample to neutralise a fixed dose of virus and prevent the appearance of a readily observable CPE in susceptible cells grown in culture. Test parameters are well-defined.

The VNT was recently used to determine serological cross-reactivity between the vaccine strains and field isolates (Lloyd-Jones et al., 2017). Previous studies (Martin and Chapman, 1961, Sutmoller and Vieira, 1980, Robiolo et al., 2010), showed that VN titres recorded after vaccination correlated with protection against FMDV challenge in cattle. This test has also been compared with others (Hamblin et al., 1987). The VNT is a recommended test for screening potential vaccine candidates in the *AgResults* Foot and Mouth Disease Vaccine Challenge Project (Hammond et al., 2021). Its drawback is the strict need to use cell culture facilities to run the test (Hamblin et al., 1986a, Jaworski et al., 2011). This dependence on cell culture facilities has made it unrealistic for use for most African countries (Jamal and Belsham, 2013).

The test is laborious, rendering large-scale serological testing difficult (Asfor et al., 2020). This test, together with the other serological tests such as the LPBE or SPCE, detect immunoglobulins directed against the capsid or structural proteins of the virus and cannot distinguish antibodies induced by vaccination from those elicited by infection with the live virus (Armstrong, 1997, Golding et al., 1976, Mackay et al., 2001, Hamblin et al., 1986a). This renders serological differential diagnosis of FMD difficult in countries where vaccination is practised, like in Zambia, because vaccinated animals cannot be distinguished from infected animals simply by the VNT. Antibodies against the NSP of FMDV as replaced serological markers for identifying FMDV-infected animals are therefore supported in vaccinating countries (Chen et al., 2011, Mattion et al., 2004).

1.6.2.2.2. Solid-phase competitive ELISA

The SPCE detects antibodies against structural proteins of FMDV (Alexandersen et al., 2003b, Hamblin et al., 1986b). It is serotype-specific and detects antibodies from vaccination and infection (Chénard et al., 2003, Paiba et al., 2004). Together with VNT and LPBE, the SPCE is one of the recommended tests for determining the FMD antibody status of livestock (WOAH, 2022a). The method described by Paiba et al. (2004) can detect antibodies against each of the seven serotypes of FMDV. In the SPCE, the purified antigen is directly adsorbed to the solid phase, and because of virus deformation, internal epitopes are exposed. Unlike the VNT, which needs maintenance of cells and handling of infectious FMDV in high containment laboratories (Jaworski et al., 2011, Hamblin et al., 1986a), SPCE assays are easier, faster to perform and can make use of inactivated FMDV antigens (Li et al., 2012, WOAH, 2022a). They can, therefore, be set up quite easily in any laboratory (Paiba et al., 2004). A study by Li et al. (2012) demonstrated that the SPCEs for the six FMDV non-O serotypes as both screening and the titration assays were sensitive, specific, speedy, robust and warrant consideration for

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adoption in routine serology to replace the LPBE. However, this study observed that the SPCE failed to detect antibodies in animals vaccinated with a SAT-2 vaccine. A similar discrepancy was observed by Mackay et al. (2001). These observations could be attributed to a limitation of SPCE in having antigens and blocking antibodies, which are fixed and usually of unknown antigenic relevance (beyond serotype) (Paton et al., 2019). Therefore, the VNT and LPBE are superior in this aspect as they use homologous viruses in most cases (Li et al., 2012). However, the SPCE is more specific but as sensitive as the LPBE (Mackay et al., 2001; Paiba et al., 2004). More recently, commercially available SPCEs have become alternatives to the LPBE for SP serology and use for routine PVM at the population level (Paton et al., 2019, FAO-OIE, 2016), and studies have shown their potential in the determination of immunogenicity (Chénard et al., 2003).

1.6.2.2.3. Liquid-phase blocking ELISA

The LPBE was developed to detect FMDV antibodies because of the drawbacks of conventional VNTs (Lazarus et al., 2010). It detects FMDV antibodies in serum and can be used as either a single dilution screening assay or a quantitative titration assay, determining the endpoint of each serum sample (Hamblin et al., 1986b, Hamblin et al., 1986a). The test is highly sensitive, provided that the virus or antigen used in the test is closely matched to the strain circulating in the field. It has been used as a prescribed test, as it is suitable for confirming previous or ongoing infections in non-vaccinated animals and for monitoring the immunity provided by vaccination in the field when purified vaccines are used (Bronsvoort et al., 2008, Hamblin et al., 1986b). The LPBE can detect antibodies against all seven FMD serotypes using polyclonal rabbit and guinea pig IgG antibodies to detect residual FMD antigen following *in vitro* incubation of test serum and FMD antigen (the "liquid phase") (Lazarus et al., 2010). However, low-titre false-positive reactions can be expected in a small proportion of the sera in LPBE (WOAH, 2022a). Since the inactivated antigen has variable stability, this test has low specificity (Jain et al., 2018). Further, the LPBE has been found to produce a significant number of false positive results when used as a screening test (Chénard et al., 2003). The performance of the SPCE was evaluated by Sammin et al. (2007) for detecting SAT-type FMD virus infection because it was introduced to replace the LPBE.

1.6.2.3. Nucleic acid detection methods

The PCR has dramatically altered how molecular studies are conducted (Erlich et al., 1991). It has become the most widely used nucleic acid-based diagnostic technique since its invention (Saiki et al., 1985). It is an extremely sensitive method widely used to identify viruses, including picornaviruses (Erlich et al., 1991, Hyypiä et al., 1989, Balanant et al., 1991). The presence of viral genomic material can be detected using RT-PCR assays. Reid et al. (2002) reported that since the advent of genetic diagnostic techniques nearly two decades ago, more than 50 different nucleic acid hybridisation and various PCR technologies have been reported to diagnose FMD. Currently, laboratory diagnosis of FMD mostly depends on the detection of viral RNA by RT-PCR (Callens and De Clercq, 1997, Reid et al., 1999). Meyer et al. (1991) reported methods for the early detection of FMDV RNA in epithelial tissues, cell culture

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isolates and samples using universal primers applicable for all seven serotypes. The RT-PCR assay can also be used to amplify genome fragments of FMDV in diagnostic materials, including milk, serum and probang samples (WOAH, 2022a). Conventional RT-PCR can avail serotype-specific results (Reid et al., 1999) for FMD diagnosis.

RT-PCR assays are not suitable for on-site screening. Therefore, samples collected from animals in the field or at quarantine stations for this assay are sent to central laboratories for testing (Abd El Wahed et al., 2013a). Previously, results from studies conducted by Reid et al. (1998) and Reid et al. (1999) reported that RT-PCR has to be used in conjunction with the routine detection methods of virus isolation and ELISA for the diagnosis and serotyping of FMD virus. However, PCR-based assays such as real-time RT-PCR (RT-qPCR) assays have gained wide acceptance due to their improved rapidity, sensitivity (> 95%) and specificity. They are, therefore, widely used for routine laboratory-based detection of FMDV (Reid et al., 2002, Callahan et al., 2002, Mackay et al., 2002, Ambagala et al., 2017). A rapid, reliable and accurate diagnostic method supports programmes that monitor and control FMD. The RT-qPCR assay uses the same procedures to extract total RNA from the test or control sample, followed by reverse transcription of the extracted RNA, as with conventional RT-PCR. However, with RT-qPCR, the PCR amplification of the RT product is performed by a different procedure (Callahan et al., 2002, Reid et al., 2002).

Five main chemistries are used for the detection of PCR products during RT-qPCR, including the DNA-binding fluorophores, the 5’ endonuclease, adjacent linear and hairpin oligoprobes, and the self-fluorescing amplicons (Hoffmann et al., 2009). This approach enables simultaneous amplification and quantification of specific nucleic acid sequences (Longjam et al., 2011). The incorporation of universal primers and fluorescent-labelled probes that recognise conserved regions within the 5’ UTR (Reid et al., 2002) or conserved gene regions within the RNA-dependent RNA polymerase gene (3Dpol) (Callahan et al., 2002) facilitates an increase in specificity for RT-qPCR compared to conventional agarose-gel-based PCR assays (Bustin, 2000). This molecular technique is replacing conventional methods such as VI and antigen detection ELISA (Ag-ELISA) for FMDV diagnosis for several reasons, which include the ease of automation (Bustin, 2000) and rapidity of the results (Reid et al., 2003, Shaw et al., 2004, Oem et al., 2005, Callahan et al., 2002).

Currently, pan-specific RT-qPCR assays for FMDV detection have been described (Callahan et al., 2002, Reid et al., 2002, Reid et al., 2009) and are well-established and widely used in endemic and FMD-free countries. The development of panels of serotype-specific RT-qPCR assays tailored to detect FMDV lineages circulating in particular regions and countries has been described (Bachanek-Bankowska et al., 2016, El Bagoury et al., 2022). However, just like RT-PCR, RT-qPCR is not suitable for onsite screening, and several attempts have been made to transfer the laboratory-based RT-qPCR assays to portable PCR equipment that can be operated in the field (Callahan et al., 2002, Madi et al., 2012, Howson et al., 2017, Mölsä et al., 2012).

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Isothermal amplification methods such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and reverse transcription-insulated isothermal PCR (RT-iiPCR) offer alternative means for rapid and sensitive detection of pathogens in clinical samples (Ambagala et al., 2017). Kasanga et al. (2014c) supported this by reporting that the performance of RT-LAMP was superior to RT-qPCR and recommended its use regarding the specific detection and surveillance of infectious diseases of humans and animals in resource-compromised developing countries.

1.6.3. Sequencing

The advent of PCR opened many doors in genetic research, including DNA analysis and identification of different genes. The diverse sequences of viral populations within individual hosts are the starting material for selection- and subsequent evolution of RNA viruses such as FMDV (Wright et al., 2011). Sequencing studies can reveal epidemiological aspects such as transmission pathways, deliver new insights into the biology and evolution of a virus and uncover genetic differences that influence host-virus interactions (Mullan et al., 2004, Radford et al., 2012). The preferred method for generating sequences currently involves RT-PCR amplification of FMDV RNA followed by nucleotide sequencing (WOAH, 2022a).

The molecular epidemiology of FMDV relies on comparing the genetic differences in the VP1 region of sequenced FMDV with those in the database at the WRLFMD, Pirbright Institute and ARC-Onderstepoort Veterinary Research (ARC-OVI) (WOAH, 2022a). Dendrograms illustrating the genomic relationship between vaccine and field strains for all seven serotypes, based on sequences from the 1D gene (which encodes the VP1 viral protein), have been published (Knowles and Samuel, 2003, FAO-WRLFMD, 2023). This coding sequence of VP1 has been extensively used for molecular epidemiological studies, as observed by its frequent use by FMD reference laboratories, which are tasked with monitoring the spread and global distribution of FMDV (Knowles and Samuel, 2003, Subramaniam et al., 2015, Banda et al., 2014, Abdul-Hamid et al., 2011b, Knowles et al., 2016a).

The selective forces at work during the emergence of FMDV are influenced by specific epidemiological and immunological aspects of host-virus interaction as well as the quasispecies composition of the viral populations (Carrillo et al., 2007). It's worthwhile to note that the VP1 coding sequence alone does not reflect all critical phenotypic traits of FMDV (Dill et al., 2017). The VP1 segment was used simply because it has been the most commonly sequenced section of the genome, but using a larger part would be more suitable and is now more viable in the era of next-generation sequencing (NGS) (Hall et al., 2013).

Following on from electron microscopy, cell culture and PCR, NGS is changing how we understand viruses, particularly in genome sequencing, evolution, ecology, discovery and transcriptomics (Radford et al., 2012). The NGS platforms promise rapid, cost-effective, and high-throughput methods for generating viral genome sequences (Logan et al., 2014). It provides greater resolution to monitor the entire sequence swarm within FMDV samples and

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the impact of transmission within and between hosts upon sub-consensus polymorphisms (King et al., 2016, Morelli et al., 2013, Orton et al., 2013). These techniques offer unprecedented "step-change" increases in the amount of sequence data that can be generated from a sample and can be applied to resequencing short viral genomes to obtain ultra-deep coverage. Therefore, NGS has the potential to provide information beyond the consensus for a viral sample by revealing nucleotide substitutions present in only a small fraction of the population (Wright et al., 2011). Retrieving whole genome consensus-level sequences provides important information for outbreak epidemiology and pathogen identification (Töpfer et al., 2013, Escobar-Gutiérrez et al., 2012). This is mostly achieved through comparing whole genome sequences which offers greater discrimination between closely related viruses and aids in reconstructing transmission pathways between locations during outbreaks (Orton et al., 2013, Juleff et al., 2013, Cottam et al., 2008a, Xu et al., 2013). Experimentally, Juleff et al. (2008) confirmed the ability of phylogeny to reproduce the transmission events between animals through whole genome consensus sequencing of FMDV. The global tracing of FMDV reported in WOAH (2022a) is also another good example of transmission tracing. It's worth noting that apparent gaps in such transmission chains have encouraged the search for unrecognised sites of infection and the discovery of such premises improves disease control (Jamal and Belsham, 2018). Maree et al. (2014) discussed that genetic sequencing and antigenic profiling of the outbreak virus are also used to identify newly emerging and re-emerging virus strains and whether available vaccine strains are likely to protect against the outbreak virus. Though the NGS is independent of prior knowledge of target sequences for primer design, it is still expensive and requires elaborate library preparation, storage and analysis of the large amounts of data generated (Logan et al., 2014, Wright et al., 2011, Shendure et al., 2008). Short reads generated by NGS may also limit the ability to identify recombination between different FMDV strains if samples could contain the parental and recombinant strains (Jamal and Belsham, 2018). However, costs will decrease as the chemistries employed mature (Radford et al., 2012).

The introduction of new fast methods such as RT-qPCR into FMD diagnostics together with viral sequence analysis allows the study of viral biodiversity and evolution, thus aiding in tracking transmission events and sources (Abdul-Hamid et al., 2011a, Wright et al., 2011, Cottam et al., 2008b). This viral biodiversity and evolution study also assures vaccine coverage of corresponding field FMDV lineages (Wright et al., 2011).

In Zambia, as in many other African countries, a combination of these diagnostic tests is used to detect and monitor FMD outbreaks. However, the lack of widespread access to advanced molecular techniques like RT-PCR means that simpler, rapid tests like antigen ELISA, serological assays, and lateral flow devices are often preferred at the field level. In a typical Zambian FMD laboratory, diagnosis of FMD is often based on serology which is done locally at CVRI. For serology, non-structural protein (NSP), SPBE and LPBE described earlier are utilised. The 3ABC NSP ELISA kits that have been used at CVRI include IDEXX, Westbrook, MN, USA, Ceditest FMDV-NS kit, Prionics Lelystad B.V and rarely Thermofisher PrioCheck®-NSP kit. Recently, the SPBE ELISA has been introduced as one of FMD diagnostic platforms at CVRI

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replacing the LPBE ELISA due to its limitations. The use of antigen ELISA was also limited at CVRI until 2018 when this test and the Real time PCR was introduced. For confirmatory diagnosis however, suspected FMD samples are routinely sent to reference laboratories of BVI, ARC-OVI and the Pirbright Institute. However, this involves transportation of samples containing infectious FMDV which represents a significant bio-security risk and is also costly. To improve FMD control, there is a growing need for better infrastructure, training, and funding to expand the availability of more sensitive and specific diagnostic methods, particularly in rural and remote areas where outbreaks often occur.

1.7. Prevention and control of foot-and-mouth disease

The fundamental components of FMD control are vaccination, biosecurity and livestock movement control (Knight-Jones et al., 2016). However, the need for continued disease surveillance to confirm the absence of disease and infection in livestock has been emphasised (Chitray et al., 2018, Metwally et al., 2023, Jamal and Belsham, 2013). Animal identification systems are also critical for effectively controlling the disease (Jamal and Belsham, 2013). Epidemiological tracing of virus transmission and conventional field investigations have also been highlighted as an integral part of any viral disease control strategy (Logan et al., 2014).

In countries free of FMD with naive livestock populations, the primary focus is on minimising the risk of virus incursions. The initial approach for controlling FMD involves stamping out, sanitation and strict quarantine measures (Paton et al., 2009). On the contrarily, in under-resourced agricultural systems like in Zambia, vaccination is the most important disease control measure employed (Knight-Jones and Rushton, 2013, Roeder et al., 2013, Taylor et al., 2002). It's worth noting, however, that vaccination is only effective when coupled with other disease control measures (Jamal and Belsham, 2013, Capozzo et al., 2023).

In southern Africa, the control of FMD has depended mainly on separating infected wildlife from susceptible domestic animals combined with vaccination in buffer zones (Sutmoller et al., 2000, Vosloo et al., 2002, Thomson et al., 2003). Usually, the control policy a country adopts depends on its FMD status and the risk of disease incursions (Paton et al., 2009). Countries with FMD-free zones without vaccination (Figure 2.3) also rely on control of animal movement and careful assessment of the risk of FMD introduction in non-infected areas (Chitray et al., 2018, Thomson et al., 2003, Namatovu et al., 2013). The control of FMD could be considered one of the significant constraints to the coexistence of livestock and wildlife in southern Africa. However, this prophylactic strategy's technical and practical limitations at the wildlife-livestock interface based on vaccination have been highlighted (Miguel et al., 2013). Furthermore, Thomson et al. (2012) stated that these control measures appear ineffective in stopping disease reoccurrence, although Hunter (1998) found that the physical separation of the wildlife species from livestock and the vaccination of domestic species were central to effective control of the disease in southern Africa. Within a country, movement restrictions and zonation disrupt domestic trade and access to rangelands (Knight-Jones et al., 2016). Establishing Transfrontier Conservation Areas (TFCA) and human encroachment into the wildlife domain can further weaken these control measures, resulting in increased

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wildlife migration and the pressure to remove fences (Tekleghiorghis et al., 2014). In Zambia, the policy on FMD control has been to prevent its occurrence and spread in the livestock population by limiting it to high-risk areas and infected wildlife populations. This is usually done through vaccinations coupled with movement restrictions. This has, however, suffered setbacks as disease outbreaks suspected to emanate from neighbouring countries have been documented (Banda et al., 2014, Rweyemamu et al., 2008b, Overby and Zyambo, 1983, Kasanga et al., 2012). Generally, if control is unsuccessful, the negative impacts of control will be experienced without obtaining the benefits; therefore, control should not be attempted unless effective control is feasible (Knight-Jones et al., 2016).

The Food and Agriculture Organization (FAO) and the WOAH developed a 15-year global control strategy in 2012 to reduce the burden of FMD in endemic and to maintain the status of FMD-free countries (FAO-WOAH, 2012). The Progressive Control Pathway for Foot-and-Mouth Disease (PCP-FMD) is a risk and evidence-based framework guiding endemic countries to progressively improve the management of FMD risks and reduce disease impacts and viral circulation (Metwally et al., 2023). Zambia is currently in PCP stage 2 and is working on modalities to maintain this status. The successes and challenges of the FAO-WOAH PCP strategy in achieving freedom from FMD by combining zoning, movement control, vaccination, and surveillance have been described (Abbas et al., 2014, Sultanov et al., 2023). In much of Africa, however, land is owned by the state and farmed communally with shared grazing. In these circumstances, a farmer can do little to prevent virus exposure if other livestock at shared grazing are infected (Knight-Jones et al., 2016). Controlling animal movement is challenging under the current production system (pastoralist and agro-pastoral) and livestock marketing systems (cross-border trade). Consequently, vaccination remains the primary method of control (Di Nardo et al., 2011, Tekleghiorghis et al., 2014).

1.7.1. Vaccination

Vaccination has played a significant role in FMD control and remains the most effective countermeasure (Ma et al., 2011). It is regarded as an efficient and cost-effective measure to prevent the transmission of this disease (Yang et al., 2022). Different approaches to designing and implementing vaccination campaigns are available (Keeling et al., 2003, Ge, 2015). Most FMDV vaccines used globally in routine vaccination are inactivated whole-virus vaccines grown in cell culture. Therefore, animals vaccinated against FMD will only develop antibodies to structural proteins (Clavijo et al., 2006).

Although currently available inactivated vaccines have helped control FMD, sustained mass vaccination campaigns, regional alliances and significant economic investments were required to achieve eradication in a relatively limited number of countries worldwide (de los Santos et al., 2018). Vaccine selection and successful vaccination campaigns rely on a deep knowledge of the epidemiology of the region where these vaccines will be used, as well as access to the appropriate diagnostic tools to underpin these campaigns (Capozzo et al., 2023). Control by vaccination is challenging due to genetic and antigenic variations among existing subtypes and the lack of information on the virus's temporal and spatial dynamics, which are

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crucial for effective vaccine matching (Sallu, 2016). Consequently, a vaccine may be effective in one region but not in another, even when the same virus strain is used. This scenario is evident, especially in southern Africa, where the possibility of controlling and eradicating FMD with existing vaccines is not feasible due to the high genetic variability within and between the FMDV SAT serotypes circulating in buffalo populations (Bastos et al., 2003b, Tekleghiorghis et al., 2014, Vosloo et al., 2005). This differs from the scenario in Europe and the greater part of South America where FMD has been eradicated with effective vaccination campaigns and stringent control measures (Freimanis et al., 2016, Bergmann et al., 2000).

In FMD-endemic countries, vaccination is the primary strategy for controlling outbreaks (Miguel et al., 2013, Hunter, 1998). However, this approach should be informed by a thorough assessment of the epidemiological and risk factors specific to each contact farm. Limitations to consider when using vaccination in endemic countries include a short duration of action, an insufficient match between the field and vaccine strains and a requirement for cold chain maintenance. Selecting FMDV vaccine candidates is challenging due to the extensive genetic and antigenic variability of the virus and the ongoing emergence of new mutants that evade the host immune response (Esteban Domingo, 1988, Haydon et al., 2001). The quality of vaccines can also vary between producers in different regions based on the fact that vaccine strains must be appropriate for the circulating strains (Jori and Etter, 2016). Therefore, knowledge of circulating FMDV in specific geographic locations is critical. In the late 1980s and 1990s, limited number of FMD outbreaks were reported in South Africa when the vaccines were synthesised from strains collected directly from Kruger National Park (KNP) buffalo populations (Thomson et al., 2013). This outcome is probably an exemplification of the importance of using appropriate circulating strains as vaccine strains as the vaccines used during that period proved protective based on the limited number of outbreaks reported. Further, Saraiva (2003), Pay (1985) found that risk of development of clinical disease in cattle exposed to buffalo infected with FMDV decreases when vaccinated with appropriately matched vaccines. Implementing an effective vaccination programme in endemic countries like Zambia also necessitates intensive molecular epidemiological surveillance of currently circulating field isolates and continuous monitoring to ensure that the vaccine strains remain protective against field viruses (Sallu, 2016). Post-vaccination monitoring (PVM) surveys are also necessary to monitor the coverage and performance of the vaccine. These routine field evaluations should be part of countries control policy and standardised practices to investigate apparent vaccine failures, including notifying the vaccine manufacturer. Such evaluation is even more essential where government-subsidized vaccines are used, like in Zambia, so that the cost-effectiveness of the control programme can be ensured (Lyons et al., 2015). However, a dependence on regular vaccination brings several problems of its own. The DIVA using serology remains a challenge, and vaccinated animals may become sub-clinically infected, thereby potentially masking an outbreak situation (Robinson and Knight-Jones, 2014, Jamal and Belsham, 2013, Namatovu et al., 2013). Nevertheless, some FMD vaccines are purified of NSP, which allows NSP serology to DIVA, which is useful when evaluating vaccination programmes (Bronsvoort et al., 2008). There is an increased demand for improved vaccines with higher potency, safety, improved DIVA capability and, most

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importantly, adequate coverage of circulating and emerging FMDV strains. In South America, a combined system of an indirect ELISA (3ABC-ELISA) with an enzyme-linked immunoelectrotransfer blot assay was successfully used to support local FMD control programs under systematic vaccination (Bergmann et al., 2000).

The control of FMD in Zambia has previously focused on the vaccination of livestock in FMD high-risk and outbreak areas (Sinkala et al., 2014b, Banda et al., 2014, Sinkala, 2015) using a regionally produced non-NSP purified vaccine. The Department of Veterinary Services (DVS) provides vaccinations every six months for cattle. Despite these efforts, some reports of FMD cases in the vaccinated areas were still reported, leaving several questions unanswered over the appropriateness of the vaccines used and the impact of the control programme. Strains used for routine vaccinations are presumed effective during vaccination response to outbreaks in Zambia, but there is no empirical data to show the efficacy of the vaccines currently in use in the various situations they have been applied (Sinkala et al., 2014b).

1.7.2. Movement control

Worldwide, animal movement significantly impacts FMD transmission patterns since it was revealed that FMD displayed geographical diversity (Yang et al., 2022) based on their geographic origin (ecological isolation or adaptation)(Di Nardo et al., 2011, Samuel and Knowles, 2001a, Foglia et al., 2021). In Africa, the epidemiology of FMD is complicated by the widespread movement of animals (Alexandersen and Mowat, 2005). Animal movement restrictions have been pivotal wherever FMD has been controlled. When effectively implemented, these restrictions have proven to be a key strategy in controlling FMD (Brown, 2003, Vosloo and Thomson, 2004). However, implementing movement restrictions in smallholder settings in poor countries is a complex challenge. Smallholders often require continued access to local markets and communal grazing, making it extremely difficult to impose restrictions (Knight-Jones et al., 2016). In Zambia, movement control has remained a considerable challenge due to the agro-pastoral production system, which includes transhumance and communal grazing (Muma et al., 2006, Muuka et al., 2012), as well as cross-border livestock trade (Banda et al., 2014, Sinkala et al., 2014b). Further, animals are kept for social reasons, including traditional practices, e.g., bridal price (Mumba et al., 2018), funerals, traditional ceremonies, Mafisa Muuka et al. (2012), etc. This entails the movement of animals from one area to another, hence disease transmission. In an outbreak scenario, however, the government of Zambia usually institutes immediate movement bans on all animals and animal products. Paton et al. (2018) had previously stated that Indirect transmission routes are the primary means for spreading FMDV in countries that effectively impose movement bans in the face of disease outbreaks.

Regarding the cross-border spread of transboundary diseases such as FMD, the movements of animals and animal products are the main risk factors involved, especially in countries where such movements are poorly regulated (Di Nardo et al., 2011). Animal identification and movement monitoring play a critical role in such circumstances. This leads to the identification of cross-border movements and market chain interactions of ruminants,

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leading to improved border and movement controls. The observed lack of movement control within countries and across international borders for both wildlife and domestic animals, especially in SSA, aggravates the problem and gives credence to the fact that FMD will remain a problem on the subcontinent for the foreseeable future (Vosloo et al., 2005).

1.7.3. Biosecurity

Maintaining sound biosecurity practices helps prevent the introduction and spread of FMDV to a particular farm or country (Sangula et al., 2011).

The implementation of measures that reduce the risk of the introduction and spread of disease agents assists in the prevention and control of FMD outbreaks. Three principal steps for biosecurity are;

- Physical segregation
- Cleaning - removing contamination
- Disinfection – Virus inactivation

Biosecurity measures fundamental to successful FMD control in the developed world can seldom be implemented effectively in many developing countries where the disease remains uncontrolled (Knight-Jones, 2014). This is because smallholder systems predominate, with dependency on common grazing and frequent livestock trading to provide income for many people (Robinson and Knight-Jones, 2014). Biosecurity is practised on-farm and at FMD control road checkpoints in Zambia.

1.7.4. Surveillance

The role of a surveillance system and the need for a well-designed, practical, and effective system to achieve and document disease control progress has been emphasised by Metwally et al. (2023). This is especially critical when disease control is tailored in line with the FAO-WOAH PCP for the Control of FMD, as being implemented in Zambia. Inadequate disease surveillance has been previously attributed to one of the reasons for missing disease presence in a population (Dawe et al., 1994b, Dawe et al., 1994a). Maree et al. (2014) noted the need for local and regional surveillance programmes to monitor FMDV circulating in wildlife and livestock populations. Furthermore, as FMD tends to occur in waves of infection with quiescent intervals, an absence of disease cannot be taken as an assurance of an effective vaccination programme (FAO-OIE, 2016) and an effective disease control programme. It may in fact represent a probable source of further rounds of infection, inter-epidemic survival of the virus and spread of disease(Barnett and Cox, 1999). Monitoring of immunity acquired either from vaccination and/or infection following an outbreak is therefore required, and this can only be achieved through surveillance. In Zambia, veterinary field staff conduct active and passive surveillance through clinical inspections and collect serum and at times tissue samples during active disease outbreaks. Tissue samples are used for virus detection, while serum samples are used for serological examination.

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Adopting a risk-based approach to FMD control is essential for several reasons, including targeted resource allocation, early detection and rapid response, cost-effectiveness, enhanced surveillance, improved livestock health and productivity, and the development of sustainable control measures. By identifying high-risk areas and populations, resources can be allocated efficiently, ensuring that vulnerable regions receive the necessary attention and interventions, maximising the impact of control measures (FAO-OIE-EUFMD, 2013). Focusing on high-risk areas allows for early detection of outbreaks, enabling rapid responses that can prevent the disease from spreading to other regions, thereby reducing its overall impact (FAO-OIE, 2016). Mitigating risks in high-priority zones is also more cost-effective than a blanket approach, minimising unnecessary expenditure on low-risk areas and promoting better financial management of FMD control programs (FAO-OIE-EUFMD, 2020). Moreover, closely monitoring high-risk areas improves surveillance and data collection, helping to understand disease dynamics and adjust strategies accordingly (FAO-OIE-EUFMD, 2020, FAO-OIE-EUFMD, 2013). By protecting high-risk populations, livestock health and productivity are maintained, which is crucial for the livelihoods of farmers and the agricultural economy (FAO-OIE-EUFMD, 2020). Additionally, a risk-based approach supports the development of sustainable control measures tailored to specific risks and conditions, contributing to long-term success in FMD management (FAO-OIE-EUFMD, 2013). Overall, this approach ensures that FMD control efforts are strategic, effective, and sustainable, not only protecting livestock but also supporting the livelihoods of those who depend on them.

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Chapter 2. Characterisation of Foot-and-Mouth Disease Viruses in Zambia - Implications for the Epidemiology of the Disease in Southern Africa

This chapter has been published as (Banda, Frank et al. "Characterization of Foot-and-Mouth Disease Viruses in Zambia-Implications for the Epidemiology of the Disease in Southern Africa." *Viruses* vol. 13,11 2195. 31 Oct. 2021, doi:10.3390/v13112195) (Appendix 1).

2.1. Abstract

The livestock industry supports the livelihood and nutritional security of at least 42% of people in the SADC region. However, the presence of animal diseases such as FMD poses a significant threat to the development of this industry. Samples collected from FMD outbreaks in Zambia during 2015 – 2020, comprising epithelial tissue samples (*n* = 47) and sera (*n* = 120), were analysed. The diagnosis was also carried out on vesicular epithelium samples (n = 7) collected from Nfooma in Katima Mulilo Urban, Namibia. Foot-and-mouth disease virus was serotyped in 30 samples, while 92 sera samples tested positive on NSP-ELISA. Phylogenetic analysis revealed notable changes in the epidemiology of FMD in Zambia and Namibia, which included: (i) introduction of a novel FMDV SAT-3 (topotype II) causing FMD cases in cattle in Western Province; (ii) emergence of FMDV serotype O (topotype O/EA-2) in Central, Southern, Copperbelt, Western, Lusaka Provinces (iii) introduction of an FMDV serotype O in Namibia and, (iv) new outbreaks due to FMDV SAT -2 (topotypes I) in Eastern Zambia. Together, these data describe eight different epizootics that occurred in Zambia, four of which were outside the known FMD high-risk areas and with one spreading into neighbouring Namibia. This study highlights the complex epidemiology of FMD in Zambia, where the country represents an interface between East Africa (Pool 4) and Southern Africa (Pool 6). This is the first detection of serotype O in Namibia, and together with the Zambian cases, represents the first time that this serotype has been detected in the Southern African FMD endemic pool since 2000, when a virus of Asian origin (O/ME SA/PanAsia) caused an outbreak in South Africa. These changing viral dynamics and the latest incursion of serotype O pose a new threat for the region, and the potential onward spread of O/EA-2 will now need to be closely monitored since serotype O vaccines are not widely used in the SADC region.

2.2. Introduction

Foot-and-mouth disease is a highly contagious transboundary animal disease (TAD) that is a major concern to livestock industries throughout the world. The disease is caused by *Aphthovirus vesiculae*, which affects cloven-hoofed animals. In addition to the ability of FMDV to spread rapidly within domesticated livestock, the virus can be maintained within populations of certain wildlife species, such as free-ranging African buffalo, which do not show obvious signs of disease and can act as virus reservoirs capable of transmitting the virus to livestock (Alexandersen et al., 2003b, Sangula et al., 2010, Thomson and Bastos, 2004). The

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involvement of wildlife in the epidemiology of FMD provides a challenge to control FMD in SSA (Weaver et al., 2013). The FMDV genome contains a single open reading frame that encodes four structural viral proteins (VP1–VP4) that form the virus capsid and eight NSPs involved in virus replication. Viral protein 1 sequences are often used for epidemiological investigations (Knowles and Samuel, 2003) to characterise FMDV within the seven serotypes (A, O, C, SAT-1, SAT-2, SAT-3 and Asia-1) and into genetically discrete topotypes. Globally, there are seven endemic pools of infection, which often require tailored diagnostics and vaccines (Freimanis et al., 2016, Paton et al., 2009). The disease circulates in Asia, Africa, and parts of South America, where outbreaks lead to livestock production losses and disruptions to international trade (Knight-Jones and Rushton, 2013). In Africa, five serotypes (O, A, SAT-1, SAT-2, SAT-3) are present, but their distribution varies. In Southern Africa (Pool 6), three serotypes (SAT 1-3) are endemic, with sporadic incursions of serotype O and A recorded in Northern Zambia as a consequence of spill over from Pool 4 (East Africa) (Kasanga et al., 2014b, Overby and Zyambo, 1983, Rweyemamu et al., 2008b). The distribution of these diverse serotypes and subtypes of FMDV is subject to change and requires continuous monitoring (Rweyemamu et al., 2008b, Paton et al., 2005).

Foot-and-mouth disease remains a major sanitary concern for beef producers in the SADC region because the disease is a key constraint to accessing high-value export markets. The occurrence of FMD results in damaging consequences for the livelihoods of local farmers due to the disease's impacts on productivity, food security and loss of income (Maree et al., 2014). Furthermore, in Zambia, FMD impedes the utilisation of livestock and wildlife resources in the country, where 80% of the population relies on agriculture for food and income generation (Government-of-Zambia, 2011). In this context, FMD in the country has been likened to a disease of poverty because of its negative effects and is the reason for low economic growth (Perry and Hedger, 1984, Sinkala et al., 2014b).

In Zambia, FMD was first reported in 1933 (Morris, 1934), and sporadic disease outbreaks have been reported from three high-risk areas (Perry and Hedger, 1984, Chilonda et al., 1999). These defined high-risk areas (Figure 2.4) are (A) the Northern part of the country on the border with Tanzania (Nakonde, Mbala and Mpulungu districts), (B) the Kafue Flats on the border between Central and Southern provinces (Namwala, Mumbwa, Itezhi tezhi and parts of Monze, Choma and Mazabuka districts) and (C) the Southern parts of Western and Southern Provinces (from Kazungula/Livingstone to a point beyond Sesheke district, where the border with Angola turns to the north-west).

Namibia maintains an 'FMD-free without vaccination' zone officially recognised by the WOAH, located south of the Veterinary Cordon Fence (VCF). The VCF, initially established in 1897 to protect farms in the south of the country from rinderpest, now provides a barrier to protect the high-value commercial export markets in southern Namibia from FMD that circulates in the eastern Zambezi region (communal grazing land) in the northern part of the country. The Northern Communal Areas of Namibia have no official WOAH status for FMD. Foot-and-mouth disease outbreaks detected recently in the north of Namibia include continued cases

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due to serotype SAT-2 (topotype III) in 2021, as well as outbreaks due to serotype SAT-3 (topotype II) in 2019 (https://www.wrlfmd.org/southern-africa/namibia#panel-4082).

An unprecedented rise in FMD outbreaks in livestock since 2004 has been observed in Southern Africa (Thobokwe et al., 2010, Thomson et al., 2012). The reasons for this are most likely multifactorial, but *prima facie* evidence points to the poor performance of prophylactic vaccination programmes (Thomson, 2020). In Zambia, a rise in FMD reports was recorded in the country's Western, Eastern, Southern and Central Provinces from 2015 to 2020. The main risk factors have been reported elsewhere (Hamoonga et al., 2014, Sinkala, 2015), while the molecular epidemiology of FMD in the country has not been fully elucidated. Previous FMD outbreaks in Zambia have been difficult to prevent and control (Chilonda et al., 1999) and have not been systematically studied to gain insight into outbreak causation or transmission patterns and predict when the next outbreak would likely occur (Sinkala, 2015). This information is necessary so that appropriate preventive measures can be instituted. This study aimed to characterise the FMDV that has caused recent cases in Zambia. Here, we also describe the detection of serotype O in cattle in Namibia, representing the first occurrence of this serotype in the country. This is to provide data to help understand the epidemiology of the disease in Southern Africa.

2.3. Materials and methods

2.3.1. Study area

The study region encompassed the whole of Zambia, where specific sampling areas were purposely selected based on suspected FMD cases reported to the DVS during the study period of 2015 to 2020. In Namibia, suspected FMD cases were reported in the Zambezi Region (Figure 2.1).

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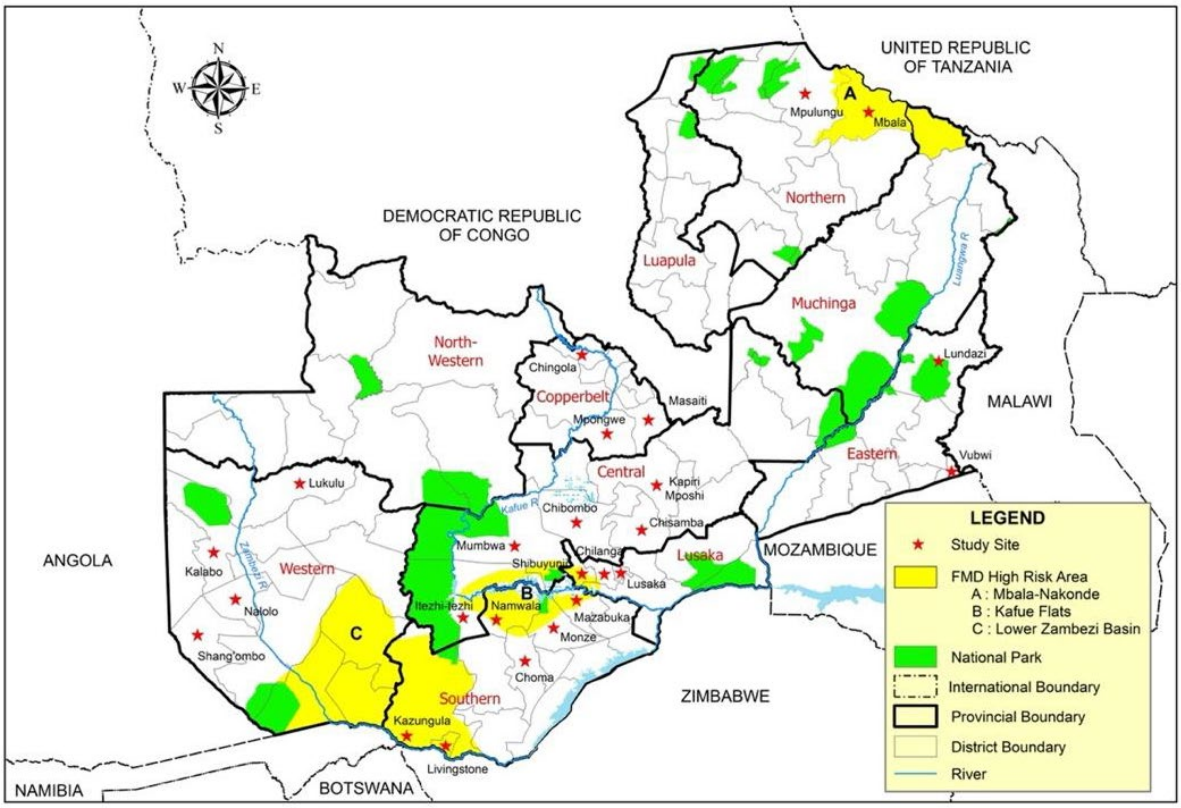


Figure 2.1 Foot-and-mouth disease high-risk areas and study sites in Zambia and Namibia.

2.3.2. Sample collection

In Zambia, 75 kraals and farms were visited during FMD outbreak investigations. After clinical examination for typical FMD lesions, fresh epithelial tissue (*n* = 47) , vesicular fluid (*n* = 2) and blood samples (*n* = 120) from suspect FMD cases in these outbreak areas were collected. The FMD cases in Namibia were detected in the Zambezi Region during June – July 2021. The initial report (on 1 June 2021) involved suspect FMD cases in a cattle herd of 144 animals (in Katima Rural Constituency (geo-coordinates: −17.7816°S, 24.3347°E) where animals exhibited clinical signs indicative of FMD. A further suspicion of FMD was raised in cattle (on 13 July 2021) at Nfooma in Katima Mulilo Urban (geo-coordinates: −17.5000°S, 24.2666°E) were vesicular epithelium samples (n = 7) were collected. The tissue specimens and blood samples were collected, as described elsewhere (Kitching and Donaldson, 1987). Briefly, at least one gram of epithelial tissue was collected from an unruptured or recently ruptured vesicle and placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer (pH 7.2 – 7.6). Whole blood was collected in plain vacutainer tubes (Becton, Dickson and Company, New Jersey, USA), and the serum was separated once the blood was clotted. Epithelial tissues and serum samples collected in the Zambian study were kept refrigerated at 4 °C or on ice for epithelial tissue until received at the CVRI in Zambia, where samples were stored at −20 °C. The samples were packaged and shipped to the WOAH Sub-Saharan Africa Regional Reference Laboratory (SSARRL) for FMD (Botswana Vaccine Institute, Gaborone, Botswana) and the FAO WRLFMD and WOAH Reference Laboratory, The Pirbright Institute, United Kingdom) for VI, NSP-ELISA, antigen-ELISA, RT-qPCR and VP1 sequencing. Vesicular

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epithelium samples (n = 7) collected on 15 July 2021 from Nfooma in the Zambezi region of Namibia were submitted to SSARRL where FMDV diagnosis was carried out as previously described (Teye et al., 2019).

2.3.3. Laboratory analysis of samples

At CVRI, all sera samples were tested for FMDV NSP-specific antibodies using a multi-species antibody test kit (IDEXX, Westbrook, Maine, USA) by following the manufacturer's instructions. Representative epithelial samples were prepared and tested for the presence of FMDV by thawing and blot drying them, followed by grinding in phosphate-buffered saline (PBS) using sterile sand in a pestle and mortar in a 10% suspension. The suspensions were tested using a sandwich ELISA (IZSLER Biotechnology Laboratory, Brescia, Italy) for detection and serotyping of FMDV antigens by following the manufacturer's instructions. At the WRLFMD, VI was carried out on primary BTy cells (Snowdon, 1966) or IB-RS-2 cell cultures (De Castro, 1964), and an Ag-ELISA was performed on cell cultures showing cytopathic effect to identify the serotype (Ferris and Dawson, 1988, Roeder and Le Blanc Smith, 1987). Lamb kidney cells were used for VI at the BVI. The FMDV-specific RT-qPCR assays were performed on the samples (Callahan et al., 2002, Shaw et al., 2007). Samples with detectable FMDV genomes were further characterised by VP1 sequencing (Knowles et al., 2016c). Complete VP1 nucleotide sequences were aligned using BioEdit 7.0.5.3 (Thompson, 1994, Hall, 1999). Optimal nucleotide substitution models were computed for each serotype using MEGA 7 (Kumar, 2016). The maximum likelihood algorithm was used to construct phylogenetic trees employing MEGA 7. One thousand bootstrap pseudo-replicates were used to assess branching confidence.

The Namibian samples were first thawed at room temperature and then blotted and dried on absorbent paper. A 10 % suspension was prepared by grinding 1 g of the sample in a sterile mortar and pestle with a small volume of tissue culture medium (10% Minimum Essential Medium (MEM 10Å~), 10% lactalbumin hydrolysate, 4.5% sodium bicarbonate, 1% calf serum, 0.2% penicillin and sterile distilled water). The suspension was clarified by centrifugation at 2000 g for 10 min at 4°C, and 500 µl of the suspension was inoculated onto primary lamb kidney cell cultures in 25 cm2 culture flasks and incubated for 1 h at 37°C. Fresh cell culture medium (15 ml) was added, incubated at 37°C, and monitored for CPE for 48 h. Samples were considered negative for VI if no CPE was observed after the third passage. An in-house sandwich ELISA using serotype-specific rabbit and guinea pig antisera for FMDV serotypes O, A, SAT-1, SAT-2 and SAT-3 (Pirbright Institute, UK, Ferris and Dawson (1988) was used to detect FMDV antigens and to define the serotype of the CPE-positive samples. FMDV sequences encoding VP1 were generated for serotype O positive samples using a published RT-PCR amplification and sequencing method (Knowles et al., 2016c). Briefly, the RT-PCR amplification used the following primers: O-1C244F 5'-GCA GCA AAA CAC ATG TCA AAC ACC TT-3', and EUR−2B52R_5'-GAC ATG TCC TCC TGC ATC TGG TTG AT-3' and cycle-sequencing adopted O-1C244F and NK72 5'-GAA GGG CCC AGG GTT GGA CTC-3'. The 1D nucleotide sequences encoding VP1 (633 nucleotides) were compared with other related serotype O viruses collected from the region. In addition to published sequences, the comparative data

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included unpublished sequences recently obtained at the FAO WRLFMD, (Pirbright, UK) for 33 samples received from Zambia. Collection locations and dates for these samples are outlined in Table 2.1. At the WRLFMD, field isolates were generated in primary BTy cell cultures and typed using an Ag-ELISA (Ferris and Dawson, 1988). The VP1 encoding regions were amplified using two separate RT-PCR reactions with primers O-1C244F and EUR-2B52R as previously described (Knowles et al., 2016c), or with FMD-3161F and FMD-4303R (Dill et al., 2017). Cycle sequencing adopted the same primers in separate reactions (Knowles et al., 2016b). New sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers OM259982–OM260015 (Table 2.1). The complete VP1 encoding nucleotide sequences were aligned using BioEdit 7.0.5.3 (Hall, 1999) and ClustalW1.83 (Thompson, 1994). To construct a maximum likelihood (ML) phylogeny (Nei and Kumar, 2000), the data set was tested for 24 common nucleotide substitution models using MEGA 7 (Kumar, 2016). The model with the lowest Bayesian information criterion (BIC) score (Tamura 3- parameter with gamma distribution) (Tamura, 1992) was chosen to construct the tree. The robustness of the tree topology was assessed with 1000 bootstrap pseudo-replicates.

Table 2.1 Sample details of O/EA-2 viruses collected recently from Zambia and Namibia (2018 - 2021). All samples were collected from cattle except ZAM/1/2020, ZAM/2/2020 and ZAM/18/2019, which were from pigs.

Virus name	GenBank number	Date collected	Sampling location	Province/ Region	Country
O/ZAM/1/2018	MZ486060	24/03/2018	Chisamba	Central	Zambia
O/ZAM/2/2018	MZ486061	24/03/2018	Chisamba	Central	Zambia
O/ZAM/3/2018	MZ486062	02/04/2018	Chisamba	Central	Zambia
O/ZAM/1/2019	MZ486065	18/01/2019	Chisamba	Central	Zambia
O/ZAM/2/2019	MZ486066	11/02/2019	Ufwenuka, Monze	Southern	Zambia
O/ZAM/3/2019	MZ486067	11/02/2019	Ufwenuka, Monze	Southern	Zambia
O/ZAM/4/2019	MZ486068	11/03/2019	Magoye, Mazabuka	Southern	Zambia
O/ZAM/7/2019	MZ486069	30/03/2019	Kasako, Mazabuka	Southern	Zambia
O/ZAM/8/2019	MZ486070	30/03/2019	Kasako, Mazabuka	Southern	Zambia
O/ZAM/15/2019	OM259982†	31/05/2019	Namwala	Southern	Zambia
O/ZAM/16/2019	OM259983†	31/05/2019	Namwala	Southern	Zambia
O/ZAM/18/2019	OM259984†	05/06/2019	Monze	Southern	Zambia
O/ZAM07/2019*	MZ486072	28/06/2019	Musenga, Chingola	Copperbelt	Zambia
O/ZAM/20/2019	OM259985†	28/06/2019	Makeni	Lusaka	Zambia
O/ZAM/21/2019	OM259986†	28/06/2019	Makeni	Lusaka	Zambia
O/ZAM/24/2019	OM259987†	17/07/2019	Kafue	Lusaka	Zambia
O/ZAM/26/2019	OM259988†	17/07/2019	Chibombo	Central	Zambia
O/ZAM/27/2019	OM259989†	17/07/2019	Chibombo	Central	Zambia
O/ZAM/28/2019	OM259990†	07/08/2019	Chikankata	Southern	Zambia
O/ZAM/54/2019	OM259991†	01/09/2019	Chikankata	Southern	Zambia
O/ZAM/55/2019	OM259992†	06/09/2019	Kabwe	Central	Zambia
O/ZAM/56/2019	OM259993†	06/09/2019	Kabwe	Central	Zambia
O/ZAM/57/2019	OM259994†	01/10/2019	Mpande	Northern	Zambia
O/ZAM/58/2019	OM259995†	01/10/2019	Sinazongwe	Southern	Zambia
O/ZAM/60/2019	OM259996†	17/10/2019	Kasempa	North-western	Zambia
O/ZAM/62/2019	OM259997†	01/12/2019	Itezi tezi	Central	Zambia
O/ZAM/1/2020	OM259998†	05/02/2020	Chilanga	Lusaka	Zambia
O/ZAM/2/2020	OM259999†	05/02/2020	Chilanga	Lusaka	Zambia
O/ZAM/3/2020	OM260000†	16/03/2020	Namwala	Southern	Zambia
O/ZAM/4/2020	OM260001†	16/03/2020	Namwala	Southern	Zambia
O/ZAM/6/2020	OM260002†	15/05/2020	Kalomo	Southern	Zambia
O/ZAM/7/2020	OM260003†	15/05/2020	Kalomo	Southern	Zambia

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O/ZAM/8/2020	OM260004†	10/08/2020	Kazungula	Southern	Zambia
O/ZAM/9/2020	OM260005†	10/08/2020	Kazungula	Southern	Zambia
O/ZAM/10/2020	OM260006†	10/08/2020	Kazungula	Southern	Zambia
O/ZAM/11/2020	OM260007†	18/08/2020	Makeni	Lusaka	Zambia
O/ZAM/12/2020	OM260008†	18/08/2020	Makeni	Lusaka	Zambia
O/ZAM/14/2020	OM260009†	28/10/2020	Nkeyema	Western	Zambia
O/ZAM/15/2020	OM260010†	28/10/2020	Nkeyema	Western	Zambia
O/ZAM/16/2020	OM260011†	28/10/2020	Nkeyema	Western	Zambia
O/ZAM/7/2021	OM260012†	18/03/2021	Kalomo	Southern	Zambia
O/ZAM/9/2021	OM260013†	08/05/2021	Mwandi	Western	Zambia
O/ZAM/11/2021	OM260014†	12/05/2021	Mwandi	Western	Zambia
O/NAM10/2021*		15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia
O/NAM11/2021*		15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia
O/NAM13/2021*	OM260015†	15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia
O/NAM14/2021*		15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia

* Not a WRLFMD isolate number
† New sequence data presented in this report

2.4. Results

2.4.1. Epidemiology and clinical observations

Twenty-five FMD outbreaks were recorded during the study period in Zambia, of which 32% (8) were primary outbreaks and 68% (17) were outbreak extensions (Figure 2.2 and Table 2.2). Out of the eight primary outbreaks, four (50%) were reported outside the three known high-risk areas in Zambia: in October 2015, from Shang'ombo district of Western Zambia; in March 2018 and January 2019, from the Chisamba district of Central Zambia, and finally during March 2019, from Lundazi and Vubwi districts of Eastern Zambia. The other four primary outbreaks were reported in February 2015 from Mpulungu and Mbala districts of Northern Zambia, whilst Kawimbe Veterinary Camp of Mbala district reported outbreaks in October and March 2018.

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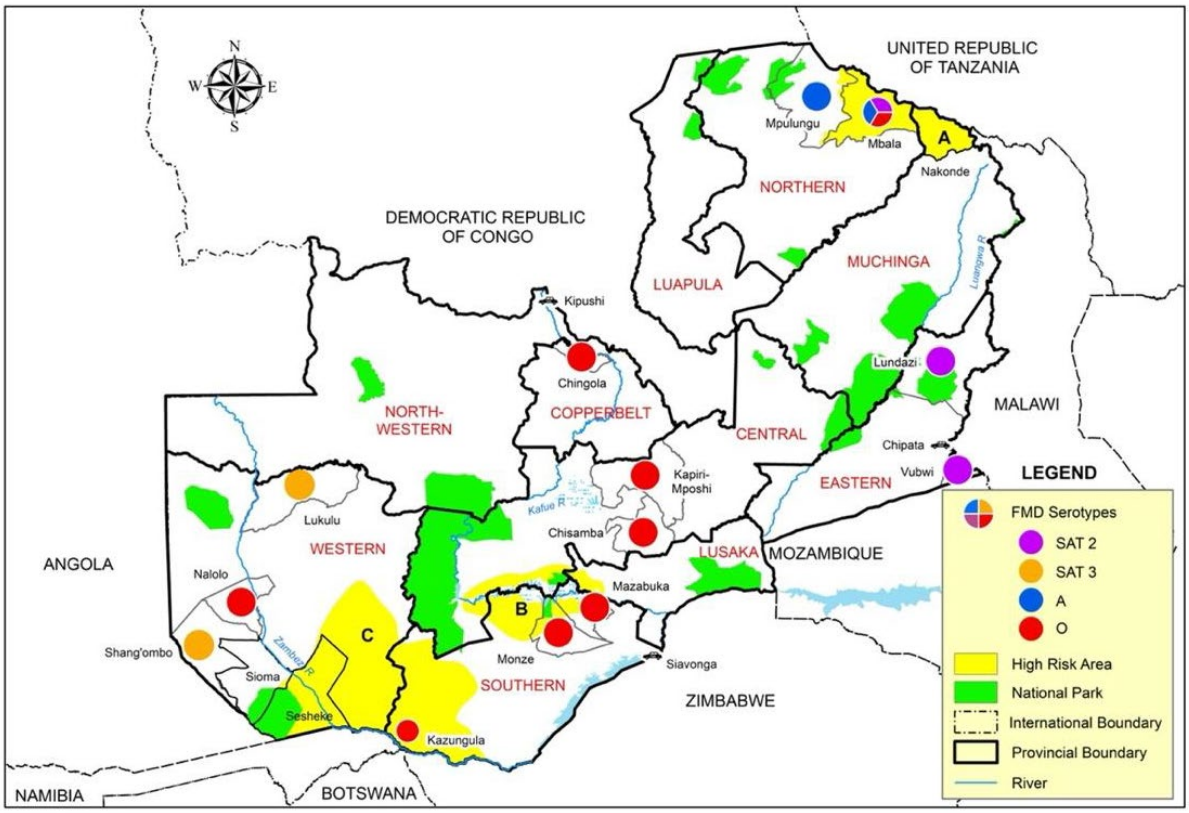


Figure 2.2 Foot-and-mouth disease virus serotypes detected in Zambia between 2015 and 2022.

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Table 2.2 Details of Zambian tissue samples described in this study (2015 – 2020).

CVRI Ref No.	Date Collected	Species	Vet Camp/Village	District, Province	NSP	Serotype	WRLFMD Ref. No.	BVI Ref. No.	RT-qPCR	Serotype	Topotype	Lineage
MM01	02.4.2015	Bovine	Mupata Village	Mpulungu, Northern	Positive	ND	ZAM/1/2015		FMDV-GD	A	AFRICA	G-I
HS 01	28.2.2015	Bovine	Chimula Village	Mbala, Northern	Positive	ND	ZAM/2/2015	ZAM03/15	FMDV-GD	SAT 2	IV	-
Sh 01	23.10.2015	Bovine	Central	Shangoomb, Western	Positive	ND	ZAM/3/2015	ZAM09/15	FMDV-GD	SAT 3	II (WZ)	-
LK1	18.5.2017	Bovine	Mbanga & Mulongo	Lukulu, Western	Positive	ND	ZAM/1/2017	ZAM08/17	FMDV-GD	SAT 3	II (WZ)	-
K1	04.1.2018	Bovine	Kaluwe & Siluwe	Kalabo, Western	Positive	ND						
Chisa 16-380	24.3.2018	Bovine	Chisamba	Chisamba, Central	Negative	ND	ZAM/1/2018	-	FMDV-GD	O	EA-2	-
Chisa 16-169	24.3.2018	Bovine	Chisamba	Chisamba, Central	Negative	ND	ZAM/2/2018	-	FMDV-GD	O	EA-2	-
BBR-1	24.3.2018	Bovine	Chisamba	Chisamba, Central	Negative	ND	ZAM/3/2018	-	FMDV-GD	O	EA-2	-
TS 01	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	ZAM/4/2018	-	FMDV-GD	A	AFRICA	G-I
ES 01	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND						
ES 02	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	ZAM/5/2018	-	FMDV-GD	A	AFRICA	G-I
GS 01	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	ZAM/6/2018	-	FMDV-GD	A	AFRICA	G-I
ES03	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	-	ZAM37/18	FMDV-GD	A	AFRICA	G-I
VS 02	27.3.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	-	ZAM10/18	FMDV-GD	O	EA-2	-
VS 05	27.3.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	-	ZAM12/18	FMDV-GD	O	EA-2	-
ZRC 1	18.1.2019	Bovine	Chisamba Central	Chisamba, Central	Positive	ND	ZAM/1/2019	-	FMDV-GD	O	EA-2	-
CVRI67/19	17.7.2019Bovine			Chibombo, Central	Positive	ND						
CVRI04/19	11.2.2019	Bovine	Ufwenuka	Monze, Southern	Positive	O	ZAM/2/2019	-	FMDV-GD	O	EA-2	-
CVRI01/19	11.2.2019	Bovine	Ufwenuka	Monze, Southern	Positive	O	ZAM/3/2019	-	FMDV-GD	O	EA-2	-
CVRI08/19	11.3.2019	Bovine	Magoye	Mazabuka, Southern	Positive	O	ZAM/4/2019	-	FMDV-GD	O	EA-2	-

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17562	16.3.2019	Bovine		Chibombo, Central		-	ZAM/5/2019	-	NGD	-	-	-
17496	17.3.2019	Bovine		Chibombo, Central		-	ZAM/6/2019	-	NGD	-	-	-
CVRI16/19	30.3.2019	Bovine	Kasako	Mazabuka, Southern	Positive	O	ZAM/7/2019	-	FMDV-GD	O	EA-2	-
CVRI15/19	30.3.2019	Bovine	Kasako	Mazabuka, Southern	Positive	O	ZAM/8/2019	-	FMDV-GD	O	EA-2	-
CVRI22/19	02.4.2019	Bovine	Mwase	Lundazi, Eastern	Positive	SAT-2	ZAM/9/2019	-	FMDV-GD	SAT 2	I	-
CVRI23/19	02.4.2019	Bovine	Mwase	Lundazi, Eastern	Positive	SAT-2	ZAM/10/2019	-	FMDV-GD	SAT 2	I	-
CVRI20/19	03.4.2019	Bovine	Zozwe & Mlawe	Vumbwi, Eastern	Positive	SAT-2	ZAM/11/2019	-	FMDV-GD	SAT 2	I	-
CVRI21/19	03.4.2019	Bovine	Mbande	Vumbwi, Eastern	Positive	SAT-2	ZAM/12/2019	-	FMDV-GD	SAT 2	I	-
CVRI25/19	03.5.2019	Bovine	Feni	Chipata, Eastern	Positive	SAT-2	-	-				
CVRI37/19	16.6.2019	Bovine	Lwashimba	K/ Mposhi, Central	Positive	O	-	ZAM06/19	FMDV-GD	O	EA-2	-
CVRI55/19	28.6.2019	Bovine	Musenga	Chingola, Copperbelt	Positive	O	-	ZAM07/19	FMDV-GD	O	EA-2	-
CVRI29/19	20.5.2019	Bovine	Chitongo	Namwala, Southern	Positive	O	-	-		O		
CVRI30/19	20.5.2019	Bovine	Chitongo	Namwala, Southern	Positive	O	-	-		O		
CVRI31/19	05.6.2019	Porcine	Hamangaba	Monze, Southern	Positive	O	-	-		O		
CVRI32/19	05.6.2019	Porcine	Hamangaba	Monze, Southern	Positive	O	-	-		O		
CVRI61/19	12.6.2019	Bovine	Sunrise (Kasupe)	Chilanga, Lusaka	Positive	O	-	-		O		
CVRI36/20	14.2.2020	Porcine	Makeni	Chilanga, Lusaka	Positive	O	-	-		O		
CVRI01/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	ZAM03/20	A.ves-GD	O	EA-2	-
CVRI02/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	-		O		
CVRI05/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	-		O		
CVRI06/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	-		O		
CVRI13/20	17.4.2020	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI14/20	17.4.2020	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI17/20	17.4.2020	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI15/20	17.4.2021	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI29/20	13.8.2020	Bovine	Makeni	Chilanga, Lusaka	Positive	O	-	-		O		

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CVRI25/20	10.3.2020	Bovine	Nyawa	Kazungula, Southern	Positive	O	-	-	O

FMDV – Foot-and-mouth disease virus, BVI-Botswana Vaccine Institute, CVRI-Central Veterinary Research Institute, GD-Genome Detected, ND-Not done, NSP-non-structural

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Affected cattle showed typical clinical signs of FMD, which included drooling, nasal discharge, the grinding of teeth, and mouth lesions on the tongue, dental pad and gums. Most animals recovered within two to three weeks. Vesicles were observed as whitish areas that ruptured when pressure was applied, and erosions were observed on the feet. Vesicles were also apparent on the mammary glands in some dairy animals, and one instance of teat sloughing was observed. Pathognomonic clinical signs of FMD were observed in pigs and piglets at Hamangaba and Makeni veterinary camps in Monze and Chilanga districts, respectively. Animals were seen with white lesions and blisters on the coronary bands of the hoof and snout. There was a loss of appetite, and the piglets showed mild lameness and reluctance to move. Severe lesions of the heel pad area were observed. In Monze, the horn of the hoof in one piglet separated and shed, as has been observed elsewhere (Alexandersen et al., 2003b).

Of the blood samples collected in Zambia, 110 (92%) were bovine samples, and 10 (8%) were swine samples. A total of 92 (77%) were positive for antibodies against FMDV NSP, with a percentage inhibition range of 60.8 to 95.9% (Table 2.2). Despite testing positive with the RT-qPCR assay, samples collected from Chisamba 2018 and Itezhi Tezhi were negative when tested with the NSP-ELISA at initial sampling. However, subsequent samples collected after 14 days were positive. This was probably due to the lack of NSP antibodies during the early stages of the disease at the time of initial collection. All pig samples tested positive with the NSP-ELISA and were typed as serotype O with the Ag-ELISA. Samples collected from the Kazungula district Nyawa camp also tested positive with the RT-qPCR assay and were typed as serotype O with the Ag-ELISA. Twenty-six samples contained detectable FMDV genomes and could be serotyped, while no virus genome was detected in the remainder of the samples. FMDV-positive samples were further characterised by VP1 sequencing.

From the 144 animals in Katima Rural Constituency of Namibia, 100 (69.4%) showed clinical signs of FMD, including lameness, salivation and frothing of the mouth, blisters in the mouth, and hooves. In Nfooma, 70 out of 87 (80.5%) animals also exhibited typical clinical signs indicative of FMD. Testing at the SSARRL for these samples yielded FMDV-positive results for five out of seven samples using VI, all characterised as serotype O using the antigen ELISA.

2.4.2. Phylogenetic analyses

The most appropriate nucleotide substitution models for each serotype were found to be the Tamura-Nei (TN-93) model, gamma-distributed with invariant sites (G+I) for type O; the Hasegawa-Kishino-Yano (HKY) model, G+I, for types A and SAT2; and the General Time Reversible (GTR) model, G+I, for serotype SAT-3.

The study revealed that FMDV SAT-3 was responsible for the outbreak reported in Beshe Vet Camp in the Shangombo district of Western Province in 2015 (isolate ZAM/3/2015 and two additional sequences for isolates from BVI). Further outbreaks linked to these cases were later reported in all districts of Western Province except for Mwandi district (Veterinary-Services, 2017). Sequence data supported this close relationship, where nucleotide (nt) identities between ZAM/3/2015 and ZAM/1/2017 were 98.0%. Beyond the genetic identity between

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Zambia FMDV sequences, ZAM/3/2015 was most closely related to SAT3/BOT/P3/98 NXA-9 (88.6% nt identity) and SAT3/BOT/P3/98 NXA-6 (88.3% nt identity) (Figure 2.3)

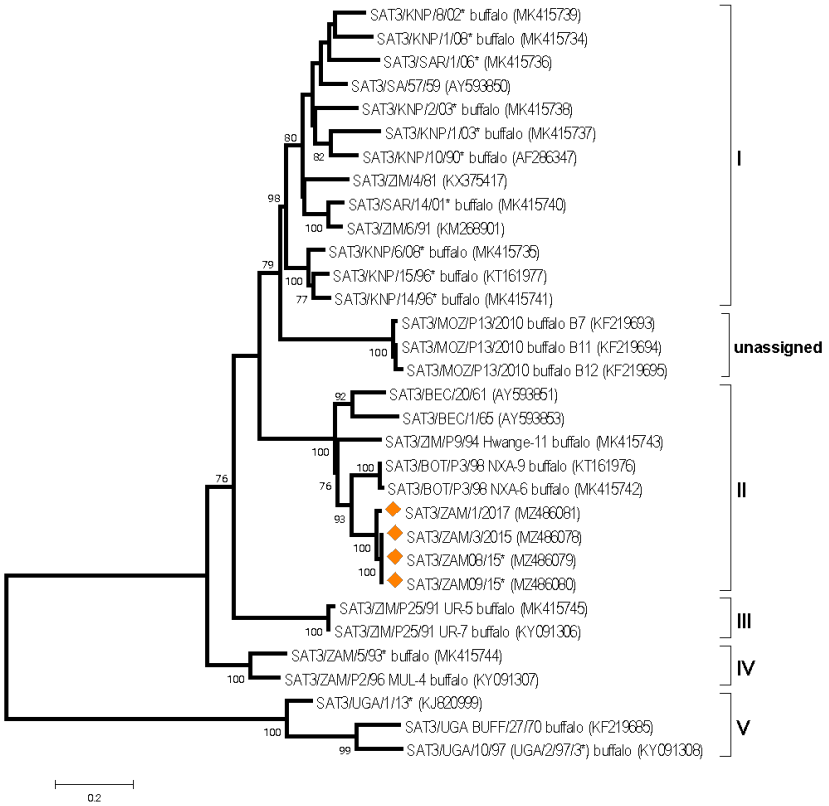


Figure 2.3 Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2015 foot-and-mouth disease virus SAT-3 serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses. * Reference number not assigned by the WRLFMD.

In the Northern Province, samples from Chimula Village in Mbala (ZAM/2/2015) were serotyped as SAT-2 and clustered within topotype IV, related to viruses circulating in East Africa (Pool 4) (Figure 2.4). In contrast, the VP1 sequences of four additional SAT-2 viruses (ZAM/9-12/2019) from Lundazi and Vubwi Districts of Eastern Zambia were characterised as belonging to topotype I, with closest relationships with SAT2/MAL02/19 and SAT2/MAL01/17 (96.6–98.3% nt identity) collected in Malawi (Figure 2.4).

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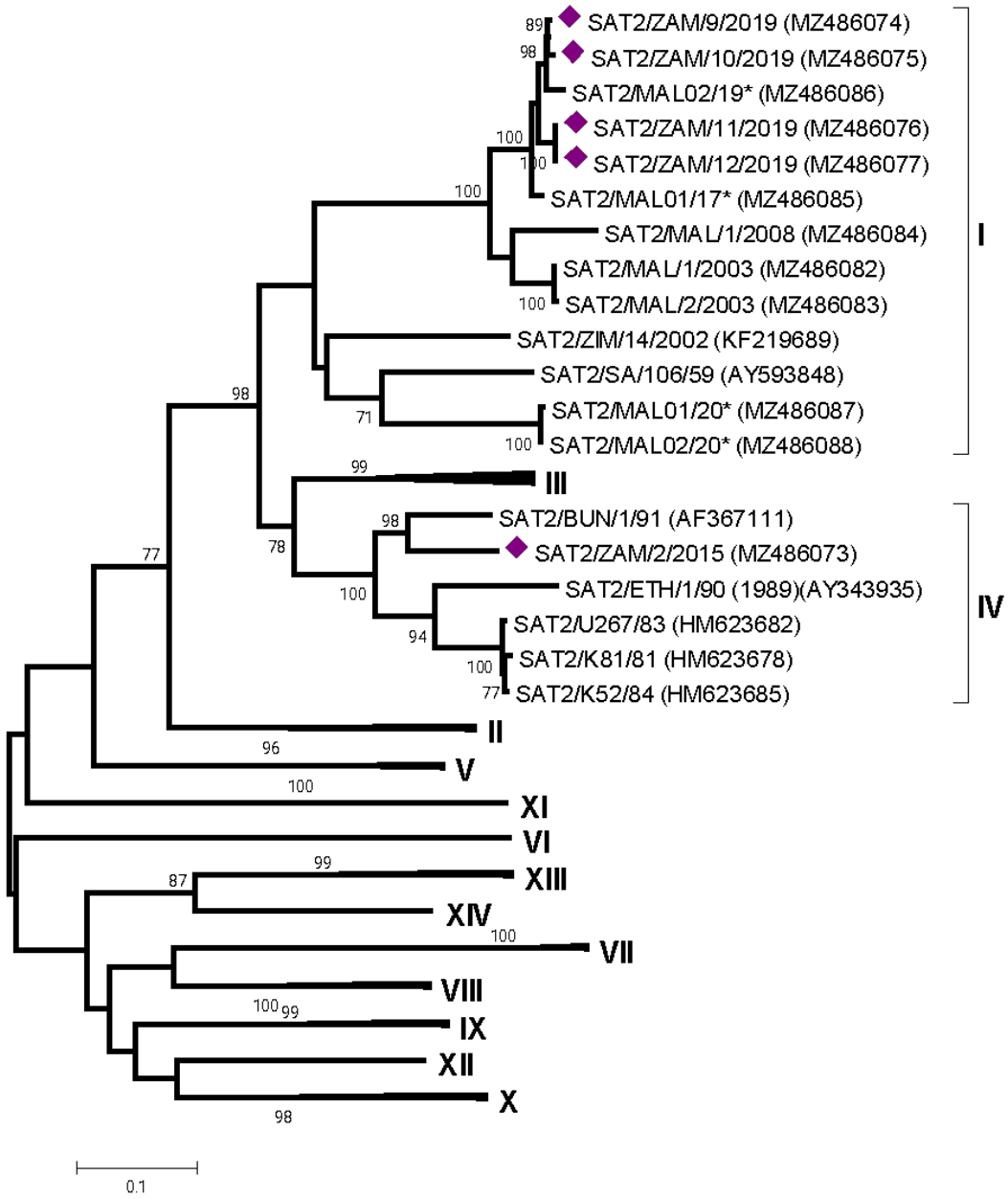


Figure 2.4 Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2015 and 2019 foot-and-mouth disease virus SAT-2 serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses. * Reference number not assigned by the WRLFMD.

This study detected five serotype A (genotype AFRICA/G-I) isolates from outbreaks that occurred in 2015 and 2018; ZAM/1/2015 from Mpulungu, which was genetically distinct to four identical sequences (ZAM/4-6/2018 and A/ZAM37/18 from the Mbala, Northern Province) (Figure 2.5). Viruses from both clades were closely related to sequences from FMDV isolates collected in Tanzania (95 – 96% nt identity).

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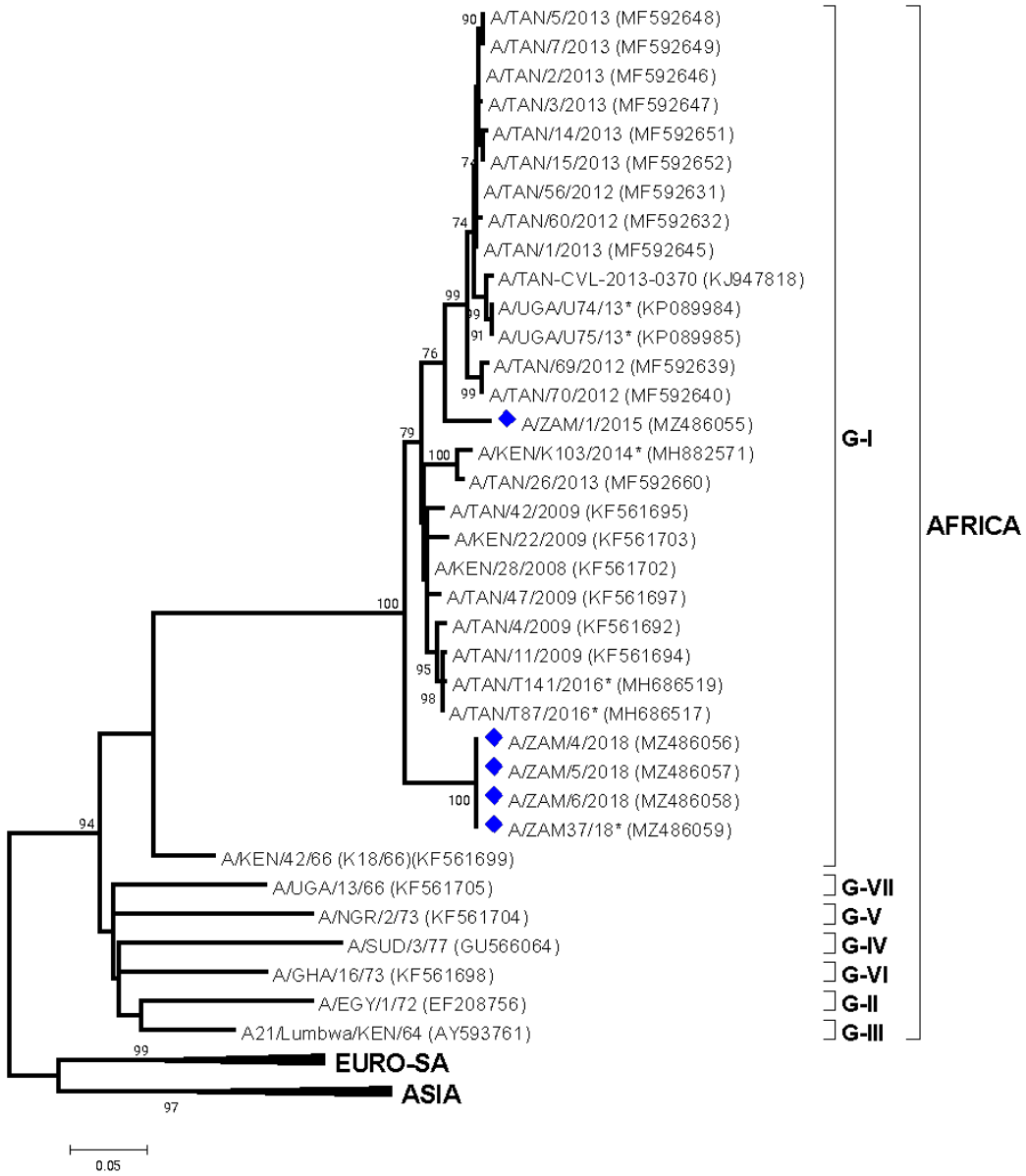


Figure 2.5 Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2015 and 2018 foot-and-mouth disease virus serotype A serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses. * Reference number not assigned by the WRLFMD.

All the serotype O viruses isolated in this study (Table 2.2) clustered within the East Africa 2 (EA-2) topotype, most closely related to viruses collected in Tanzania: O/TAN-CVL-2012-0318 (95.8–95.9% nt identity) and O/TAN-CVL-2012-0321 (95.9–96.1% nt identity) (Figure 2.6). These included FMD samples collected from six provinces in Zambia (Central, Copperbelt, Lusaka, Northern, Southern, and Western Provinces). Phylogenetic analysis of O/ZAM/1/2018 from Chisamba, Central Zambia, and O/ZAM/10/2018 from Kawimbe, Mbala, Northern Zambia, revealed that the viruses responsible for these two primary outbreaks were closely related to each other (99.8% nt identity).

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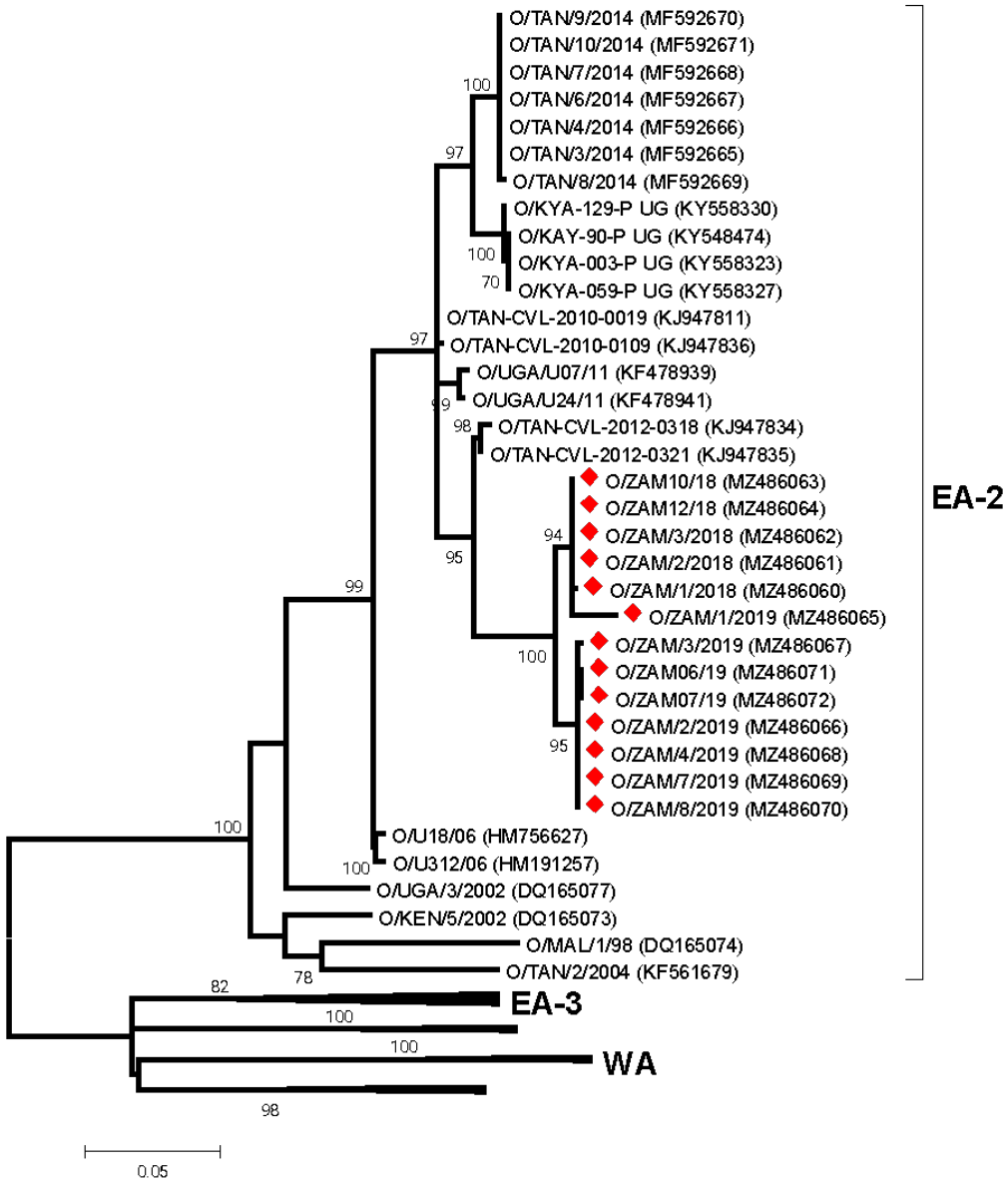


Figure 2.6 Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2018 foot-and-mouth disease virus O serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses.

The sequences encoding VP1 of four selected virus isolates from Namibia (O/NAM10/2021, O/NAM11/2021, O/NAM13/2021 and O/NAM14/2021) were identical to each other and were also categorised as belonging to the EA-2 topotype within serotype O. This analysis showed that the Namibian sequences shared closest nucleotide identity (99.5%) to two FMDV isolates collected from the Western Province of Zambia during 2021 (O/ZAM/9/2021 and O/ZAM/11/2021; (Figure 2.7).

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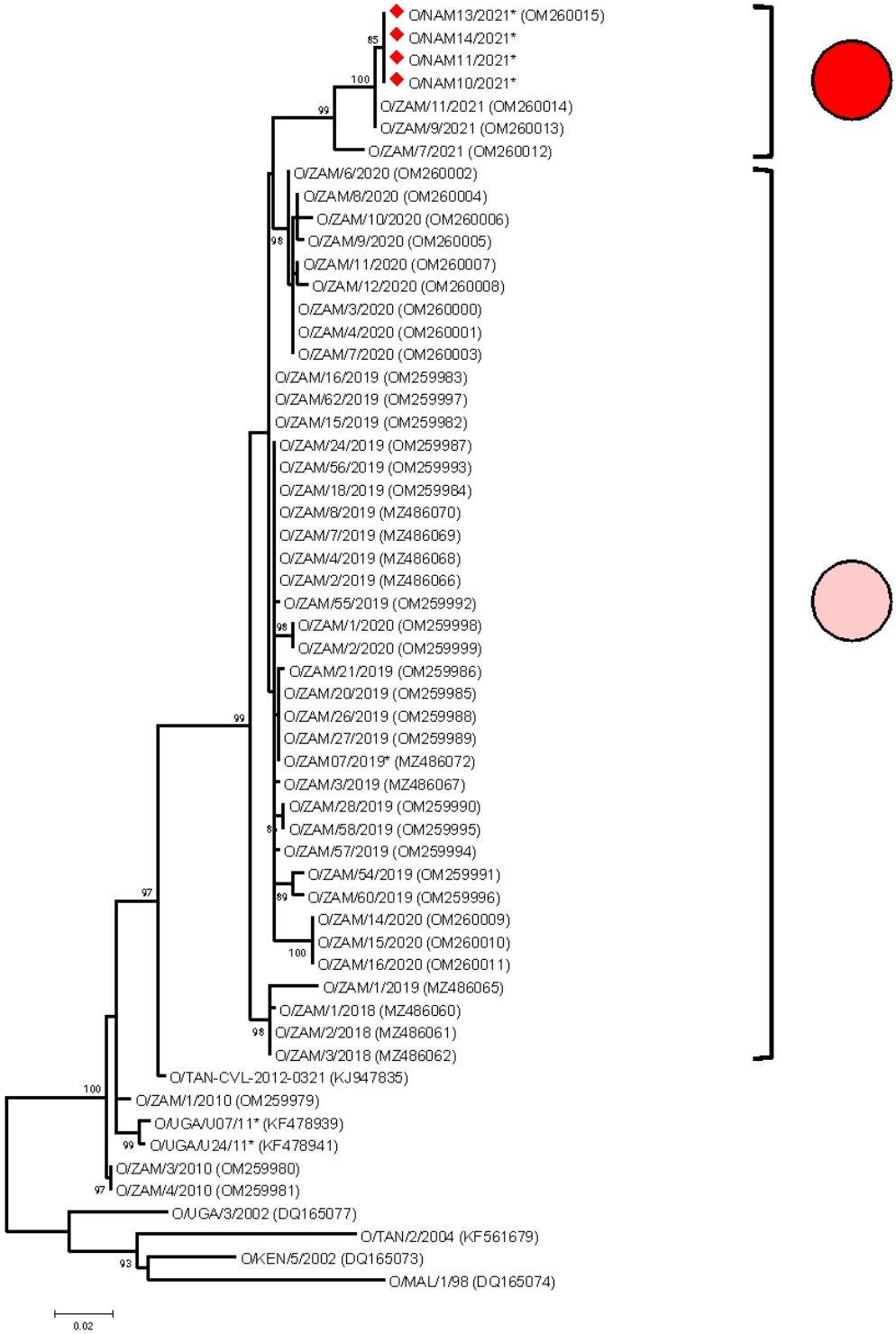


Figure 2.7 A midpoint-rooted maximum likelihood tree highlighting the monophyletic relationship of O/EA-2 FMD viruses collected from Zambia and Namibia during 2018-2021. Coloured dots represent data for samples sequenced during 2021 (●) and 2018-2020 (○). Bootstrap values of 70% and above are shown.

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2.5. Discussion

This is the first molecular epidemiology study of FMDV in Zambia to define the genetic relationships between Zambian FMDV and those collected from neighbouring countries. These results highlight that FMD continues to circulate in the three high-risk areas of FMD in Zambia; therefore, the FMD control strategies should continue to be planned and implemented with these areas in focus. Also revealed are notable changes in the epidemiology of FMD, including confirmed cases of FMDV serotype SAT-3 in cattle of Shangombo district of Western Province in 2015, the emergence of FMDV serotype O (O/EA-2) in Central, Southern, Copperbelt, Western and Lusaka Provinces and the introduction of the SAT-2 serotype into Eastern Zambia. This report also describes the molecular characterisation of a serotype O FMDV recovered from a field outbreak in the Zambezi region, Namibia, in July 2021.

In Zambia, the likelihood of FMD outbreaks in certain regions is largely influenced by livestock movement patterns and the proximity of meat markets. The factors influencing livestock movement and the drivers that contribute to the appearance of FMD in certain locations include transboundary movements across borders for trade, grazing, or seasonal migration. This is due to Zambia's shared borders with several countries, many of which have FMD-endemic regions within their border communities. Livestock, especially cattle, often move within these communities for grazing and traditional practices, posing a risk of FMD viral transmission since there is usually inadequate disease surveillance in such areas. Informal cross-border trade, particularly in areas where veterinary controls are weak, as observed at Kasesya border post between Zambia and Tanzania, also exacerbates the spread of FMD across regions and neighbouring countries. With regards to internal livestock movements, seasonal transhumance, trade, traditional and agricultural purposes are common drivers of the disease in the country. In pastoral and agropastoral systems, where cattle are essential for livelihoods, animals are often moved over large distances for grazing. These practices are particularly widespread in the southern province, home to the country's largest livestock population. These migrations, which are also prevalent in drought-prone areas, increase animal movement through FMD-endemic zones, spreading FMD to previously unaffected areas when animals mix in communal grazing lands. On the other front, major cities and towns in Zambia serve as hubs for meat markets and abattoirs, as there is high demand for beef and other livestock products in these areas due to urbanisation and population growth. This increases the volume of livestock movement from rural areas, which are the biggest source of livestock. Before being transported, weekly or monthly cattle markets are held in these rural districts, particularly in regions with strong cattle farming communities, such as southern, western, and eastern Zambia. These rural livestock markets often involve the mixing of livestock from different herds and areas, creating opportunities for the spread of FMD if animals harbouring the virus are present. Informal trade routes are mostly utilised to trek animals to these markets. With the limitation of veterinary controls and veterinary services, monitoring of these livestock movements is difficult, thus exacerbating the spread of FMD. Further, the likelihood of increasing FMD outbreak spread across regions is certain

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during long-distance transportation and at abattoirs as animals await slaughter. Inadequate biosecurity measures at such facilities make it easier for FMD to spread. Sometimes animals are held in feedlots, where they mix with those from other regions and markets before being sent to abattoirs. This can further intensify the spread of FMD. Overall, the occurrence of FMD in Zambia is mainly driven by livestock movement, cross-border activities, seasonal grazing, wildlife interactions, and biosecurity gaps. Border regions, livestock markets, and cattle farming areas are the most affected, making enhanced surveillance, stricter controls, and improved biosecurity in high-risk areas crucial for effective FMD control.

With regards to the reporting of FMDV SAT-3 in Zambia, this virus has only been isolated previously in Zambia from wild buffalo, although infection due to this serotype was once diagnosed serologically in cattle (Chilonda et al., 1999, Sinkala, 2015). This study describes the first FMDV serotype SAT-3 outbreak in domesticated cattle, believed to have been initiated by an illegal livestock movement from a neighbouring country. According to Dr. Banda, these outbreaks may have involved buffalo from the Sioma National Game Park as cattle were seen grazing with buffalo (Dr. F. Banda, personal communication, 5 March 2016). This national park in the Shangombo and Sioma districts is a protected area in the Kavango Zambezi (KAZA) TFCA, where transboundary animal movement is common and promoted, especially for wildlife. As reported, most SAT serotype outbreaks have occurred in and around the KAZA TFCA, where approximately 1.5 million people and their livestock reside (Maree et al., 2014, Thomson et al., 2013). Although two sequences (SAT3/BOT/P3/98 NXA-9 and SAT3/BOT/P3/98 NXA-6) collected from buffalo in north-western Botswana (Nxaraga Lagoon, Lower Boro floodplain) were clustered with the SAT-3 sequences from Zambia (Figure 2.3), direct testing of the buffalo-cattle transmission hypothesis requires further sampling of buffalo populations resident in Zambia.

The FMDV SAT-2 identified in this study clustered within two topotypes (I and IV) (Figure 3.4). These two topotypes represent FMDV that circulate in two different endemic pools (Pool 4 for topotype IV and Pool 6 for topotype I, respectively), and these differences demonstrated that the SAT-2 outbreaks in Eastern and Northern Provinces were not epidemiologically linked. Eastern Province recorded only one previous FMD case in Egichikeni Veterinary Camp of Lundazi District in 2001 (Sinkala, 2015, Lubinga, 2000). The FMD cases in 2019 were first reported from the Vubwi district, followed by Lundazi and lastly Chipata in May 2019. This Province shares borders with Malawi and Mozambique, and the close relationship between the outbreak viruses from Zambia and Malawi provides evidence for an epidemiological connection between FMD cases in these two countries. There is no natural barrier between the border areas of these countries, and it has been reported that livestock from the Malawian side are herded into Zambia, where they graze with Zambian livestock (Siankuku, 2019). This outbreak was resolved following two rounds of vaccinations with a bivalent vaccine (serotypes SAT-2 and O) targeting the cattle population at risk.

For serotype A, the sequences recovered in this study represent two genetic clades within the A/AFRICA/G-I genotype, representing viruses collected from 2015 and 2018, respectively.

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These data suggest that there were two separate serotypes A introductions into northern Zambia from a neighbouring country in East Africa.

The O/EA-2 topotype originates from East Africa (FMD endemic Pool 4; (Paton et al., 2009), where it has been detected in countries such as Burundi, the Democratic Republic of the Congo, Kenya, Tanzania and Uganda (Kasanga et al., 2014b, Namatovu et al., 2015, Kerfua et al., 2019, Wekesa et al., 2015). The O/EA-2 topotype has also previously appeared transiently in the northern parts of some Southern African countries, such as Malawi, where an isolate collected in 1998 shared a close genetic relationship with FMDV from Tanzania (Kasanga et al., 2014b). The first confirmed serotype O outbreak in Zambia was in the Mbala District of Northern Province in 1976 (Perry and Hedger, 1984, Mweene et al., 1996). Since then, serotype O outbreaks have only been reported in this province in three instances (1982, 2000 and 2010). Sequence data for VP1 encoding sequences for O/EA-2 viruses collected in 2010 close to the border with Tanzania are shown in Figure 3.7 (O/ZAM1/2010, O/ZAM/3/2010 and O/ZAM/4/2010. This study reports sequence data for further topotype O/EA-2 outbreaks in the Northern Province and the wider circulation of this topotype into other Zambian provinces and the Namibian Zambezi region. Although these sequences represent what was considered two primary outbreaks, their close sequence identity demonstrates that they are intimately connected, supporting suspicions that animals had been moved illegally from Northern Zambia (Mbala) into Central Zambia (Chisamba, a previously FMD-free area) in March 2018. The precise transmission routes are unknown, but the abattoir's closure in the Mbala district of Northern Zambia may have incentivised stock owners to move their animals southwards in search of market access. From the Chisamba district, the topotype O/EA-2 spread into other districts in Zambia, where sequences have been recovered from cases in the Monze district of Southern Zambia. This was after a disease quiescence of 11 months when a herd of cattle were moved illegally from Chisamba (Dr. F. Mulenga, personal communication, 10 April 2019). Foot-and-mouth disease cases due to topotype O/EA-2 have been detected subsequently in Copperbelt and Western provinces, and further serotype O samples characterised by Ag-ELISA have been collected from Central, Lusaka and North-Western Provinces. From Monze (Southern Province), sequence data confirmed the spread of topotype O/EA-2 to Mazabuka, from where there were reports that FMD spread to the Kafue Flats, an area where animals congregate during the transhumance grazing, a common practice in Southern and Western provinces of Zambia (Mumba et al., 2018, Muuka et al., 2012).

These emerging strains of FMDV in Zambia have had significant economic and social impacts in the country, whose rural livestock communities rely heavily on livestock for their livelihoods, threatening food security and income, pushing vulnerable households into poverty. The reduced livestock productivity has caused lower availability of meat and milk, impacting the nutritional status of communities that depend on these foods (Sikombe et al., 2015). A major economic setback occurred at a commercial dairy farm in Chisamba, where a significant reduction in milk production was recorded, resulting in substantial financial losses for the company. Restrictions on the movement of livestock and livestock products on the

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other end also led to substantial financial losses for farmers and the broader agricultural sector. This was evident in Southern Province, where cattle prices fell by approximately 30% due to oversupply and reduced demand during the outbreak (Mumba, 2019).

Phylogenetic analyses (Figure 2.7) highlight the transboundary connectivity between Zambia and Namibia, and understanding epidemiological connections that underpin the recent spread of the O/EA-2 topotype in Zambia and Namibia is a priority for new studies. By themselves, these sequence analyses cannot reconstruct the specific epidemiological routes by which O/EA-2 has been introduced into Namibia; however, the Katima Mulilo Bridge, which was built in 2004 to carry the Trans-Caprivi-Highway (Walvis Bay-Ndola-Lubumbashi Development Road) across the Zambezi River could provide an obvious pathway. Across the border in Zambia, the proximity to international border crossing points and major roads was identified in a previous study as a risk factor for FMD outbreaks (Hamoonga et al., 2014). They were also reports of farmers from Namibia preferring large sized herds of bulls from Zambia in exchange of Namibian Heifers. Further work is now required to quantify the risks associated with these transboundary animal movements and to develop controls that reduce the likelihood of further FMD incursions into the region. Further, there have been reports that O/EA-2 FMD infection in naïve animals is more severe with higher morbidity, a feature of these outbreaks that should be closely monitored if this topotype spreads to the other SADC states.

The detection of O/EA-2 in the Zambezi Region in the Caprivi Strip in north-east Namibia represents the first-time serotype O has been detected in the country. This incursion now poses a new threat to Namibia's FMD-endemic region and other countries, such as Angola, Botswana and Zimbabwe. Botswana also maintains an official WOAH FMD-free without vaccination zone in the south, but like Namibia, it has no WOAH status in the northern part of the country. Introducing a new serotype or an antigenically novel variant can pose challenges for regional FMD control initiatives, as was recently documented by the rapid spread of the A/ASIA/G-VII lineage in Asia (Bachanek-Bankowska et al., 2018). The introduction of serotype O, therefore, poses significant challenges to regional FMD control efforts in Southern Africa. One key issue is the lack of widespread vaccination against FMD serotype O, which is typically necessary for effective control. Additionally, limited cooperation among Southern African countries, particularly in sharing information, resources, and strategies, may need to be strengthened to better manage and contain the spread of serotype O. On the economic front, the detection of FMD serotype O can lead to immediate trade restrictions on livestock and livestock products, with countries importing these products imposing bans or stringent checks to prevent the spread of the disease. This may result in substantial economic losses for livestock producers and exporters in Namibia and neighbouring countries like South Africa (Sihlobo, 2023). In FMD-endemic regions of Southern Africa, onward transmission of O/EA-2 may go unnoticed due to the significant under-reporting of the disease. In these areas, serotype O-specific immunity is likely to be very low since FMD vaccines, where these are used in Southern African countries, do not typically contain a serotype O component.

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This study also demonstrates the movement of FMDV serotypes and topotypes among different territories and the importance of surveillance in rationally designing vaccination campaigns in other regions. Although presented too late to assist with curbing the spread of FMD in Zambia, the data presented accentuates challenges faced in FMD control as previously outlined (Maree et al., 2014, Sinkala et al., 2014b), with the time factor being critical. Not reporting or late reporting of the disease leads to poor diagnostic ability and makes the disease investigation devoid of the epidemiological information required for effective disease management. These findings should, therefore, motivate field teams and laboratory scientists to increase surveillance within the region to rapidly detect further outbreaks due to this topotype. Increased stakeholder engagement is necessary and should be encouraged during FMD outbreaks, as disease control has proved effective with this strategy in some outbreak areas of Zambia. Secondly, the lack of adequate diagnostic facilities in most endemic countries like Zambia raises the argument that virus analysis in endemic settings must be locally matched with commensurate investment in FMD diagnostic infrastructure, including sample collection and processing. In addition, rapid field diagnostic tests close to outbreak areas are necessary for phylogenetic analysis to have a tangible impact on curtailing circulating viruses during disease outbreaks. Now that this serotype has been identified, new resources will be required to ensure that laboratories in the region can correctly identify new clinical cases due to serotype O and detect serotype O-specific antibodies in sera collected as part of surveillance activities. It is therefore proposed that the planned risk-based FMD control strategy for Zambia and the region takes into account the sound epidemiological assessment of the incidence and distribution of FMD provided in this work. This includes identifying risk sources as primary or secondary endemic ecosystems to design FMD control programmes effectively. We acknowledge the limitation of this study in terms of sample size, which could affect the generalizability of the findings. Additionally, the diversity of samples might not fully represent all regions or animal populations in Zambia. In terms of temporal scope, this study covers a specific period (2015-2020), which might not capture long-term trends or changes in the epidemiology of FMD in Zambia. We are also aware that the accuracy and sensitivity of the diagnostic methods used (e.g., serotyping, NSP-ELISA) could also influence the results, as limitations in these methods might lead to underreporting or misclassification of FMD cases.

The introduction and circulation of novel FMDV in Zambia and Namibia may have a bearing on the epidemiology of FMD in the region. Zambia represents an interface between endemic Pool 4 (East Africa) and Pool 6 (Southern Africa), and the spread of O/EA-2 in Zambia and Namibia is of particular concern, as it could alter the epidemiological risks for the SADC region. Furthermore, the emergence of serotype O as a risk for SADC countries has impacted the selection and costs associated with FMD vaccination since trivalent vaccines (containing only SAT-1, SAT-2 and SAT-3 vaccine strains) are the most frequently used in the region. Foot-and-mouth disease vaccines selected for use in the region should now consider these risks, and vaccination campaigns may need to absorb the extra costs of adding a serotype O antigen component to the deployed vaccines.

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2.6. Conclusion

This study describes the molecular epidemiology of FMDV collected from Zambia between 2015 and 2020, highlighting the recent spread of serotypes O, A, SAT-2 and SAT-3 viruses in the country. The study also highlights a new FMD threat for countries in Southern Africa through the molecular characteristics of a serotype O FMDV recovered from Namibia in 2021. These results reinforce the importance of synchronising surveillance and disease control strategies where the emergence of new FMDV serotypes and lineages impact vaccine selection for the region to ensure that data is shared between countries to monitor this dynamic situation.

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Chapter 3. A Scoping Review of Foot-and-Mouth Disease Virus Southern African Territories Serotypes Diagnostic Tests

3.1. Introduction

Foot-and-mouth disease, caused by *Aphthovirus vesiculae,* is highly contagious. This is due to the ability of the virus to gain entry and initiate infection via a variety of sites, the small infective dose, the short incubation period, and the release of the virus before the onset of clinical signs (Abd El Wahed et al., 2013b). Clinical signs in sheep and goats are often mild and difficult to observe, and several other vesicular virus infections cannot be distinguished from FMDV infections clinically (Fernández et al., 2008, Mowat et al., 1972), making clinical diagnosis unreliable. It is, however, critical that diagnosis is carried out promptly before the virus spreads, and this underscores the need for rapid, sensitive and specific diagnosis of the disease.

There are seven serotypes of FMDV (O, A, C, Asia-1, SAT-1, SAT-2 and SAT-3) with seven endemic pools of infection worldwide, often requiring tailored diagnostics and vaccines (Freimanis et al., 2016, Paton et al., 2009). Although FMDV SAT serotypes are primarily restricted to Africa, SAT-1 and SAT-2 serotype incursions have been recorded outside Africa, mainly in the Middle East and due most likely through live animal importation from Africa (Tekleghiorghis et al., 2014). In Southern Africa, FMD outbreaks reported during the last 20 years have been restricted to those caused by the three SAT serotypes (Maree et al., 2014), with the SAT-2 serotype responsible for almost half of the FMD outbreaks in domestic animals (Thomson and Bastos, 2004).

To control and eradicate FMD in endemic regions, the accurate diagnosis of FMDV infection is of utmost importance as it complements the established prevention and control strategies (Sumption et al., 2012, Maree et al., 2014). Muzykina et al. (2024) stated that an effective strategy in the battle against infectious diseases lies in constantly monitoring their spread through point-of-care tests and laboratory methods. However, most diagnostic tests, including those utilised for FMD, have shortcomings, especially when applied in environments that occur in Zambia and the larger African countries. This calls for continuous review. The FMD diagnostic tests currently being used in Zambia have been observed to have limited specificity and sensitivity required to detect all cases, particularly in areas where multiple strains of the FMD virus circulate. Obtaining false negatives from such diagnostic tests can lead to undetected outbreaks, while false positives can cause unnecessary culling and economic loss. Further, FMDV SAT serotypes are known to evolve rapidly with a great propensity for mutations (Longjam et al., 2011), with the SAT-1 and SAT-2 serotypes displaying greater antigenic variation compared to the Euro-Asian serotypes (Maree et al., 2011, Esterhuysen et al., 1988, Reeve et al., 2010). This vast antigenic variation leads to the emergence of new topotypes and lineages, rendering available diagnostic methods less

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sensitive and specific, necessitating gaps in surveillance. The genetic diversity of FMD virus strains in Africa can also lead to cross-reactivity in tests, making it difficult to differentiate between different serotypes or closely related viruses. Available diagnostic tests have also been noted to have inadequate serotyping and strain identification characteristics as they are not optimised to identify all seven FMDV strains, leading to incomplete serotyping coverage. A limit in the availability of high-tech laboratories required for specialised tests like virus isolation or molecular testing has also been observed in Zambia and the region, leading to delays in outbreak confirmation. It is, therefore, imperative to continuously monitor FMD diagnostic tests and ensure that available tests can accurately detect circulating FMDV strains.

Several reviews of FMD diagnostic assays have been conducted (Longjam et al., 2011, Wong et al., 2020, Poonsuk et al., 2018), but no review has specifically targeted FMDV SAT serotypes diagnostics. A scoping review of these diagnostic assays was undertaken, as it is critical to determine the most sensitive and specific assays, provide comprehensive analyses to add to the current knowledge base, and provide guidance about areas lacking knowledge.

The aim was to perform a scoping review of FMDV SAT serotype diagnostic tests to identify the most sensitive and specific assays. This study did not consider other FMDV serotypes (i.e., O, A, C, and Asia-1).

The goals included: i) conduct a search using four electronic bibliographic databases, ii) collate relevant information related to diagnostic assays for FMDV, specifically for SAT serotypes, iii) compare the diagnostic sensitivities and specificities of the assays, and iv) highlight research gaps based on the current scientific literature.

3.2. Materials and methods

The study consisted of a scoping review. The population, interest, and context (PICO) framework was modified to address the research topic and questions (Schardt et al., 2007). The focus was on diagnostic assays for FMDV SAT serotypes. The context was to establish the available literature on these assays' diagnostic sensitivity and specificity.

The knowledge synthesis methodologies (Young et al., 2014) were applied to deliver an overview of published research on diagnostic tests for FMDV SAT serotypes diagnostic tests. Peer-reviewed research was quantitatively characterised with respect to the author, date of publication, reference type, animal species involved, sample type, diagnostic type, and sensitivity and specificity of the diagnostic tests. The information collected through this synthesis process produced an evidence map of the current scientific literature and identified knowledge gaps and limitations of the studies.

We used the methodological and reporting quality guidelines proposed by an extension of the Preferred Reporting Items for Systematic Reviews (PRISMA) designed for Scoping Reviews (PRISMA-ScR) to conduct and report all aspects of this literature synthesis (Tricco et al., 2018). The PRISMA-ScR provides standardised definitions and guidelines for scoping reviews and is

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the most suitable method for synthesising evidence (Tricco et al., 2018). Scoping reviews are commonly used for 'reconnaissance'. They are, therefore, instrumental when a body of literature has not yet been comprehensively reviewed or exhibits a complex or heterogeneous nature that is not amenable to a more precise systematic review of the evidence (Peters et al., 2015). This study was implemented systematically as follows:

- Develop the research topic with relevant questions.
- Conduct a literature search by researching and identifying relevant publications.
- Screen and sort search results.
- Data extraction and analyses.

The questions addressed by this review were defined as follows:

- Which diagnostic assays are used to diagnose FMDV SAT serotypes?
- Which assay is the most appropriate for each intended purpose?
- Which assay is the most sensitive and specific?
- Which assay is furthermost on the assay validation pathway?

3.2.1. Eligibility criteria

Two members of the research team set the eligibility criteria. One member initiated and completed the screening process under the constant supervision of the other. A three-stage screening process was implemented to evaluate the relevance of publications obtained during the search process.

3.2.2. Information sources

A search strategy was designed and run in four electronic bibliographic databases (Google Scholar, PubMed, Scopus, and Web of Science). No language, date, subject, or type filters were used during the searches, which allowed for a comprehensive search and reduced limitations on publications obtained. All databases were searched using the topic search function, which searched each publication's titles, abstracts, and keywords.

3.2.3. Literature search

Medical Subject Headings (MeSH) and truncated free-text terms were combined using Boolean and proximity operators to balance sensitivity and specificity while searching for the studies. Another strategy used was snowballing (citation screening) to identify additional references from included studies and published literature reviews that fell closely within the scope of this evidence synthesis.

A base search string was developed using terms deemed relevant and descriptive of the publications required for inclusion in this scoping review. "South* African territor* was used as a search term to limit results to SAT serotypes. The search terms for foot-and-mouth disease were ("foot-and-mouth" OR FMD OR FMDV). The following base search string was

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adapted to meet the requirements of the individual database search engines; ("foot-and-mouth" OR FMD OR FMDV) AND (SAT OR "South* African territor*") AND (Evaluation OR development OR validation OR optimisation OR optimisation OR diagnostic OR sensitivity OR specificity OR accuracy OR Diagnosis OR Detection OR Screening) AND (assay OR test OR ELISA OR PCR OR "lateral flow"). Only articles in English were included, whilst no limit was included on publication dates. Articles were first screened using titles and abstracts, followed by a review of the full text of articles identified as possibly relevant. Assessment of bibliographies for included articles was done for further eligible publications.

3.2.4. Citation management

During the search process, the obtained publications were imported into EndNote X8 (Clarivate Analytics, Philadelphia, PA, USA). Duplicate publications were removed via EndNote's automated duplicate screening process, and several more duplicates were removed manually. All publications underwent manual title and abstract screening for relevance and then full-text screening. An explicit inclusion was only decided after the reviewer had read the full texts. The reasons for exclusion after this stage were documented for traceability and transparency.

3.2.5. Inclusion and exclusion criteria

Publications were eligible to be included in the study if the full-text article was written in English and if they described generally or specifically FMDV SAT serotype diagnostic tests. This included diagnostic tests, which involved only SAT tests or were combined with other serotypes. A publication was excluded if it involved diagnostic tests which did not cover any SAT serotype.

3.2.6. Selection of sources of evidence

3.2.6.1. Title and abstract relevance screening

The initial screening level involved a review of only the title and the abstract of publications. Publications without keywords referring to FMD OR foot-and-mouth, FMDV or SAT in their title, abstract and keywords were excluded. Upon first review, several inapplicable publications were obtained due to search terms having similar meanings, different search algorithms, different truncation rules, and other database settings over which the user had limited control. Through this process, many immaterial publications were excluded.

3.2.6.2. Full-text screening

Electronic search outputs identified full texts of relevant publications by title and abstract screening before importation into EndNote X8 (Clarivate Analytics, Philadelphia, PA, USA). Most of these text articles were obtained by searching the titles in Google Scholar linked to the University of Pretoria (UP) library service. Scopus, Web of Science, and PubMed references were obtained directly via the UP-library's database search function. Following the

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removal of duplicate studies, the titles and abstracts were used to screen the reports. Full-text articles were retrieved to assess the final inclusion based on the eligibility criteria, and if the criteria were not met, the publications were excluded at this step. A decision to include an article was only made after reading the full texts by an individual reviewer, with reasons for exclusion after this stage documented for traceability and transparency. Data extraction only commenced following the construction of a final set of full-text publications.

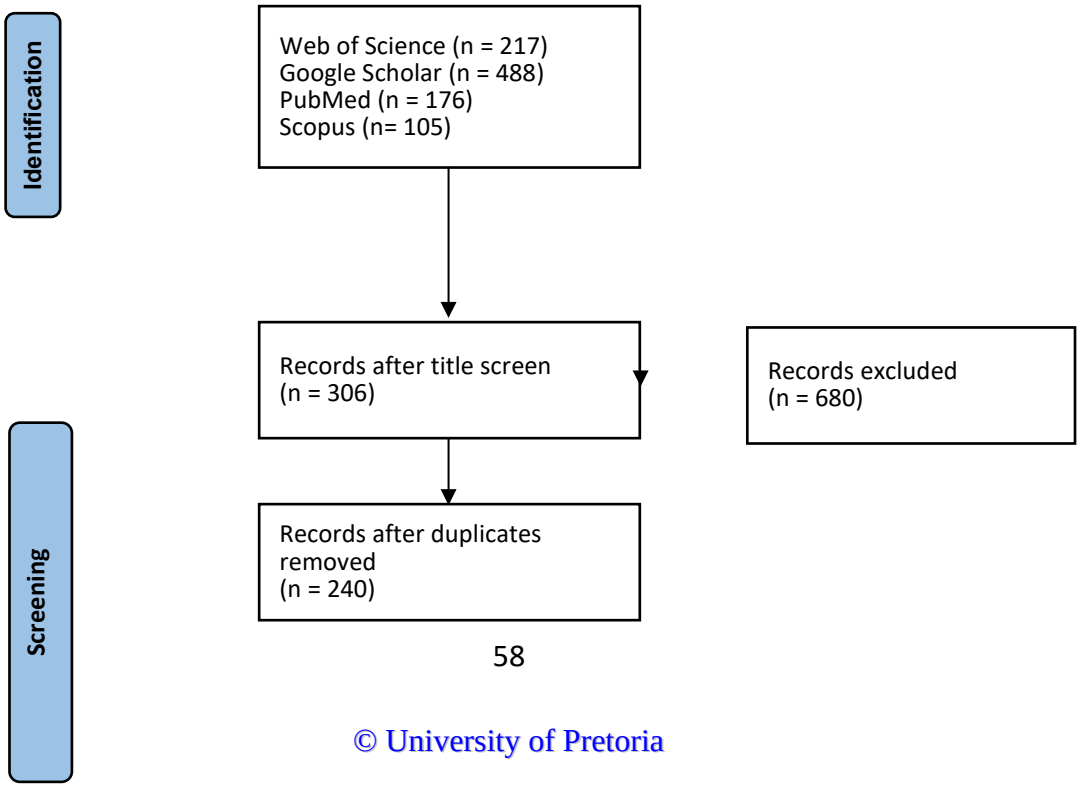
3.2.7. Data extraction

The reviewer recorded data from each publication using a pre-designed standardised form in a Microsoft Excel spreadsheet. Specific data included author/s, year of publication, the assay's purpose, the sample's origin, sample type, type of assay, target serotype, sensitivity and specificity. The data extraction form is available in Appendix 8.6.

3.3. Results

3.3.1. Article selection

A total of 986 articles were captured during the initial search, and 746 were excluded after eliminating duplicates and screening titles. After screening abstracts against the eligibility criteria, 141 articles were excluded. Most articles excluded reported data on tests this review and review papers did not consider. Ninety-nine articles entered the full-text screening process. During the full-text screening, three more publications were identified and obtained via snowballing and added to the articles assessed in detail. Ten full-text articles were unavailable in English (one in Hindi, two in Egyptian and seven in Chinese), four were not peer-reviewed, whilst three had no full-text articles. Nine more publications were excluded for reasons which included no SAT serotypes reported in the study. Therefore, from the 102 full-text manuscripts assessed in detail, 76 articles were retained for the knowledge synthesis (Figure 3.1).



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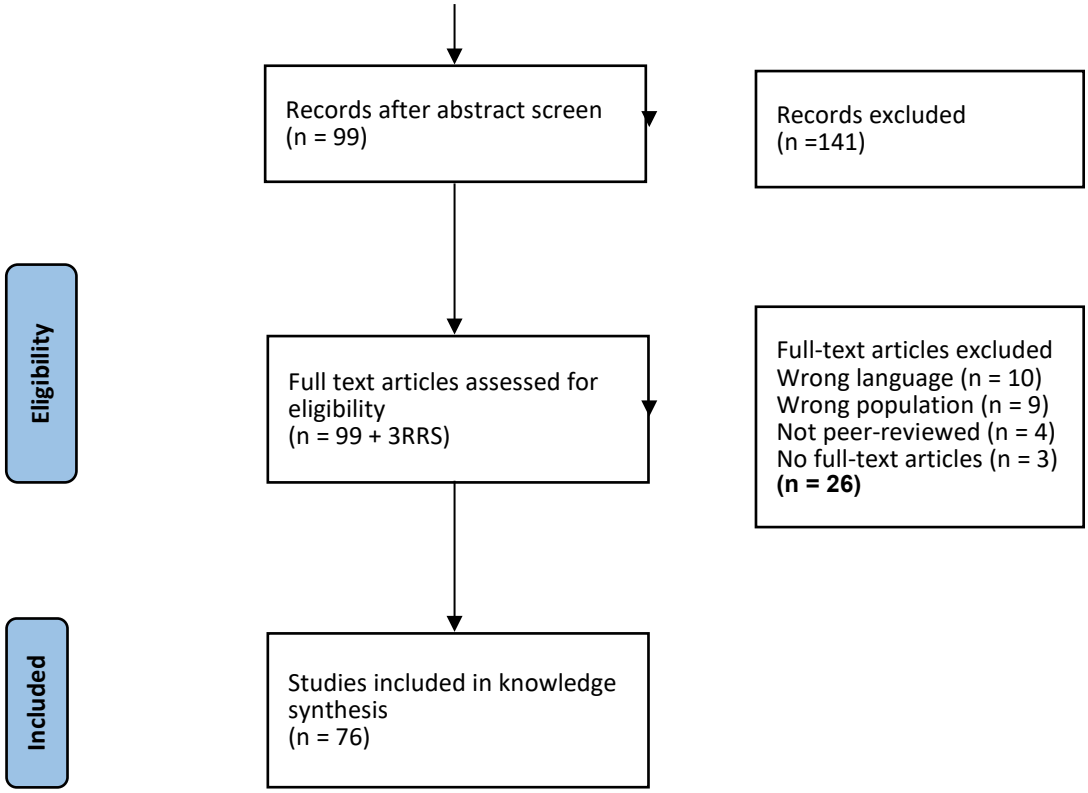


Figure 3.1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of the literature search, screening, assessing eligibility, and article selection, RRS—Reverse Reference Search.

3.3.2. Synthesis of results

3.3.2.1. General characteristics of publications

Figure 3.2 indicates the range of publication dates for the articles in the knowledge synthesis. Thirty per cent (23/76) of publications were published from January 2015 to December 2022, and 50% (38/76) of studies were published between 2009 and the end of 2022. The most publications, five in a year, were in 2004, 2007, and 2018.

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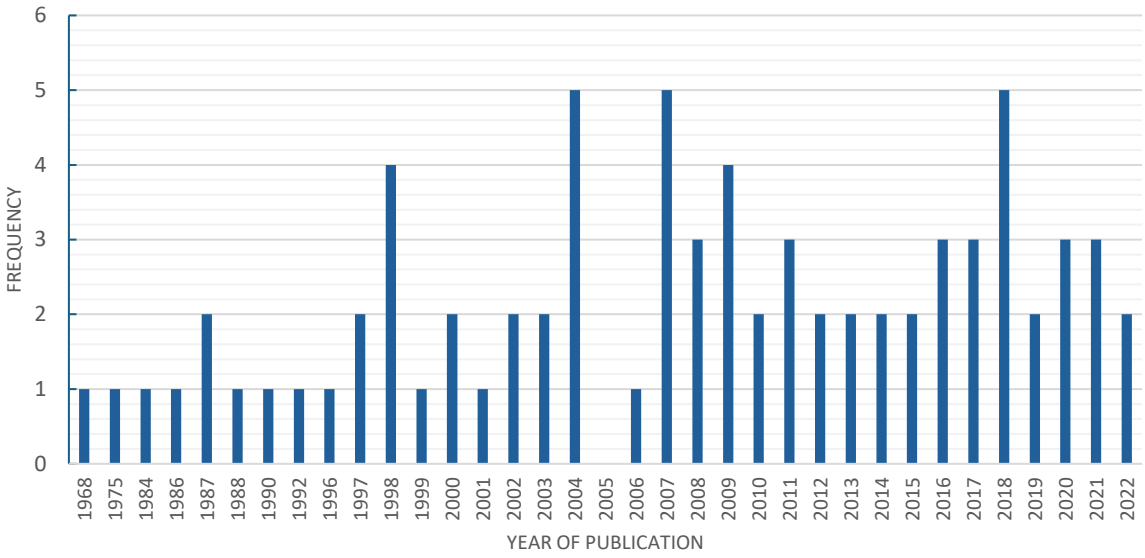


Figure 3.2 The number of publications reporting on foot-and-mouth disease virus SAT serotype diagnostic assays from 1968 to 2022.

3.3.2.2. Characteristics of assays

Thirty-nine (51%) of the assay methods used in the knowledge synthesis were conventional serological assays, which detect antibodies to structural proteins and are serotype-specific. Nineteen (25%) were pan-serotype diagnostic assays, and eighteen (23%) were both serotype-specific and serotyping assays (Figure 3.3).

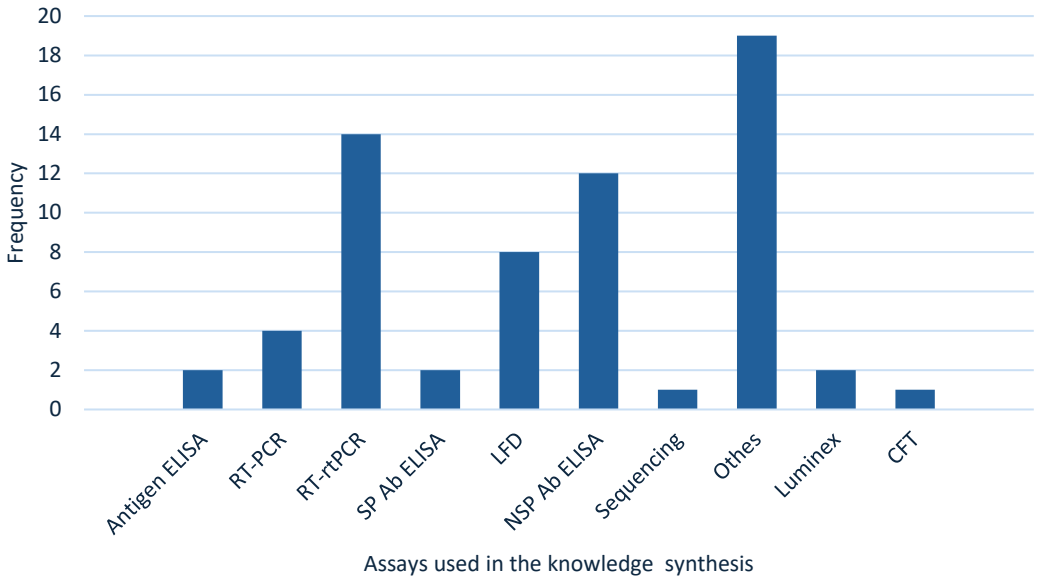


Figure 3.3 The frequency of the assay type reported in the knowledge synthesis, CFT - complement fixation test, LFD - lateral flow device, NSP Ab ELISA - non-structural protein antibody ELISA, RT-PCR - reverse-transcriptase polymerase chain reaction, RT-qPCR - real-time reverse transcriptase-polymerase chain reaction, SP Ab ELISA - structural protein antibody ELISA.

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Detecting FMDV and the corresponding antibodies is crucial for controlling outbreaks. Fifty-three (69.7 %) of the assays in this review detected the virus whilst 23 (30.3%) detected antibodies. Of the fifty three virus detecting assays, fourteen assays were real-time reverse transcriptase-polymerase chain reaction, thirteen 13 reverse-transcriptase polymerase chain reaction, four antigen ELISA, eight lateral flow device and one sequencing. The knowledge synthesis also reviewed 13 other virus detecting assays from assays categorised as others (Figure 3.3). With regards to assays detecting antibodies, twelve assays were NSP Ab ELISA, two SP Ab ELISA, one CFT and two Luminex. Six antibody detection assays were also observed from those assays categorised as others.

The most common reason for developing assays in the knowledge synthesis was to confirm clinical cases (Table 3.1). Sixty (79%) of the studies were developed only in the laboratory only, and sixteen (21%) also involved testing of field samples.

Table 3.1 The purpose of the assay development in the articles reviewed in the scoping review.

	Purpose	Total publications
1	Confirmation of clinical cases	55
2	Individual animal freedom from infection prior to movement	9
3	Prevalence of infection – surveillance	13
4	Population freedom from infection	13
5	Immune status in individual animals or populations post-vaccination	13
6	Contribute to eradication policies	6

3.3.3. Specificity and sensitivity of the foot-and-mouth disease virus SAT assays

From the 76 articles retained for the knowledge synthesis, only 12 focused exclusively on FMDV SAT diagnosis (Table 3.2). Most articles reported their assays to have a specificity of 100%, while their sensitivity varied.

Table 3.2 Specificity and sensitivity of assays focusing on foot-and-mouth disease virus SAT diagnosis only.

	Article reference	Type of test	Sensitivity	Specificity	Comments
1	Booth et al., 1975	Serum neutralisation tests and HI	63% and 57%, respectively	100%	Targeted SAT-2 only Haemagglutination limited applicability with SAT-2 strains
2	Bronsvoort et al., 2008	NSP ELISA-Cedi test	87.70%	87.30%	Looked at buffaloes Suggested that Cedi TEST is a useful tool for screening buffalo for infection with the various serotypes of FMDV
3	Callens et al., 1997	Multi-primer RT-PCR mixes used in conjunction with a consensus RT-PCR			Looked at all serotypes but targeted SATs Results were compatible with the standard techniques for FMDV detection and serotyping (P32 and P33 Primers)
4	Chitray et al., 2018	NSP ELISA-SAT specific NSP ELISA	76%	96% at a 95% confidence	Looked at all serotypes but targeted SATs Only SAT only ELISA ever developed

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5	Elmenofy et al., 2020	NSP ELISA- IB capsid protein-based			Targeted SAT-2 only 1B capsid protein-based indirect ELISA was able to detect anti-FMDV SAT-2 antibodies
6	Ferris et al., 2010	Lateral flow device	88%	99%	Targeted SAT-2 only Achieved validating an LFD for the detection of FMDV SAT-2 to address the shortcoming of the 1F10 LFD.
7	Hussein et al., 2021	SAT-2-VIP sensor	LOD of 0.1 ng/mL.	No cross-selectivity was noticed	Targeted SAT-2 only 70-fold more sensitivity than the reference RT-PCR
8	Liu et al., 2018	FMDV SAT-D8 one-step RT-PCR	4×10^2 copies/µL for DNA.	No cross-reaction with all these virus samples	A FMDV SAT-D8 one-step RT-PCR is a promising method for primary screening for FMDV SAT serotypes
9	Sammin et al., 2007	NSP ELISA, solid-phase competition ELISA		70%-90%	Looked at virus, SP and NSP antibodies RT-qPCR can be at least as sensitive as VI in detecting persistent infections with SAT serotypes Commercially available NSP test kits can be used for the serodiagnosis of SAT-1 and SAT-2 infections in cattle
10	Sorensen et al., 1992	SP Ab ELISA	3A 96% 3ABC 92%	100%	Presented comparative results with the liquid-phase blocking ELISA (LP-ELISA). Resource-saving and proved to be a reliable and precise method for the assessment of antibody levels.
11	Yang et al., 2021	LFD strip	SAT 1 - 51.90% SAT 3 -62%	100%	First-ever LFD for SAT-1 and SAT-3 Has excellent potential for the rapid identification of the FMDV SAT-1 and SAT-3 viruses
12	Yang et al., 2019	LFD strip	64%	100%	Targeted SAT-2 only Have great potential for the quick identification of FMDV SAT-2 viruses on-site

3.3.4. Assay validation

Validation, a process that determines the fitness of an assay that has been properly developed, optimised and standardised for an intended purpose, is critical, especially for infectious diseases, to ensure that assays are applied and interpreted in a scientifically robust and defensible manner (Colling and Gardner, 2021). From the 76 articles retained for knowledge synthesis, only ten assays were designated to have been "validated for the original intended purpose(s)" and have therefore achieved international recognition conferred by the World Organisation for Animal Health (WOAH) as outlined in the assay development and validation pathway (WOAH, 2023). Unfortunately, none of the validated test methods were specifically developed for FMDV SAT serotype diagnosis only.

3.3.5. Limitations

This research study has several limitations. Firstly, it was predisposed to database bias as only four multidisciplinary databases were interrogated during the search process. If other databases were interrogated, additional publications may have been obtained (Pham et al., 2014). Additionally, relying solely on publications from databases may introduce temporal bias, potentially leading to the exclusion of older publications not available in those

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databases, thus affecting the comprehensiveness and objectivity of the findings. Since this scoping review relied on scientific publications, it was susceptible to publication bias influenced by factors such as the author's career status, institution, language, country, study outcome, research topic and timeline (Song et al., 2013, Song et al., 2010). The study might also be limited by the range of diagnostic tests reviewed, potentially excluding newer or less commonly used methods. Further, the variability in the methodologies and outcomes of the included studies can make it challenging to draw consistent conclusions.

3.4. Discussion

In this review, we used methodological and reporting quality guidelines proposed by PRISMA-ScR to find relevant information related to the characteristics of diagnostic assays for FMDV SAT diagnosis. Data from these articles were retrieved from public databases, and as far as the authors are aware, this is the first time such a topic has been examined. Recommendations are outlined below based on the general characteristics of reported publications to provide a foundation for more focused analyses in future research projects.

Publications were identified during the search process by interrogating the database using the search string and then via a reverse reference search process. This is because articles had specific words in their titles, abstracts or keyword lists or the correct combination of words between the three categories for the database algorithm to include the publications in the search results. A total of 76 articles met our inclusion criteria, and our findings suggested a tendency toward the increase in number of reported studies retrieved in the last two decades. More than 50% of studies were published between 2009 and the end of 2022, indicating the scientific community is showing increased interest in this area. In particular, among the highly variable FMDV SAT serotypes (Carrillo et al., 2007), SAT-2 diagnosis received the most attention, attesting its significance as a serotype with the highest incidence among the SAT serotypes (Bastos et al., 2003a, Vosloo et al., 2006).

The indispensable role of the WOAH/FAO World Reference Laboratory for FMD in both diagnosis and research of this disease was attested to by 50% of works in the articles obtained through this synthesis process being conducted by this institute. Even though this review also reported 55% of the samples utilised as originating from the Pirbright Institute, it was clear that these samples were just submitted to this laboratory and not obtained from within the United Kingdom, where the laboratories are located.

More than half of the assays (51%) from the knowledge synthesis were conventional serological assays, which detect structural proteins. The structural proteins assist in differentiating between vaccinated and infected animals, although they cannot distinguish between the two groups with complete confidence. Furthermore, it has been established that to detect FMDV infection, conventional techniques such as CFT, serum neutralisation tests and ELISA are either not precisely specific or lack an optimum degree of sensitivity (Longjam et al., 2011). This observation cannot be overlooked when screening a herd for FMDV. Advances towards faster detection and serotyping to compensate for some weaknesses of

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traditional methods such as virus isolation (VI), Ag-detection ELISA, pan-reactive real-time reverse transcription–polymerase chain reaction have been published (Alexandersen et al., 2003b, Remond et al., 2002, Reid et al., 2002, Callahan et al., 2002). This review noted that 69.7 % of the assays focused on agent detection and were purposed for confirmation of clinical cases (Table 3.1). A similar scenario was observed for the twelve assays targeting SAT serotypes (Table 3.2) a quest which could be likened to supporting advances of faster detection and serotyping of FMD. Diagnostic assays specifically designed for on-site diagnosis (e.g., the lateral flow immunochromatographic test strips) are also gaining prominence in FMD diagnosis (Wong et al., 2020). Identification of serotypes, combined with phylogenetic analyses, assist in determining the outbreak's origin and monitor onward dissemination (Foglia et al., 2021). Fast and reliable characterisation of circulating viruses critically enables epidemiological analyses and vaccine choice (Sumption et al., 2012). However, a closer look at the above eight assays revealed that none had 100% specificity, though some reported 100% sensitivity (Table 4.2). This could partly be attributed to the high inherent genetic variability of FMDV, especially for SAT serotypes but also due stochasticity of laboratory tests. However, Foglia et al. (2021) have previously reported that the diagnostic and serotyping performances approached 100% when some assays were combined.

For the standardisation of developed assays with a focus on agent detection, VI (Alexandersen et al., 2003b, WOAH, 2022a) and Ag-detection ELISA (Ag-ELISA) (Remond et al., 2002, WOAH, 2022a) remain the most used methods. As previously reported by Foglia et al. (2021), (Reid et al., 2000) the Ag-ELISA remains the most user-friendly assay with the advantage of typing capability. However, it still cannot be used to type FMDV SAT 3, and to detect viruses from poor-quality field samples. The RT-qPCRs emerged as the most used assay in this review. Although its use is reported as growing exponentially and rapidly replacing conventional PCR and other established diagnostic methods such as Ag-ELISA and VI (Longjam et al., 2011), its use remains confined to pan-serotype detection of FMDV. This lack of serotyping capabilities was also previously reported by Foglia et al. (2021), hence its non-appearance among the twelve assays focusing on SAT diagnosis. However, RT-qPCR has been previously reported to be at least as sensitive as virus isolation in detecting persistent infections with SAT serotypes, particularly when it has been optimised for the most likely virus strain/serotype in circulation (Sammin et al., 2007). Significant progress has recently been observed in developing a PCR technique specifically for diagnosing FMD SAT serotype. The FMDV SAT-D8 one-step RT-PCR has been reported as a promising method for primary screening for FMDV SAT serotypes (Liu et al., 2018).

Eight articles in this review focused on LFD, with two (Yang et al., 2019, Yang et al., 2021) targeting SAT serotypes diagnosis specifically with a 100% specificity (Table 4.2). This first-ever reported SAT-1 and SAT-3 test strip, combined with the previously developed SAT-2 strip (Yang et al., 2019), can be used for quick diagnosis in endemic countries in Africa (Yang et al., 2021). According to our review, the LFD test reported by Ferris et al. (2010) was the first ever LFD developed for SAT diagnosis. Generally, LFDs have the advantage of not requiring the aid of an external device, which significantly lowers the cost per test. There are commercially

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available LFDs for detecting FMDV antigens (Ferris et al., 2009). Still, WOAH has not yet received a validation dossier for these tests to enable their inclusion on the WOAH test register (WOAH, 2022a).

An evaluation of the performance of serological tests for detecting SAT serotype infection, particularly ELISAs for antibodies to NSPs of FMDV and SPCE for serotypes SAT-1 and SAT-2, was conducted (Sammin et al., 2007). This study noted that available NSP test kits, can be used to sero diagnose SAT-1- and SAT-2 serotypes in cattle by detecting between 70 and 90 per cent of cattle in which persistent FMD virus infection was demonstrated (Sammin et al., 2007). Previously, however, Sorensen et al. (1992) reported on a blocking ELISA for detecting antibodies to FMDV SAT-1, SAT-2 and SAT-3. This study reviewed comparative results with the LP-ELISA (Hamblin et al., 1987) and distinguished between vaccinated and infected cattle but not between carriers and non-carriers.

The first SAT-targeted 3ABC NSP ELISA ever developed was also reported in this review (Table 3.2). This assay, which was validated by doing a comparison with PrioCheck, was an advantageous test for routinely detecting anti-3ABC antibodies in sub-Saharan Africa for the FMDV SAT serotypes (Chitray et al., 2018). Previously, Bronsvoort et al. (2008) suggested that Cedi TEST is a useful tool for screening buffalo for infection with the various serotypes of FMDV. The PrioCheck remains the most sensitive and specific NSP ELISA according to this review as attested by being the most used for validation purposes.

These findings will enhance FMD control strategies as solutions outlined for improved detection and serotyping of FMDV will allow for quicker identification of outbreaks, enabling faster implementation of control measures such as quarantine and movement restrictions (Chestley et al., 2022). The need for creating diagnostic assays with higher sensitivity and specificity which can simultaneously confirm virus's presence and type in a single reaction, would ensure streamlining of the diagnostic process and further assist in enhancing disease surveillance. Even though not yet validated, LFDSs stand out effective in improving FMD control strategies due to their cost effectiveness and turnaround time in the disease diagnosis process, thus an area of focus for development of better diagnostic tools to enhance FMD control strategies. These tests are also highly recommended for use in resource limited countries like Zambia. On the other front, integrating diagnostic data with geographic information systems can help in mapping the spread of FMD and identifying hotspots, aiding in more effective surveillance and control (USDA, 2013). All in all, by leveraging these findings, endemic regions can significantly enhance their FMD control strategies, develop more effective diagnostic tools, and improve their surveillance efforts, ultimately reducing the impact of FMD on livestock and economies.

3.5. Conclusion

Previous reviews on FMD diagnostic tests (Longjam et al., 2011) reported that no diagnostic test had been developed to confirm the virus's presence and type in a single reaction. According to data generated from this review, the status quo remains the same. The

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combination of multiple assays remains the only solution for improved detection and serotyping of FMDV as reported by Foglia et al. (2021). However, the commercially available and widely used PrioCheck®-NSP kit proved to be an excellent NSP kit for detecting SAT NSP antibodies and is therefore recommended for SAT serotype diagnosis.

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Chapter 4. The Immunogenicity of a Foot-and-Mouth Disease Virus Serotype O Vaccine in Cattle Herds in Zambia

This chapter has been published as (Banda, Frank et al. "The Immunogenicity of a Foot-and-Mouth Disease Virus Serotype O Vaccine in Commercial and Subsistence Cattle Herds in Zambia." *Vaccines* vol. 11,12 1818. 5 Dec. 2023, doi:10.3390/vaccines11121818) (Appendix 3)

4.1. Abstract

The recent introduction of foot-and-mouth disease virus serotype O (O/EA-2 topotype) in Southern Africa has changed the epidemiology of the disease and vaccine requirements of the region. Commercial and subsistence cattle herds in Zambia were vaccinated with an FMDV serotype O Manisa vaccine according to a double- or single-dose vaccination schedule. Heterologous antibody responses induced by this vaccine against a representative O/EA-2 virus from Zambia were determined. Virus neutralisation tests showed double-dosed cattle had a mean reciprocal log virus neutralisation titre of 2.02 (standard error [SE] = 0.16, n = 9) for commercial herds and 1.65 (SE = 0.17, n = 5) for subsistence herds 56 days after the first vaccination (dpv). Significantly lower mean titres were observed for single-dosed commercial herds (0.90, SE = 0.08, n = 9) and subsistence herds (1.15, SE = 0.18, n = 3) 56 dpv. Significantly lower mean titres were also observed for single-dosed animals from a similar project on a trivalent vaccine (SAT-1, SAT-2, Serotype O) conducted in 2018. A comparison of results and those generated by SPCE tests showed a statistically significant positive correlation by Cohen's kappa coefficient. Therefore, SPCE might be used to assess the immunogenicity of vaccines in place of VNT. Furthermore, for this vaccine and field strain, a vaccination regime employing a two-dose primary course and revaccination after four to six months is likely to be appropriate.

4.2. Introduction

Foot-and-mouth disease is one of the most economically important infectious livestock diseases in the SADC due to its impact on livestock productivity and resulting international trade restrictions on live animals and livestock products. The causative agent, *Aphthovirus vesiculae*), is contagious and antigenically diverse, with six currently circulating serotypes that do not cross-protect. This role of virus diversity in the epidemiological dynamics of FMD in Africa has been described (Woldemariyam et al., 2023).

There has been an escalation in FMD outbreaks in the past decade in southern Africa (Chitray et al., 2018), with Zambia and Namibia reporting novel serotype O incursions due to the O/EA-2 topotype (Banda et al., 2022). Before 2018, FMD was confined to three FMD high-risk areas of Zambia (Overby and Zyambo, 1983, Chilonda et al., 1999, Mweene et al., 1996), but, in

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2018, an FMDV serotype O outbreak was reported in the Chisamba District of Central Zambia (Banda et al., 2022). This disease spread to all the provinces in Zambia except the Luapula Province. A subsequent spillover of this outbreak to the Zambezi Region of Namibia during June–July 2021 was also recorded (Banda et al., 2022). Further spread of this lineage into Malawi and northern Mozambique was reported in 2022 (WRLFMD, FAO, 2023).

Vaccination campaigns together with stringent control measures have been used to eradicate FMD from Europe and most of South America (Bergmann et al., 2000, Leforban and Gerbier, 2002); in endemic regions, vaccination, especially with good quality FMD vaccines, is an important means of FMD control and can help prevent losses in stock production and reduce the overall incidence of the disease (Hunter, 1998). Post-vaccination monitoring (PVM) guidelines have been published to advise countries on principles and suggested procedures for monitoring various aspects of FMD vaccination (FAO-OIE, 2016). Estimating the immunity of the population targeted for protection by vaccination is the core of PVM, as it is a key indicator of how well vaccination has been carried out and whether or not protection against infection is likely. Further, it's important to quantify the risk of NSP positivity in vaccinates (which is useful when interpreting serosurveys), i.e., quantify the % NSP positive after vaccination or after repeated vaccinations.

In Southern Africa, FMD vaccination has targeted livestock mainly for prophylaxis and to prevent the spread of the disease using trivalent vaccines containing SAT-1, SAT-2, and SAT-3 antigens (Fana et al., 2021). The introduction of serotype O into the region means that new vaccines are needed to control outbreaks due to this serotype. As no empirical data is available to show the appropriateness of serotype O vaccines in Zambia, this study used the principles of the PVM guidelines to assess the immunogenicity of an imported serotype O vaccine in Zambia.

The specific objectives of this study were to evaluate FMDV-specific antibody responses after a single dose and a second booster dose in vaccinated cattle and to compare results obtained by the VNT and a commercial SPCE kit.

4.3. Materials and methods

4.3.1. Study design

In 2020, field studies were conducted in commercial cattle (Study A) and subsistence cattle (Study B) to assess the immunogenicity of an imported serotype O vaccine using either a one- or two-dose primary vaccination protocol. Study A animals were Friesian cattle from a commercial dairy farm in the Momboshi area of the Chisamba District, Zambia, whilst Study B animals were mixed breeds in herds from different subsistence farmers in multiple sublocations in the Rufunsa District, Zambia. For the 2018 project, a similar setup was implemented to that of the 2020 project, and both commercial and subsistence cattle were utilised. However, in this project, an imported Tri-Valent FMD vaccine (SAT-1, SAT-2 and O) vaccine was assessed. Eight farms were selected for this project: four from the Chisamba

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district and four from the Chibombo district. There was no previous history of any FMD vaccination and no history of FMD outbreaks in the last two years in selected areas of both the 2020 and 2018 projects. Selected animals were six to twelve months old at vaccination, including males and females. All animals were individually identified by ear tags to ensure accurate follow-up. Consent was obtained from the farmers to use their animals in the studies, and owners were advised not to move their animals out of the farms during the study period. The 2020 project was approved under research ethics committee number REC 061-19 from the University of Pretoria, South Africa.

The protocol followed the FAO and WOAH PVM guidelines Section 3.3 (FAO-OIE, 2016) (Figure 4.1). For the 2020 project, in Study A, 69 animals were recruited, including ten unvaccinated controls, to act as sentinels for FMDV exposure, whilst in Study B, 55 animals were recruited, including five unvaccinated controls. Sera from 32 cattle were randomly selected and tested for antibodies to FMDV for each of the five time points (0–168 days after the first vaccination (dpv). Of the vaccinated animals in both studies, ten were given a second dose 28 dpv. A second dose is recommended by the vaccine manufacturer and previous research on immunologically naïve animals (i.e., with no previous FMDV exposure or vaccination) (Doel, 1999). Blood samples were collected in plain Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) at first vaccination (0 dpv), 28 dpv (time of second vaccination), 56 dpv, 112 dpv, and 168 dpv. Not all samples were tested for FMDV antibodies because the budget for this project was limited. From Chisamba, samples from nine single-dose and nine double-dose cattle and samples from four unvaccinated controls were tested. From Rufunsa, samples from three single-dose, five double-dose, and two unvaccinated control cattle were tested. In the 2018 project, 49 animals were selected for the studies. Still, the sample size was increased to 80 animals to compensate for possible withdrawals, previous exposure to the virus or problems with sample analysis. A total of 20 animals were selected from each farm. Sixteen animals from the selected 20 animals from each farm were vaccinated, whilst four were left as controls and were not vaccinated. Of the vaccinated animals, ten were given a second dose 28 days post-vaccination (dpv), whilst the blood collection procedures and intervals followed as in the 2020 study. As in the 2020 project, not all samples were tested for FMDV antibodies because of budget limitations. In this project, five single-dose, five double-dose cattle and five unvaccinated controls from each farm were tested.

├—► **2020 PROJECT**
| ├—► ***Study A (Chisamba)***
| | • 69 animals total
| | • 10 unvaccinated controls (sentinels)
| | • 10 vaccinated animals received second dose at 28 dpv
| ***Samples tested*** :
| | - 9 single-dose
| | - 9 double-dose
| | - 4 unvaccinated

| └—► ***Study B (Rufunsa)***

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- 55 animals total
- 5 unvaccinated controls
- 10 vaccinated animals received second dose at 28 dpv

Samples tested:

- 3 single-dose
- 5 double-dose
- 2 unvaccinated

Sampling Schedule (both studies)

- Day 0 dpv – first vaccination
- Day 28 dpv – second vaccination (for subset)
- Day 56 dpv
- Day 112 dpv
- Day 168 dpv

2018 PROJECT

- 80 animals total (increased from 49)
- 20 animals/farm × 4 farms
- 16 vaccinated, 4 controls per farm
- 10 vaccinated animals per farm received second dose at 28 dpv

Sampling schedule: same as 2020

Samples tested (per farm):

- 5 single-dose
- 5 double-dose
- 5 unvaccinated

2020 PROJECT

Study A (Chisamba)

- 69 animals total
- 10 unvaccinated controls (sentinels)
- 10 vaccinated animals received second dose at 28 dpv

Samples tested :

- 9 single-dose
- 9 double-dose
- 4 unvaccinated

Study B (Rufunsa)

- 55 animals total
- 5 unvaccinated controls
- 10 vaccinated animals received second dose at 28 dpv

Samples tested:

- 3 single-dose
- 5 double-dose
- 2 unvaccinated

Sampling Schedule (both studies)

- Day 0 dpv – first vaccination
- Day 28 dpv – second vaccination (for subset)
- Day 56 dpv

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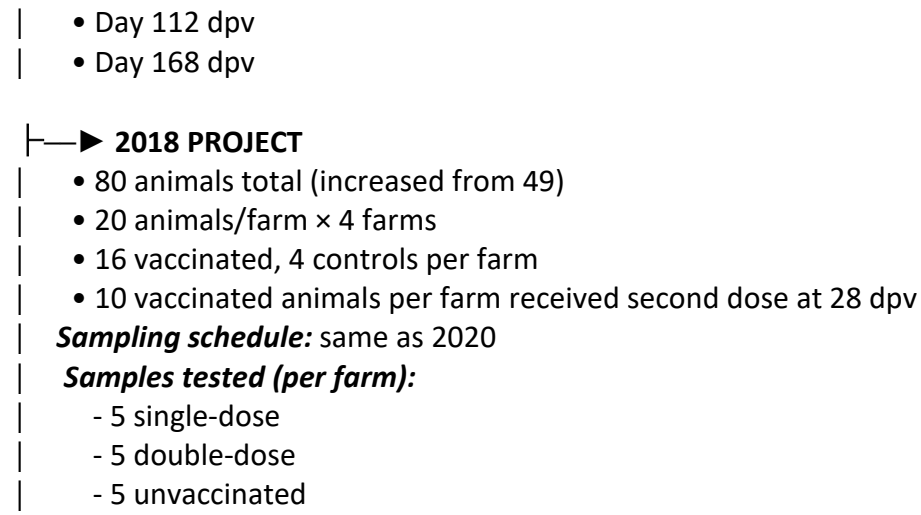


Figure 4.1 Workflow for study design.

4.3.2. Vaccine

In the 2020 project, the animals were vaccinated with a monovalent FMDV serotype O Manisa vaccine of at least three PD50, containing purified inactivated FMDV antigen and aluminium hydroxide with saponin as an adjuvant obtained from the BVI. This aqueous adjuvanted vaccine is indicated for use in cattle, buffalo, sheep, and goats. Each dose (1 mL) was given according to the recommendations from the vaccine manufacturer. In a primary vaccination course, two injections three to four weeks apart are recommended, whilst a booster dose is advisable every four to six months, depending on the risk and following local practice.

In the 2018 project, the animals were vaccinated with a three PD50 Tri-Valent FMD vaccine (SAT-1, SAT-2 and O). This aqueous adjuvanted vaccine is also indicated for use in cattle, buffalo, sheep, and goats as the monovalent vaccine utilised in the 2020 project. Each dose (3 mL) was given according to the recommendations from the vaccine manufacturer. In a primary vaccination course, two injections three to four weeks apart are also recommended for this vaccine, with a booster dose advisable every four to six months depending on the risk and following local practice similar to recommendations for the monovalent vaccine used in the 2020 project.

4.3.3. Serological testing

All samples from both projects (2020 and 2018) were shipped on ice to the CVRI, Zambia, and, on arrival, sera were separated and then stored at 20°C until testing. All samples were tested with an FMDV NSP ELISA kit (IDEXX, Westbrook, MN, USA) according to the manufacturer's instructions at the CVRI to evaluate if natural exposure to FMDV had occurred during the study period. Antibodies to viral NSP are considered reliable evidence of previous or current viral replication in the host, irrespective of vaccination status (WOAH, 2022a, Barnett et al., 2015, Colling et al., 2014). Test results for the NSP ELISA are expressed as a percentage positivity relative to the strong positive control [(optical density of test or control wells/optical

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density of strong positive control) × 100] (Bruderer et al., 2004). Values < 20% are considered negative, values ≥ 20% and < 30% are considered suspect, and values ≥ 30% are considered positive.

For the 2020 project, samples were also tested using a solid phase competitive ELISA (SPCE) or antibodies specific to FMDV serotype O structural proteins (IZSLER Biotechnology Laboratory, Brescia, Italy). This test detects antibodies elicited by vaccination and natural infection. A percentage inhibition for this test is calculated for each well (100 − [optical density of each test or control value/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and a specific murine monoclonal antibody for the FMDV antigen on the ELISA plate. Using the semi-quantitative method applied in this test, sera are considered positive when inhibition is ≥ 70% and negative when inhibition is < 70% at 1:10 dilution. A second dilution (1:30) of strongly positive sera samples (≥ 80% inhibition at 1:10 dilution) indicates the level of antibodies with strongly positive sera showing ≥ 80% inhibition at both 1:10 and 1:30 dilutions.

For both the 2020 and 2018 projects, samples were shipped on dry ice to the FAO WRLFMD, Pirbright, UK, for testing by VNT, as described previously (Chen et al., 2011, WOAH, 2022a). A representative Zambian field isolate from the O/EA-2 topotype (O/ZAM/7/2021) was used in the VNT to derive field strain-specific (heterologous) titres for the 2020 project. Earlier comparison of the neutralisation of O Manisa and O/ZAM/7/2021 viruses by O Manisa post-vaccination sera had shown an antigenic similarity score (r1 matching value) of 0.47, where 1.0 is a perfect match and values greater than or equal to 0.3 are considered indicative of an acceptable match for field vaccination (WRLFMD, 2021). For the 2018 project, a representative Zambian field isolate from the O/EA-2 topotype (O/ZAM/3/2018) was used in the VNT to derive field strain-specific (heterologous) titres for serotype O, whilst (SAT 2/ZAM/2/2015) was used to derive field strain-specific (heterologous) titres for SAT-2. The post-vaccination VNT titres to heterologous viruses associated with post-vaccination cross-protection have been evaluated. Using VNT, an indicator of likely heterologous cross-protection is considered to be a $\log_{10}$ reciprocal titre of 1.5 (1:32) after a single-dose vaccination with serum collected 21 days later (FAO-WRLFMD, 2022, Hammond et al., 2021).

4.3.4. Data analysis

Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and SPSS Statistics v29 (IBM, Armonk, NY, USA) were used to analyse the data.

4.4. Results

During the 2020 project implementation period, one animal died in Study A, and five cattle died or went missing in Study B. The animal that died in Study A belonged to the unvaccinated control group. From Study B, deaths were observed at different time intervals during the study, with one animal dying from each group, i.e., unvaccinated controls, single-dosed, and double-dosed. Their deaths were attributed to East Coast fever, a tick-borne disease

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prevalent in some areas due to poor dipping practices, especially by farmers from Study B. This was supported by pathological signs, which included fever, enlarged lymph nodes, anorexia, laboured breathing, corneal opacity, nasal discharge, diarrhoea, and anaemia. The two missing animals could have been sold by the farmers, as in this sector, animals serve as a source of income, food, and social security (Perry et al., 1984).

Although three cattle tested weakly positive for NSP antibodies at single time points in the study, all animals eventually tested negative for NSP antibodies, and there was no evidence of clinical FMD in the project areas.

There were differences in the VNT titres between cattle receiving one and two doses of vaccine (Table S1 and Figure 5.1). The mean logs of the reciprocal VNT titres 56 dpv were 1.15 (standard error of the mean [SE] = 0.18, n = 3) and 1.65 (SE = 0.17, n = 5) for subsistence cattle receiving 1 and 2 doses of vaccine, respectively, and 0.90 (SE = 0.08, n = 9) and 2.02 (SE = 0.16, n = 9) for commercial cattle receiving 1 and 2 doses of vaccine, respectively. The mean titre obtained from the samples of unvaccinated animals was 1.01 $\log_{10}$ (range 0.6 – 1.2). Only one subsistence animal (out of three) and no commercial animals (out of nine) that were vaccinated once developed VNT titres by 56 dpv above the threshold value of 1.5 to be considered protective, but three subsistence animals (out of five) and seven commercial animals (out of nine) that were vaccinated twice developed VNT titres by 56 dpv to be considered protective.

The pattern of structural proteins binding antibody responses largely mirrored those for neutralising antibodies. Peak responses in double-dose cattle were detected 56 days after the first vaccination. The mean SPCE percentage inhibitions 56 dpv were 25.7 (SE = 12.6, n = 3) and 86.6 (SE = 8.8, n = 5) for cattle from Rufunsa receiving one and two doses of vaccine, respectively, and 14.1 (SE = 5.6, n = 9) and 60.6 (SE = 8.6, n = 9) for cattle from Chisamba receiving one and two doses of vaccine, respectively. None of the animals from Rufunsa (out of three) and only one animal from Chisamba (out of nine) that were vaccinated once developed antibodies by 56 dpv above the threshold value of 70% inhibition to be considered positive, but four animals from Rufunsa (out of five) and all animals from Chisamba (out of nine) that were vaccinated twice developed antibodies by 56 dpv to be considered positive (Table S1, Figure 4.2). A one-sided independent samples *t*-test where equal variance was assumed showed a significant difference in antibodies 56 dpv between cattle given one and two vaccine doses (Rufunsa: $p = 0.003$; Chisamba: $p < 0.001$).

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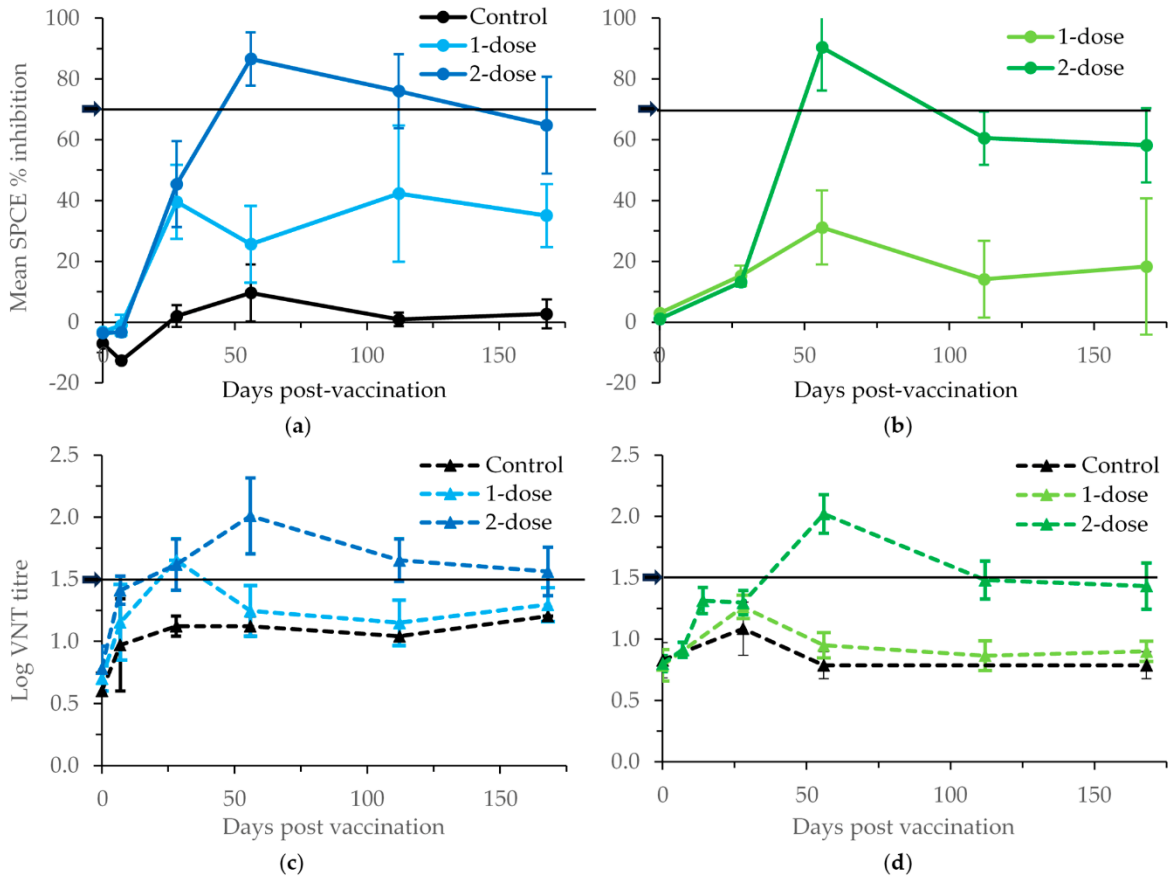


Figure 4.2 The mean solid-phase competition ELISA (SPCE) % inhibition at 1:10 serum dilution (a,b) and virus neutralisation test titres (c,d) in subsistence (a,c) and commercial (b,d) cattle herds vaccinated against FMDV serotype O at day 0 only (1-dose) or again 28 days after the first dose (2-dose). Control animals were not vaccinated. Values greater than the threshold values indicated on the y-axis by arrows are considered protective.

A Spearman's rank-order correlation determined the relationship between the log of the reciprocal of the virus neutralisation titres and the SPCE % inhibition results (Figure 4.3). A positive correlation between the VNT and SPCE results was statistically significant ($\rho = 0.598$ for 1:10 dilution, and $\rho = 0.562$ for 1:30 dilution of SPCE % inhibition results, $p < 0.001$ for both dilutions).

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Statistical analysis by Cohen's kappa coefficient showed a moderate agreement (Landis and Koch, 1977, Altman, 1999) between the two assays (κ = 0.516, 95% CI, 0.365 to 0.667, $p < 0.001$) using positive titre thresholds of ≥ 1:32 for the VNT and ≥ 70% inhibition for the SPCE.

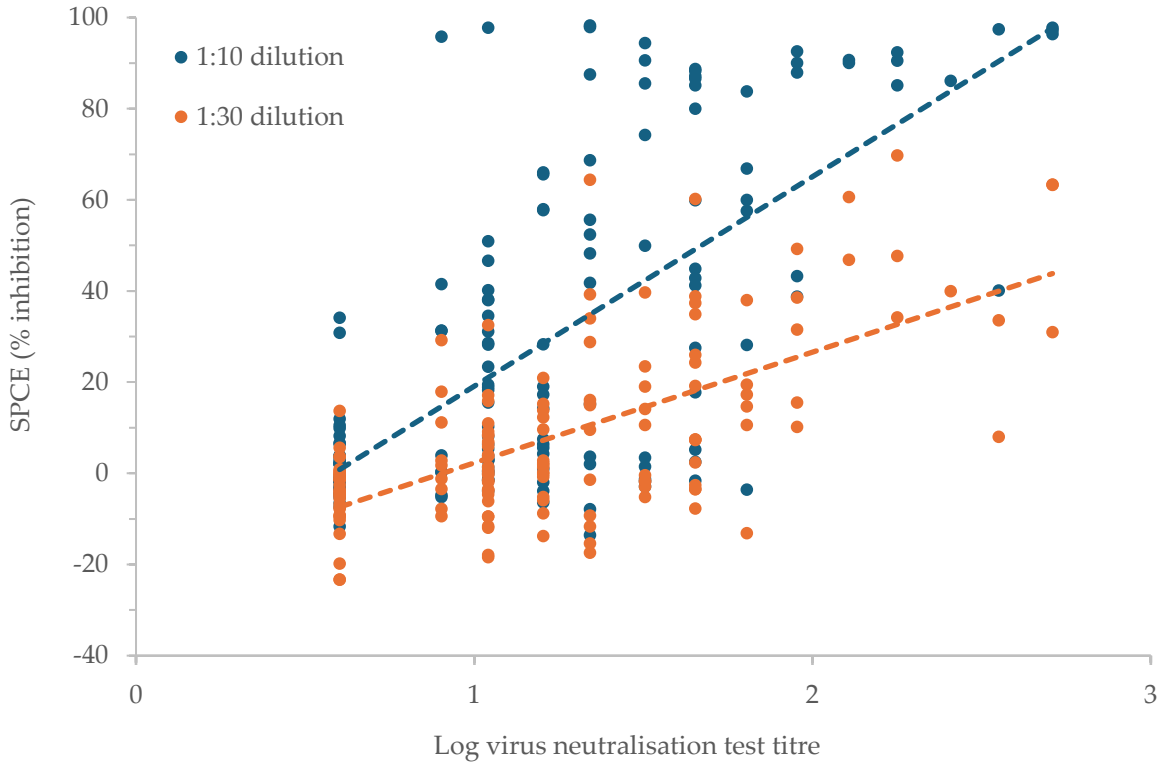


Figure 4.3 Positive correlation between log10 virus neutralisation titres and 1:10 (blue, R2 = 0.446) and 1:30 (orange, R2 = 0.426) solid-phase competition ELISA (SPCE) % inhibition in cattle vaccinated against FMDV serotype O.

In the 2018 project, ten animals recruited for the study at Ashwood farms died within the first two visits. The deaths were attributed to poor management and not related to FMD. This farm was eventually excluded from the study.

Generally, more positive reactors were picked up from several animals in the Chisamba district on 3 ABC ELISA during the 2018 project implementation period, whilst all animals from farms in the Chibombo district were negative throughout. Twenty-five samples from five different animals consistently NSP negative throughout the study period were tested by VNT. There were differences in the VNT titres between cattle receiving one and two vaccine doses (Figure 4.4 and Figure 4.5) in this project, just as observed in the 2020 project.

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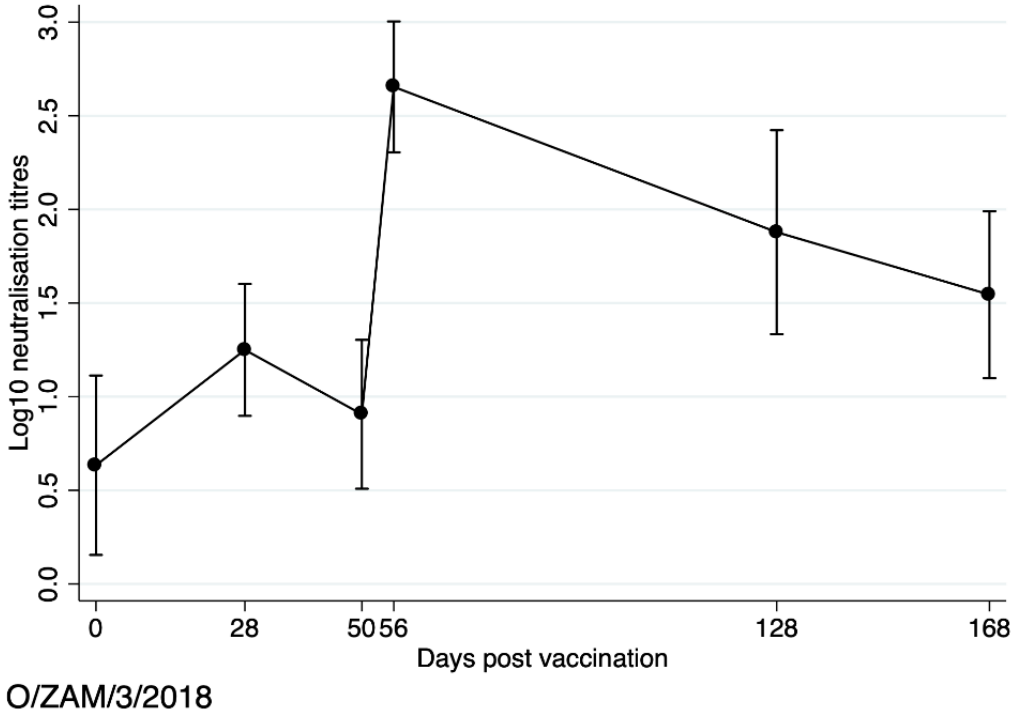


Figure 4.4 Log_{10} virus neutralisation titres at the different time points post-vaccination using the BVI vaccine for Zambian strains of serotype O lineage in the 2018 project.

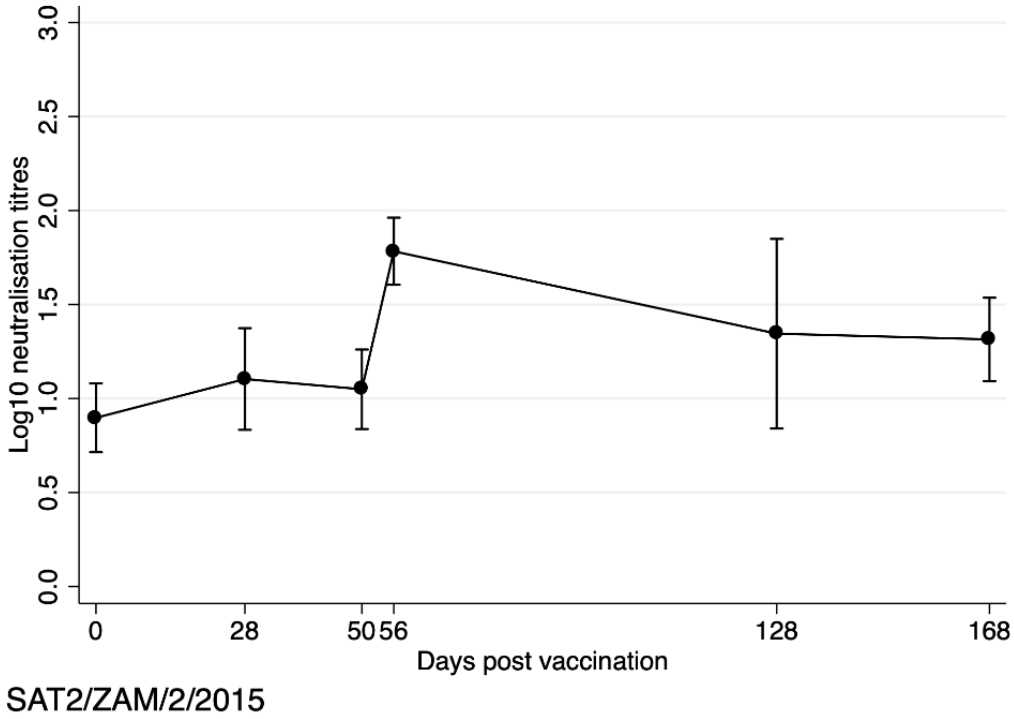


Figure 4.5 Log_{10} virus neutralisation titres at different time points post-vaccination using the BVI vaccine for Zambian serotype SAT-2 lineage strains in the 2018 project.

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The ranges around each point are 95% confidence intervals, and data were analysed using interval regression, which accounts for censored data (i.e., data points above or below a threshold value) and points falling within ranges (as is the case for VNT data). From these data, the significant points deduced were:

i. There were dramatic differences between the SAT-2 and O serotypes, with the SAT-2 component being much less immunogenic than O.
ii. For the O serotype, there was a modest response to a single dose but a strong anamnestic response, showing the importance of a second dose in a primary course with this vaccine.
iii. Even with a second dose, the response to the SAT-2 vaccination remains relatively poor (< 2.0 $\log_{10}$) throughout the study period.

4.5. Discussion

In both the 2020 and 2018 projects, the immunogenicity of imported FMD vaccine was evaluated to determine its suitability for use in Zambia and other Southern African countries where serotype O has been described as becoming important (Gobiye et al., 2023). The evaluated vaccine is already widely used in sub-Saharan Africa and was an obvious candidate for controlling the FMDV serotype O outbreaks reported in the Southern African region. An estimation of the proportion of animals with a detectable level of antibodies above a designated threshold in a population after vaccination of cattle with Trivalent vaccine SAT-1, SAT-2 and serotype O vaccine under field conditions was also conducted. Foot-and-mouth disease virus-specific antibody responses were measured after one- or two-dose primary courses of the vaccine. The study also compared results obtained by the VNT and a commercial SPCE kit to evaluate simple-to-use tests to support PVM studies at a population level in settings where high-containment facilities are unavailable.

The performance of an FMD vaccine can be assessed in vaccination-challenge studies according to the protocols defined in the WOAH Terrestrial Manual (WOAH, 2022a). However, due to several reasons, including ethical considerations and costs, *in vitro* studies are recommended (WOAH, 2022a). The relationship between serology and protection in FMD-vaccinated cattle has been previously studied by correlating the antibody titres at the point of challenge with the outcome, i.e., protected or not protected (FAO-WRLFMD, 2022, Gubbins et al., 2022, Brehm et al., 2008). A correlation between neutralising antibody titre and protection against homologous challenge is well established (Barnett et al., 2003, van Bekkum et al., 1969). The testing and analysis of day-of-challenge sera from vaccination- and challenge-cross-protection studies have long established an association between in vitro neutralising antibody titres to the challenge viruses and *in vivo* clinical cross-protection (Gubbins et al., 2022). Cross-strain protection between different vaccines and challenge viruses also have similar relationships, although much less data are available from these studies (Gubbins et al., 2022, FAO-WRLFMD, 2022). A recommended simple approach to assess cross-protection is to measure the amount of antibodies that vaccinated animals have

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against the field virus of concern (Gubbins et al., 2022). Against this background, these studies examined day-of-challenge antibody titres to heterologous challenge viruses.

Apart from the three weakly positive samples observed at one time point in the 2020 project implementation period, neither seroconversion to FMDV NSP nor clinical signs were observed throughout this study, suggesting the FMDV-specific antibody titres detected were vaccine-induced. The observed mean reciprocal log virus neutralisation titre of 2.02 for commercial cattle from Chisamba and 1.65 for subsistence cattle from Rufunsa in second-dosed cattle (Table S1 and Figure 5.1) imply that the aqueous adjuvanted vaccine used in this study produced neutralisation titres likely to offer protection against the target lineages. However, the observed mean titres from single-dosed cattle from Chisamba (0.90) and Rufunsa (1.15) 56 days after the first vaccination suggest that the vaccine given in this way was not able to reach the recommended protective titres as described (FAO-WRLFMD, 2022, Hammond et al., 2021). This emphasises the value of the second vaccine dose in this context, but its importance is likely to vary according to the vaccine's potency and its antigenic match to the strain against which protection is required. After the primary course of vaccination, the manufacturer of this vaccine recommends a booster at four to six months, which also seems in line with the results obtained here on the duration of the antibody responses found via VNT and ELISA.

Similar observations were obtained on the duration of the antibody responses found via VNT in the 2018 project. The titres at four months for serotype O were sufficient to offer protection against the target lineages but the confidence intervals were wide, so the results were inconclusive, and a bigger sample size was required. The six-month titres were low, and a booster dose was needed for this time (maybe earlier). It cannot be concluded that the differences between the O and SAT-2 serotypes were related directly to the vaccine quality since these were not the vaccine strains but heterologous strains deemed relevant to Zambia. Furthermore, the low SAT-2 serotype titres could be due to either low potency, poor match (or both), poor vaccine storage or other user-related problems. Assuming minimal user-related problems since vaccination was conducted under controlled conditions, a concern was raised about the SAT-2 serotype component of the vaccine, assuming the strain used is still the dominant threat to the country. Apart from these general trends, care must be taken not to overinterpret the findings, as predicting protection from antibody titres cannot be carried out with precision due to several uncontrollable variables (FAO-WRLFMD, 2022). Other studies have also shown that antibodies alone are not responsible for protection but correlate with the animal's immune response (van Bekkum et al., 1969).

Previous studies have correlated potency test outcomes with pre-challenge antibody titres measured using the VNT and LPBE (Barnett et al., 2003, Robiolo et al., 2010). More recently, commercially available SPCEs have become alternatives to the LPBE for SP serology and use for routine PVM at the population level (Paton et al., 2019, FAO-OIE, 2016). A limitation of SPCE in having antigens and blocking antibodies which are fixed and usually of unknown antigenic relevance (beyond serotype) has, however, been discussed (Paton et al., 2019), and this, together with antigen instability (Ludi et al., 2022) may account for differences in results

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compared to VNT. Nevertheless, studies have shown the potential of SPCE in determining immunogenicity (Chénard et al., 2003). The moderate correlation in results between the two test methods (i.e., VNT and SPCE) in this study suggests that the SPCE could be used in assessing the immunogenicity of vaccines where high-level biocontainment facilities are not available to conduct VNT. However, the VNT is likely to be superior at predicting cross-protection to specific field strains according to their antigenic match.

We recommend the implementation of booster dose vaccination every four to six months as a standard in Zambia and the region to increase herd immunity, thus contributing to effective control of FMD outbreaks. Further, the field evaluation in commercial and subsistence cattle heads is crucial for tailoring vaccination programs to different farming systems in Zambia and the region, ensuring broader and more effective coverage. Lastly, the suggested alternative testing method of SPCE, which is more accessible for assessing vaccine efficacy in the field, facilitates more frequent and widespread monitoring. Implementing a robust vaccination regime based on the study's findings can ensure reduced FMD incidence, thereby improving livestock productivity and economic stability in Zambia. The vaccination regime in the region should now consider the incorporation of serotype O in its vaccination plan.

4.6. Conclusion

This study confirms the value of PVM testing and shows that for these vaccines and field strains utilised, a vaccination regime employing a two-dose primary course and revaccination after four to six months is likely to be appropriate, as recommended by the vaccine manufacturer. Countries, especially sub-Saharan countries with diverse FMDV strains and inconsistent vaccine quality, are encouraged to implement PVM studies that can account for variations in vaccine potency and antigenic match.

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Chapter 5. Conclusions

This study aimed to address key objectives related to the epidemiology, diagnostics, and vaccination strategies of Foot and Mouth Disease Virus (FMDV) in Zambia, with a particular focus on its persistent challenges, emerging risks, and recommendations for improving disease control efforts.

The investigation into the genetic diversity and phylogenetic relationships of FMDV from 2015-2021 outbreaks in Zambia reveals crucial epidemiological implications. The study highlighted the continued circulation of FMDV serotypes such as SAT-2, SAT-3, and the newly emerged O/EA-2 in various regions of Zambia, emphasizing the need for targeted and adaptive control measures. The identification of new FMDV serotypes, particularly in areas that were previously free of the disease, underscores the shifting dynamics of FMD transmission and the necessity for a comprehensive and dynamic approach to control strategies, including the inclusion of these newly identified strains in future vaccination programs.

The scoping review of diagnostic techniques for SAT FMDV serotypes pointed to significant gaps in Zambia's diagnostic capabilities, particularly regarding the SAT serotypes. The study advocates for the adoption of more efficient, accessible, and cost-effective diagnostic methods such as RT-qPCR, Ag-ELISA, and LFD test strips for early detection and timely control. Furthermore, the findings stress the need for improved serological diagnostics to differentiate between vaccinated and infected animals, which is essential for targeted control measures. This calls for urgent improvements in Zambia's diagnostic infrastructure, which remains hindered by the reliance on reference laboratories and delays in disease detection.

Regarding vaccination strategies, the study's findings underscore the importance of high-quality vaccines and optimised vaccination protocols. The current bi-annual vaccination regime in Zambia is inadequate, as antibody titres tend to wane before the next dose, leaving cattle vulnerable to FMDV infection. The study recommends a two-dose primary vaccination schedule followed by revaccination every four to six months for optimal immunogenicity, particularly for the newly circulating serotype O/EA-2. Additionally, the study revealed concerns about the immunogenicity of the SAT-2 vaccine component, indicating that future vaccine research should prioritise enhancing the effectiveness of this component, especially given its low efficacy in the current vaccination regimen. Furthermore, the study highlights the importance of vaccinating all susceptible livestock species, including small ruminants, to reduce the recurrence and spread of FMDV in Zambia. To improve the cost-effectiveness of vaccination programs, multivalent vaccines should be considered in areas with multiple circulating serotypes.

Finally, this study identifies critical factors contributing to Zambia's FMD control challenges, including gaps in vaccine coverage, insufficient diagnostic tools, and inadequate vaccination monitoring systems. The examination of regional FMD control strategies underscores the vital importance of fostering cross-border cooperation and improving surveillance systems,

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particularly in Southern Africa, where transboundary animal movement significantly contributes to disease spread. The study also emphasizes the need for stricter regulations on animal movement and the promotion of community-based surveillance and control efforts, which have proven effective in other regions.

In light of these findings, it is recommended that Zambia's FMD control efforts be strengthened through improved diagnostic capabilities, enhanced vaccination strategies, and more effective disease monitoring and control systems. Regional cooperation and data sharing, along with the adoption of risk-based control measures, will be crucial in reducing the frequency and impact of FMD outbreaks and ensuring the long-term sustainability of Zambia's livestock sector. Future research should continue to focus on improving diagnostic methods, vaccine efficacy, and understanding the epidemiological dynamics of FMDV, ensuring that Zambia and the broader Southern African region are better prepared to face the ongoing threat of FMD.

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Chapter 7. Appendix

7.1. Research ethics

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Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Genetic and antigenic characterization of FMD viruses circulating in Zambia**
Project Number	**REC061-19**
Researcher / Principal Investigator	**Mr F Banda**

Dlissertation / Thesis submitted for	**Doctoral**

Supervisor	**Prof M Quan**

APPROVED	Date: **2019-10-11**
CHAIRMAN: UP Research Ethics Committee	Signature:

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7.2. Animal ethics

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Faculty of Veterinary Science
Animal Ethics Committee

4 December 2019

Approval Certificate with Conditions
New Application

AEC Reference No.: REC061-19
Title: Genetic and antigenic characterization of FMD viruses circulating in Zambia
Researcher: Mr F Banda
Student's Supervisor: Prof M Quan

Dear Mr F Banda,

The **New Application** as supported by documents received between 2019-03-26 and 2019-11-25 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-11-25.

Please note the following about your Conditional ethics approval:

1. The use of species is approved:

Species	**Number**
Cattle (Bovine)	All numbers be reported on annually
Samples	
Serum	500 (2 ml per animal)
Tissues	50 (100g each)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-12-04.
3. Any future changes on this application must be done as an AMENDMENT.
4. Please remember to use your protocol number (REC061-19) on any documents or correspondence with the AEC regarding your research.
5. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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7.3. Article 1

viruses

MDPI

Article

Characterization of Foot-and-Mouth Disease Viruses in Zambia-Implications for the Epidemiology of the Disease in Southern Africa

Frank Banda [1,2,*], Yona Sinkala [3], Liywalli Mataa [3], Phiyani Lebea [4], Tingiya Sikombe [1], Henry L. Kangwa [1], Elliot M. Fana [5], Mokganedi Mokopasetso [5], Jemma Wadsworth [6], Nick J. Knowles [6], Donald P. King [6] and Melvyn Quan [2]

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Abstract: The livestock industry supports livelihood and nutritional security of at least 42% of people in the Southern African Development Community region. However, presence of animal diseases such as foot-and-mouth disease poses a major threat to the development of this industry. Samples collected from FMD outbreaks in Zambia during 2015–2020, comprising epithelial tissues samples (n = 47) and sera (n = 120), were analysed. FMD virus was serotyped in 26 samples, while 92 sera samples tested positive on NSP-ELISA. Phylogenetic analysis revealed notable changes in the epidemiology of FMD in Zambia, which included: (i) introduction of a novel FMDV SAT-3 (topotype II) causing FMD cases in cattle in Western Province; (ii) emergence of FMDV serotype O (topotype O/EA-2) in Central, Southern, Copperbelt, Western, Lusaka Provinces; and (iii) new outbreaks due to SAT-2 (topotypes I) in Eastern Zambia. Together, these data describe eight different epizootics that occurred in Zambia, four of which were outside the known FMD high-risk areas. This study highlights the complex epidemiology of FMD in Zambia, where the country represents an interface between East Africa (Pool 4) and Southern Africa (Pool 6). These changing viral dynamics have direct impacts on FMD vaccine selection in the SADC region.

Keywords: FMD; livestock; buffalo

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious transboundary animal disease (TAD) that is a major concern to livestock industries throughout the world. The disease is caused by the FMD virus (FMDV), which affects cloven-hoofed animals. In addition to the ability of FMDV to spread rapidly within domesticated livestock, the virus can be maintained within populations of certain wildlife species, such as free-ranging African buffalo (*Syncerus caffer*), which do not show obvious signs of disease and can act as virus reservoirs capable of transmitting the virus to livestock [1–3]. The involvement of wildlife in the epidemiology of FMD provides a challenge to control FMD in sub-Saharan Africa [4].

The FMDV genome contains a single open reading frame that encodes four structural viral proteins (VP1–VP4) that form the virus capsid, and eight non-structural proteins (NSP)

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involved in the replication of the virus. VP1 sequences are often used for epidemiological investigations [5] to characterise FMD viruses within the seven serotypes (A, O, C, SAT-1, SAT-2, SAT-3 and Asia-1) and into genetically discrete topotypes. Globally, there are seven endemic pools of infection which often require tailored diagnostics and vaccines [6,7]. In Africa, five serotypes (O, A, SAT-1, SAT-2, SAT-3) are present, but their distribution varies. In Southern Africa (Pool 6), three serotypes (SAT 1-3) are endemic with sporadic incursions of serotype O and A recorded in Northern Zambia as a consequence of spillover from Pool 4 (East Africa) [8–10]. The distribution of these diverse serotypes and subtypes of FMDV is subject to change, and requires continuous monitoring [10,11].

FMD continues to be a major sanitary concern for beef producers in the Southern African Development Community (SADC) region because the disease is a key constraint to access high-value export markets. The occurrence of FMD results in damaging consequences for the livelihoods of local farmers due to the disease's impacts on productivity, food security and loss of income [12]. Furthermore, in Zambia, FMD impedes the utilisation of livestock and wildlife resources of the country, where 80% of the population rely on agriculture for food and income generation [13]. In this context, FMD in the country has been likened to a disease of poverty because of its negative effects, and is the reason for low economic growth [14,15].

In Zambia, FMD was first reported in 1933 [16], and sporadic outbreaks of the disease have been reported from three high-risk areas [14,17]. These defined high-risk areas (Figure 1) are (A) the Northern part of the country on the border with Tanzania (Nakonde, Mbala and Mpulungu districts), (B) the Kafue Flats on the border between Central and Southern provinces (Namwala, Mumbwa, Itezhi tezhi and parts of Monze, Choma and Mazabuka districts) and (C) the Southern parts of Western and Southern Provinces (from Kazungula/Livingstone to a point beyond Sesheke district, where the border with Angola turns to the north-west). An unprecedented rise in the incidence of FMD outbreaks in livestock since 2004 has been observed in Southern Africa [18,19]. The reasons for this are most likely multifactorial, but prima facie evidence points to the poor performance of prophylactic vaccination programmes [20]. In Zambia, a rise in FMD reports was recorded in Western, Eastern, Southern and Central Provinces of the country from 2015 to 2020. The main risk factors have been reported elsewhere [21,22], while the molecular epidemiology of FMD in the country has not been fully elucidated. Previous FMD outbreaks in Zambia have been difficult to prevent and control [17], and have not been systematically studied to gain insight into outbreak causation or transmission patterns, and also to predict when the next outbreak would likely occur [22]. This information is necessary so that appropriate preventive measures can be instituted. This study aimed to characterize FMD viruses causing recent cases in Zambia, in order to provide data to help understand the epidemiology of the disease in Southern Africa.

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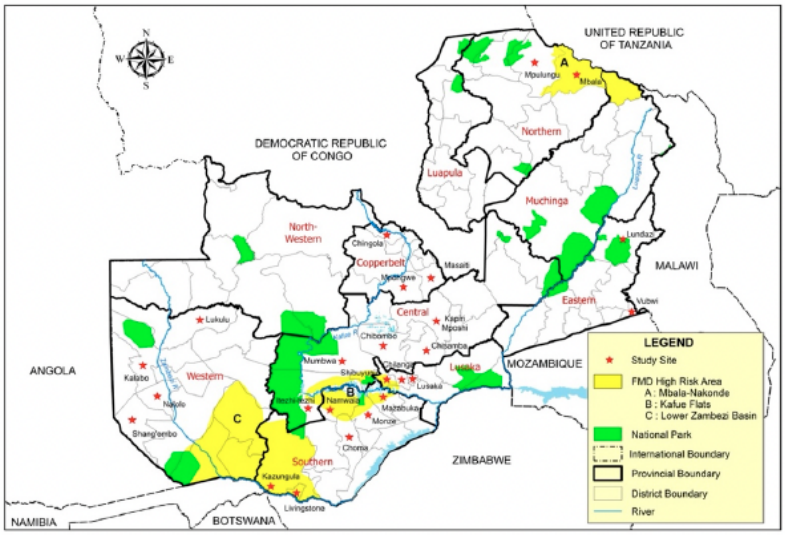


Figure 1. Foot-and-mouth disease high-risk areas and study sites in Zambia.

2. Materials and Methods

2.1. Study Area

The study region encompassed the whole of Zambia, where specific sampling areas were purposely selected based on suspected FMD cases reported to the Department of Veterinary Services during the study period of 2015 to 2020.

2.2. Sample Collection

A total of 75 kraals and farms were visited during FMD outbreak investigations. After clinical examination for the presence of typical FMD lesions, fresh epithelial tissue, vesicular fluid and blood samples from suspect FMD cases in these outbreak areas were collected. The tissue specimens and blood samples were collected, as described elsewhere [23]. Briefly, at least one gram of epithelial tissue was collected from an unruptured or recently ruptured vesicle and placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer (pH 7.2–7.6). Plain vacutainer tubes (Becton, Dickson and Company, Franklin Lakes, NJ, USA) were used to collect whole blood, and the serum was separated once the blood was clotted. Epithelial tissues and sera samples were kept refrigerated at 4 °C, or on ice for epithelial tissue until received at the Central Veterinary Research Institute (CVRI) in Zambia, where samples were stored at −20 °C. The samples were packaged and shipped to International Reference Laboratories (World Organisation for Animal Health (OIE) Reference Laboratory, Botswana Vaccine Institute (BVI) and the Food and Agriculture Organization (FAO) World Reference Laboratory for FMD (WRLFMD) and OIE Reference Laboratory, The Pirbright Institute, United Kingdom) for virus isolation, NSP-ELISA, Antigen-ELISA, real-time reverse transcription PCR (RT-qPCR) and VP1 sequencing. During the study period, 35 samples were submitted to BVI and 13 samples were submitted to WRLFMD.

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2.3. Laboratory Analysis of Samples

At CVRI, all sera samples were tested for the presence of FMDV NSP-specific antibodies using a multi-species antibody test kit (IDEXX, Westbrook, ME, USA) by following the manufacturer's instructions. Representative epithelial samples were prepared and tested for the presence of FMDV by thawing and blot drying them, followed by grinding in phosphate-buffered saline (PBS) using sterile sand in a pestle and mortar in a 10% suspension [24]. The suspensions were tested using a sandwich ELISA (IZSLER Biotechnology Laboratory, Brescia, Italy) for detection and serotyping of FMDV antigens by following the manufacturer's instructions. At the WRLFMD, virus isolation was carried out on primary bovine thyroid (BTy) cells [25] or IB-RS-2 cell cultures [26], and an Ag-ELISA was performed on cell cultures showing cytopathic effect to identify the serotype [24,27,28]. At the BVI, lamb kidney cells were used for virus isolation. FMDV-specific RT-qPCR assays were performed on the samples [29,30]. Samples with detectable FMDV genomes were further characterised by VP1 sequencing [31]. Complete VP1 nucleotide sequences were aligned using BioEdit 7.0.5.3 [32,33]. Optimal nucleotide substitution models were computed for each serotype using MEGA 7 [34]. The maximum likelihood algorithm was used to construct phylogenetic trees employing MEGA 7. One thousand bootstrap pseudo-replicates were used to assess branching confidence.

3. Results

3.1. Epidemiology and Clinical Observations

Twenty-five FMD outbreaks were recorded during the study period, of which 32% (8) were primary outbreaks and 68% (17) were outbreak extensions (Table 1 and Figure 2). Out of the eight primary outbreaks, four (50%) were reported outside the three known high-risk areas in Zambia (Figure 2): in October 2015 from Shang'ombo district of Western Zambia; in March 2018 and January 2019 in Chisamba district of Central Zambia and finally during March 2019 in Lundazi and Vubwi districts of Eastern Zambia (Figure 2). The other four primary outbreaks were reported in February 2015 from Mpulungu and Mbala districts of Northern Zambia, whilst Kawimbe Veterinary Camp of Mbala district reported outbreaks in October and March 2018.

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Table 1. Details of tissue samples described in this study (2015–2020).

CVRI Ref No.	Date Collected (DD/MM/YYYY)	Species	Vet Camp/Village	District, Province	NSP	Serotype	WRLFMD Ref. No.	BVI Ref. No.	RT-qPCR	Serotype	Topotype	Lineage
MM01	02.4.2015	Bovine	Mupata Village	Mpulungu, Northern	Positive	ND	ZAM/1/2015		FMDV-GD	A	AFRICA	G-I
HS 01	28.2.2015	Bovine	Chimula Village	Mbala, Northern	Positive	ND	ZAM/2/2015	ZAM03/15	FMDV-GD	SAT 2	IV	-
Sh 01	23.10.2015	Bovine		Shangoomb, Western	Positive	ND	ZAM/3/2015	ZAM09/15	FMDV-GD	SAT 3	II (WZ)	-
LK1	18.5.2017	Bovine	Mbanga & Mulongo	Lukulu, Western	Positive	ND	ZAM/1/2017	ZAM08/17	FMDV-GD	SAT 3	II (WZ)	-
K1	04.1.2018	Bovine	Kaluwe & Siluwe	Kalabo, Western	Positive	ND						
Chisa 16-380	24.3.2018	Bovine	Chisamba	Chisamba, Central	Negative	ND	ZAM/1/2018	-	FMDV-GD	O	EA-2	-
Chisa 16-169	24.3.2018	Bovine	Chisamba	Chisamba, Central	Negative	ND	ZAM/2/2018	-	FMDV-GD	O	EA-2	-
BBR-1	24.3.2018	Bovine	Chisamba	Chisamba, Central	Negative	ND	ZAM/3/2018	-	FMDV-GD	O	EA-2	-
TS 01	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	ZAM/4/2018	-	FMDV-GD	A	AFRICA	G-I
ES 01	24.10.2018		Kawimbe	Mbala, Northern	Positive	ND						
ES 02	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	ZAM/5/2018	-	FMDV-GD	A	AFRICA	G-I
GS 01	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	ZAM/6/2018	-	FMDV-GD	A	AFRICA	G-I
ES03	24.10.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	-	ZAM37/18	FMDV-GD	A	AFRICA	G-I
VS 02	27.3.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	-	ZAM10/18	FMDV-GD	O	EA-2	-
VS 05	27.3.2018	Bovine	Kawimbe	Mbala, Northern	Positive	ND	-	ZAM12/18	FMDV-GD	O	EA-2	-
ZRC 1	18.1.2019	Bovine	Chisamba Central	Chisamba, Central	Positive	ND	ZAM/1/2019	-	FMDV-GD	O	EA-2	-
CVRI67/19	17.7.2019			Chibombo, Central	Positive	ND						
CVRI04/19	11.2.2019	Bovine	Ufwenuka	Monze, Southern	Positive	O	ZAM/2/2019	-	FMDV-GD	O	EA-2	-
CVRI01/19	11.2.2019	Bovine	Ufwenuka	Monze, Southern	Positive	O	ZAM/3/2019	-	FMDV-GD	O	EA-2	-
CVRI08/19	11.3.2019	Bovine	Magoye	Mazabuka, Southern	Positive	O	ZAM/4/2019	-	FMDV-GD	O	EA-2	-
17562	16.3.2019			Chibombo, Central		-	ZAM/5/2019	-	NGD	-	-	-
17496	17.3.2019			Chibombo, Central		-	ZAM/6/2019	-	NGD	-	-	-
CVRI16/19	30.3.2019	Bovine	Kasako	Mazabuka, Southern	Positive	O	ZAM/7/2019	-	FMDV-GD	O	EA-2	-
CVRI15/19	30.3.2019	Bovine	Kasako	Mazabuka, Southern	Positive	O	ZAM/8/2019	-	FMDV-GD	O	EA-2	-
CVRI22/19	02.4.2019	Bovine	Mwase	Lundazi, Eastern	Positive	SAT-2	ZAM/9/2019	-	FMDV-GD	SAT 2	I	-
CVRI23/19	02.4.2019	Bovine	Mwase	Lundazi, Eastern	Positive	SAT-2	ZAM/10/2019	-	FMDV-GD	SAT 2	I	-
CVRI20/19	03.4.2019	Bovine	Zozwe & Mlawe	Vumbwi, Eastern	Positive	SAT-2	ZAM/11/2019	-	FMDV-GD	SAT 2	I	-
CVRI21/19	03.4.2019	Bovine	Mbande	Vumbwi, Eastern	Positive	SAT-2	ZAM/12/2019	-	FMDV-GD	SAT 2	I	-
CVRI25/19	03.5.2019	Bovine	Feni	Chipata, Eastern	Positive	SAT-2	-	-		SAT 2		
CVRI37/19	16.6.2019	Bovine	Lwashimba	Kapiri Mposhi, Central	Positive	O	-	ZAM06/19	FMDV-GD	O	EA-2	-
CVRI55/19	28.6.2019	Bovine	Musenga	Chingola, Copperbelt	Positive	O	-	ZAM07/19	FMDV-GD	O	EA-2	-
CVRI29/19	20.5.2019	Bovine	Chitongo	Namwala, Southern	Positive	O	-	-		O		
CVRI30/19	20.5.2019	Bovine	Chitongo	Namwala, Southern	Positive	O	-	-		O		
CVRI31/19	05.6.2019	Porcine	Hamangaba	Monze, Southern	Positive	O	-	-		O		
CVRI32/19	05.6.2019	Porcine	Hamangaba	Monze, Southern	Positive	O	-	-		O		
CVRI61/19	12.6.2019	Bovine	Sunrise (Kasupe)	Chilanga, Lusaka	Positive	O	-	-		O		
CVRI36/20	14.2.2020	Porcine	Makeni	Chilanga, Lusaka	Positive	O	-	-		O		
CVRI01/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	ZAM03/20	FMDV-GD	O	EA-2	-
CVRI02/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	-		O		
CVRI05/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	-		O		
CVRI06/20	14.1.2020	Bovine	Kaungaleuti	Nalolo, Western	Positive	O	-	-		O		
CVRI13/20	17.4.2020	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI14/20	17.4.2020	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI17/20	17.4.2020	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI15/20	17.4.2021	Bovine	Luubwe	Itezhi- Tezhi, Central	Positive	O	-	-		O		
CVRI29/20	13.8.2020	Bovine	Makeni	Chilanga, Lusaka	Positive	O	-	-		O		
CVRI25/20	10.3.2020	Bovine	Nyawa	Kazungula, Southern	Positive	O	-	-		O		

BVI-Botswana Vaccine Institute, CVRI-Central Veterinary Research Institute, GD-Genome Detected, ND-Not done, NSP-non-structural protein (3ABC ELISA), RT-qPCR-real-time PCR, Serotype (antigen ELISA), WRLFMD-World Reference Laboratory for Foot and Mouth Disease.

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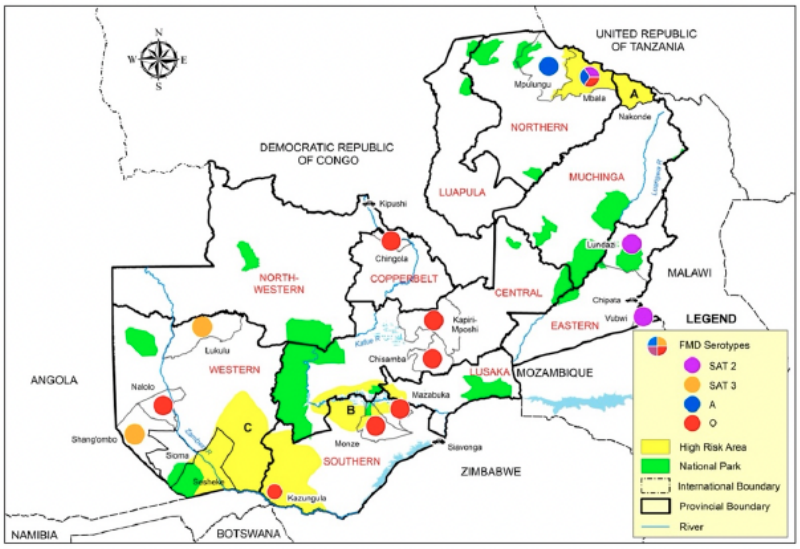


Figure 2. Foot-and-mouth disease virus serotypes detected in Zambia between 2015 and 2020.

Affected cattle showed typical clinical signs of FMD, which included drooling, nasal discharge, the grinding of teeth, and mouth lesions on the tongue, dental pad and gums. Most animals recovered within two to three weeks. Vesicles were observed as a whitish area that ruptured when pressure was applied, and erosions were observed on the feet. Vesicles were also apparent on the mammary glands in some of the dairy animals, and one instance sloughing of the teat was observed. Pathognomonic clinical signs of FMD were observed in pigs and piglets at Hamangaba and Makeni veterinary camps in Monze and Chilanga districts, respectively. Animals were seen with white lesions and blisters on the coronary bands of the hoof and snout. There was a loss of appetite, and the piglets showed mild lameness with a reluctance to move. Severe lesions of the heel pad area were observed. In Monze, the horn of the hoof in one piglet separated and shed, as has been observed elsewhere [1].

Out of the blood samples collected, 110 (92%) were bovine samples and 10 (8%) were swine samples. A total of 92 (77%) were positive for antibodies against FMDV NSPs with a percentage inhibition range of 60.8 to 95.9% (Table 1). Despite testing positive with the RT-qPCR assay, samples collected from Chisamba 2018 and Itezhi Tezhi were negative when tested with the NSP-ELISA at initial sampling, but subsequent samples collected after 14 days were positive, probably due to the lack of NSP antibodies during the early stages of disease at the time of initial collection. All pig samples tested positive with the NSP-ELISA, and were typed as serotype O with the Ag-ELISA. Samples collected from Kazungula district Nyawa camp also tested positive with the RT-qPCR assay, and were typed as serotype O with the Ag-ELISA. Twenty-six samples contained detectable FMDV genomes and could be serotyped, while no virus genome was detected in the remainder of the samples. FMDV positive samples were further characterised by VP1 sequencing.

3.2. Phylogenetic Analyses

The most appropriate nucleotide substitution models for each serotype were found to be the Tamura-Nei (TN-93) model, gamma-distributed with invariant sites (G+I) for type

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O; the Hasegawa-Kishino-Yano (HKY) model, G+I, for types A and SAT2; and the General Time Reversible (GTR) model, G+I, for serotype SAT-3.

The study revealed that FMDV SAT-3 was responsible for the outbreak reported in Beshe Vet Camp in Shangombo district of Western Province in 2015 (isolate ZAM/3/2015 and two additional sequences for isolates from BVI). Further outbreaks linked to these cases were later reported in all districts of Western Province except for Mwandi district [35]. Sequence data supported this close relationship, where nucleotide (nt) identities between ZAM/3/2015 and ZAM/1/2017 were 98.0%. Beyond the genetic identity between Zambia FMDV sequences, ZAM/3/2015 was most closely related to SAT3/BOT/P3/98 NXA-9 (88.6% nt identity) and SAT3/BOT/P3/98 NXA-6 (88.3% nt identity) (Figure 3).

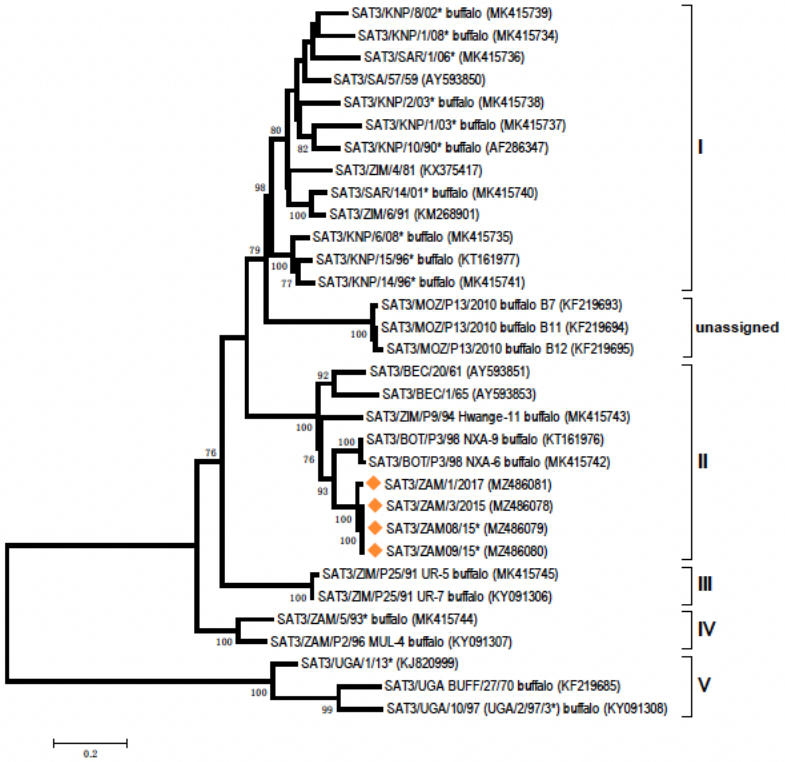


Figure 3. Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2015 foot-and-mouth disease virus SAT-3 serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses. * Reference number not assigned by the WRLFMD.

All the serotype O viruses isolated in this study (Table 1) clustered within the East Africa 2 (EA-2) topotype, most closely related to viruses collected in Tanzania: O/TAN-CVL-2012-0318 (95.8–95.9% nt identity) and O/TAN-CVL-2012-0321 (95.9–96.1% nt identity) (Figure 4). These included FMD samples collected from six provinces in Zambia (Central, Copperbelt, Lusaka, Northern, Southern, Western Provinces). Phylogenetic analysis of O/ZAM/1/2018 from Chisamba, Central Zambia and O/ZAM/10/2018 from Kawimbe, Mbala Northern Zambia revealed that the viruses responsible for these two primary outbreaks were closely related to each other (99.8% nt identity).

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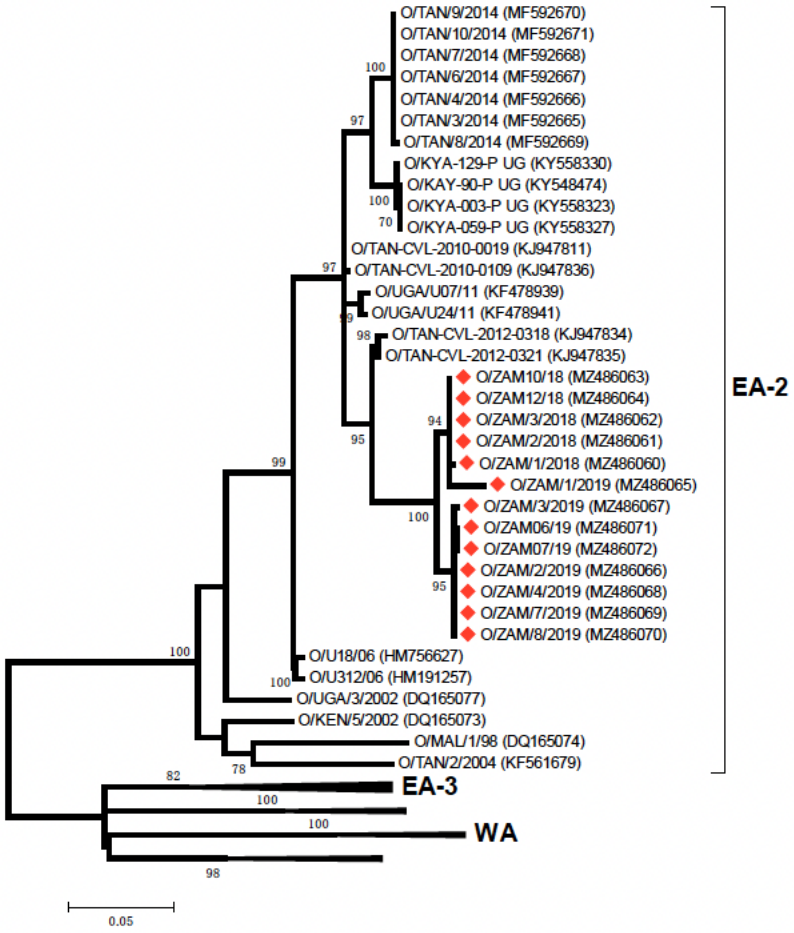


Figure 4. Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2018 foot-and-mouth disease virus O serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses.

In the Northern Province, samples from Chimula Village in Mbala (ZAM/2/2015) were serotyped as SAT-2 and clustered within topotype IV, related to viruses circulating in East Africa (Pool 4) (Figure 5). In contrast, the VP1 sequences of four additional SAT 2 viruses (ZAM/9-12/2019) from Lundazi and Vubwi Districts of Eastern Zambia were characterized as belonging to topotype I, with closest relationships with SAT2/MAL02/19 and SAT2/MAL01/17 (96.6–98.3% nt identity) collected in Malawi (Figure 5).

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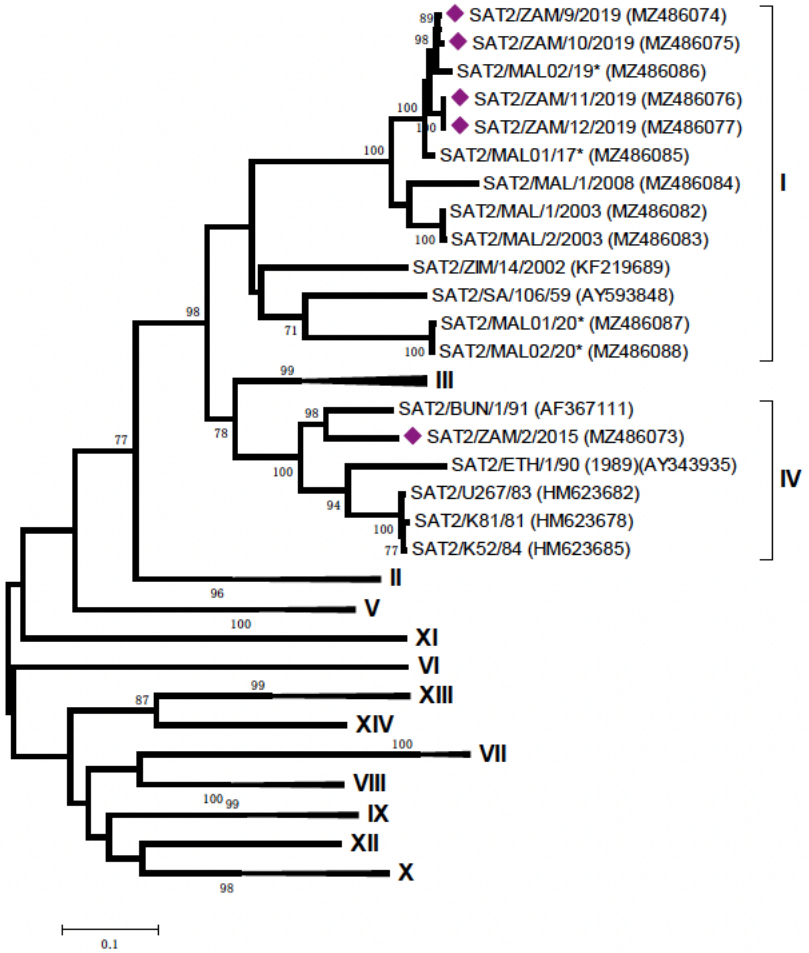


Figure 5. Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2015 and 2019 foot-and-mouth disease virus SAT-2 serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses. * Reference number not assigned by the WRLFMD.

This study detected five serotype A (genotype AFRICA/G-I) isolates from outbreaks that occurred in 2015 and 2018; ZAM/1/2015 from Mpulungu which was genetically distinct to four identical sequences (ZAM/4-6/2018 and A/ZAM37/18 from the Mbala, Northern Province) (Figure 6). Viruses from both of these clades were most closely related to sequences from FMDV isolates collected in Tanzania 95–96% nt identity.

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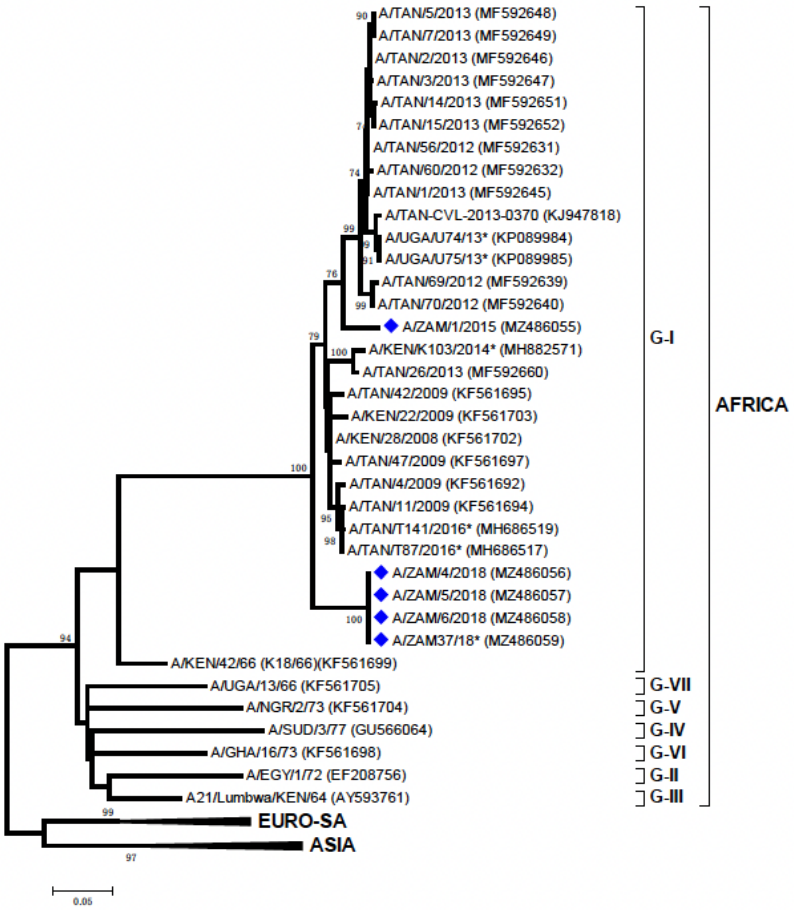


Figure 6. Midpoint-rooted maximum likelihood tree showing the relationships between the VP1 sequences of the 2015 and 2018 foot-and-mouth disease virus serotype A serotypes from Zambia (indicated with diamonds) and other contemporary and reference viruses. * Reference number not assigned by the WRLFMD.

4. Discussion

This is the first molecular epidemiology study of FMDV in Zambia undertaken to define the genetic relationships between Zambian FMD viruses and those collected from neighbouring countries. These results highlight that FMD continues to circulate in the three high-risk areas of FMD in Zambia, and also reveals notable changes in the epidemiology of FMD, including confirmed cases of FMDV serotype SAT-3 in cattle of Shangombo district of Western Province in 2015, the emergence of FMDV serotype O (O/EA-2) in Central, Southern, Copperbelt, Western and Lusaka Provinces and the introduction of the SAT-2 serotype into Eastern Zambia.

FMDV serotype SAT-3 has only been isolated previously in Zambia from wild buffalo, although infection due to this serotype was once diagnosed serologically in cattle [17,22]. This study describes the first FMDV serotype SAT-3 outbreak in domesticated cattle, believed to have been initiated by an illegal livestock movement from a neighbouring country. It has also been speculated that these outbreaks may have involved buffalo from the Sioma

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National Game Park after cattle were seen grazing with buffalo [36]. This national park, located in Shangombo and Sioma districts, is one of the protected areas in the Kavango Zambezi (KAZA) Transfrontier Conservation Area (TFCA), where transboundary animal movement is common and promoted especially for wildlife. As reported previously, most SAT serotype outbreaks have occurred in and around the KAZA TFCA, where approximately 1.5 million people and their livestock are resident [12,37]. Although two sequences (SAT3/BOT/P3/98 NXA-9 and SAT3/BOT/P3/98 NXA-6) collected from buffalo in northwestern Botswana (Nxaraga Lagoon, Lower Boro floodplain) were clustered with the SAT3 sequences from Zambia, direct testing of the buffalo-cattle transmission hypothesis requires further sampling of buffalo populations resident in Zambia.

The first confirmed serotype O outbreak in Zambia was in Mbala District of Northern Province in 1976 [14,38]. Since then, serotype O outbreaks have only been reported in this province in three instances (1982, 2000 and 2010). This study reports sequence data for further topotype O/EA-2 outbreaks in the Northern Province and the wider circulation of this topotype into other Zambian provinces. Although these sequences represent what was considered as two primary outbreaks, their close sequence identity demonstrates that they are intimately connected, supporting suspicions that animals had been moved illegally from Northern Zambia (Mbala) into Central Zambia (Chisamba, a previously FMD-free area) in March 2018. The precise transmission routes are not known, but the closure of the abattoir in the Mbala district of Northern Zambia may have incentivized stock owners to move their animals' southwards in search of market access. From the Chisamba district, the topotype O/EA-2 spread into other districts in Zambia where sequences have been recovered from cases in the Monze district of Southern Zambia after a disease quiescence of 11 months, when a herd of cattle were moved illegally from Chisamba [39]. FMD cases due to topotype O/EA-2 have been detected subsequently in Copperbelt and Western provinces, and further serotype O samples characterized by Ag-ELISA have been collected from Central, Lusaka and North-Western Provinces. From Monze (Southern Province), sequence data confirmed the spread of topotype O/EA-2 to Mazabuka, from where there were reports that FMD spread to the Kafue Flats, an area where animals congregate during the transhumance grazing, a common practice in Southern and Western provinces of Zambia [40,41]. There have been reports that O/EA-2 FMD infection in naïve animals is more severe with higher morbidity, a feature of these outbreaks that should be closely monitored if this topotype spreads to the other SADC states south of the Zambezi River.

The serotype SAT-2 FMDVs, identified in this study clustered within two topotypes (I and IV) (Figure 5). These two topotypes represent FMD viruses that circulate in two different endemic pools (Pool 4 for topotype IV and Pool 6 for topotype I, respectively), and these differences demonstrated that the SAT-2 outbreaks in Eastern and Northern Provinces were not epidemiologically linked. Eastern Province recorded only one previous FMD case in Egichikeni Veterinary Camp of Lundazi District in 2001 [22,42]. The FMD cases in 2019 were first reported from the Vubwi district, followed by Lundazi and lastly Chipata in May 2019. This Province shares borders with Malawi and Mozambique, and the close relationship between the outbreak viruses from Zambia and Malawi provides evidence for an epidemiological connection between FMD cases in these two countries. There is no natural barrier between the border areas of these countries, and it has been reported that livestock from the Malawian side are herded into Zambia, where they graze with Zambian livestock [43]. This outbreak was resolved following two rounds of vaccinations with a bivalent vaccine (serotypes SAT-2 and O) targeting the cattle population at risk.

For serotype A, the sequences recovered in this study represent two genetic clades within the A/AFRICA/G-I genotype, representing viruses collected from 2015 and 2018, respectively. These data suggest that there were two separate serotypes A introductions into northern Zambia from a neighbouring country in East Africa.

The data reported in this paper reflect the complex epidemiology of FMD in Zambia, where field outbreaks in domesticated species over five years have been due to four different serotypes (O, A, SAT-2 and SAT-3). The causes of these FMD outbreaks in

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Zambia are multifactorial, although uncontrolled livestock movement continues to play a very important role in spreading FMD viruses within the country [22,40,41]. During 2019, the potential for FMD transmission in Zambia was elevated as a consequence of a drought (November–March) [44], which resulted in a shortage of water and pasture for farmers, leading to the congregation of livestock by small ponds, a scenario which has been previously reported as contributing to the spread of infectious diseases such as FMD [21]. Furthermore, the lack of grazing pasture forced livestock keepers to sell their livestock in exchange for maize, thus facilitating livestock movement as animals moved from buyer to seller.

This paper also demonstrates the movement of FMDV serotypes and topotypes among different territories, and the importance of surveillance to rationally design vaccination campaigns in the different regions. Although presented late to assist curb the spread of FMD in Zambia, the data presented accentuates challenges faced in FMD control as previously outlined [12,15], with the time factor being predominant. Not reporting or late reporting of the disease leads to poor diagnostic ability, and furthermore makes the disease investigation devoid of the epidemiological information required for effective disease management. Increased stakeholder engagement is necessary, and should be encouraged during FMD outbreaks, as disease control proved effective with this strategy in some outbreak areas during this study. Secondly, the lack of adequate diagnostic facilities in most endemic countries like Zambia raises the argument that virus analysis in endemic settings must be locally matched with commensurate investment in FMD diagnostic infrastructure, including sample collection and processing. In addition, the use of rapid field diagnostic tests close to outbreak areas are necessary for phylogenetic analysis to have a real impact on curtailing circulating viruses during disease outbreaks. It is therefore proposed that the planned risk-based FMD control strategy for Zambia and the region takes into account the sound epidemiological assessment of the incidence and distribution of FMD provided in this paper. This includes identifying risk sources as either primary or secondary endemic eco-systems for the effective design of FMD control programs.

The introduction and circulation of novel FMD viruses in Zambia may have a bearing on the epidemiology of FMD in the country. Zambia represents an interface between endemic Pool 4 (East Africa) and Pool 6 (Southern Africa), and the spread of O/EA-2 into southern border areas in Zambia is of particular concern, as it could alter the epidemiological risks for the SADC region. Furthermore, the emergence of serotype O as a risk for SADC countries has impacted the selection, and costs associated with FMD vaccination since trivalent vaccines (containing only SAT-1, SAT-2, SAT-3 vaccine strains) are the most frequently used in the region.

5. Conclusions

This study describes the molecular epidemiology of FMD viruses collected from Zambia between 2015 and 2020 highlighting the recent spread of serotype O, A, SAT-2 and SAT-3 viruses in the country. These results reinforce the importance of synchronizing surveillance and disease control strategies where the emergence of new FMD virus serotypes and lineages impacts vaccine selection for the region to ensure that data is shared between countries to monitor this dynamic situation.

Author Contributions: F.B. and Y.S. contributed to the study conception and design. F.B., L.M., T.S., H.L.K., E.M.F. and M.M. performed material preparation and data collection. N.J.K., J.W., D.P.K. and F.B. performed data analysis. F.B. wrote the first draft of the manuscript, and all authors have commented and revised previous versions of the manuscript. Supervision, M.Q. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: No ethical approval was sought for this study, as the animal sample collected were part of standard diagnostic protocols for disease control in Zambia.

Data Availability Statement: All nucleotide sequences generated during this study have been submitted to GenBank and have been assigned the following accession numbers: MK422556-MK422569 and MK422575-MK422608.

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Conflicts of Interest: The authors declare no conflict of interest.

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7.4. Article 2

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SHORT COMMUNICATION

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The first detection of a serotype O foot-and-mouth disease virus in Namibia

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Abstract

This report describes the molecular characterization of a serotype O foot-and-mouth disease virus (FMDV) recovered from a field outbreak in the Zambezi region, Namibia during July 2021. Sequence analysis demonstrates that this FMDV belongs to the O/EA-2 topotype sharing closest nucleotide identity (99.5%) to FMD viruses collected since 2018 in Zambia. This is the first detection of serotype O in Namibia, and together with the cases that have been recently detected in southern Zambia, represent the first time that this serotype has been detected in the Southern African FMD endemic pool since 2000, when a virus of Asian origin (O/ME-SA/PanAsia) caused an outbreak in South Africa. This incursion poses a new threat for the region and the potential onward spread of O/EA-2 will now need to be closely monitored since serotype O vaccines are not widely used in Namibia, nor in neighbouring countries.

KEYWORDS
foot-and-mouth disease, serotype O, Southern Africa, transboundary

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1 | INTRODUCTION

Foot-and-mouth disease (FMD) is caused by a highly contagious virus (FMDV: family *Picornaviridae*, genus: *Aphthovirus*) that infects cloven-hooved animals. The disease circulates in Asia, Africa and parts of South America where outbreaks lead to livestock production losses and disruption to international trade (Knight-Jones & Rushton, 2013). In sub-Saharan Africa, FMD is caused by five different serotypes of FMDV: O, A, Southern African Territories (SAT) 1, SAT 2 and SAT 3 (Casey et al., 2014). Southern Africa comprises FMD endemic pool 6 (Paton et al., 2009), which encompasses Namibia, Botswana, Zimbabwe, Malawi, Mozambique and South Africa where FMD outbreaks reported during the last 20 years have been restricted to those caused by the three SAT serotype viruses (Maree et al., 2014). Zambia, although belonging to this endemic pool, represents a special case as it forms an interface between the Eastern African and Southern Africa endemic pools (Pool 4 and Pool 6, respectively). Prior to 2018, outbreaks due to serotype O were restricted to the Northern Province of Zambia (most recently detected in 2010) where there was close genetic relationship with viral sequences found in other countries in Pool 4. However, since 2018 this serotype has been detected in a number of central and southern Zambian provinces representing a new incursion of serotype O into the Southern African Pool (Banda et al., 2021).

Namibia maintains a 'FMD-free without vaccination' zone officially recognized by the World Organisation for Animal Health (OIE), located to the south of the Veterinary Cordon Fence (VCF). The VCF initially established in 1897 to protect farms in the south of the country from rinderpest, now provides a barrier to protect the high-value commercial export markets in southern Namibia from FMD that circulates in the eastern Zambezi region (communal grazing land) in the northern part of the country. The Northern Communal Areas of Namibia have no official OIE status for FMD. FMD outbreaks detected recently in the north of Namibia include continued cases due to serotype SAT 2 (topotype III) in 2021 as well as outbreaks due to serotype SAT 3 (topotype II) in 2019 (https://www.wrlfmd.org/southern-africa/namibia#panel-4082).

Here, we describe the detection of serotype O in cattle in Namibia which represents the first occurrence of this serotype in the country. New genetically related FMDV sequences for samples collected in Zambia since 2018 are also included in this analysis to help understand the epidemiological connectivity in the region. These cases represent new threats with a potential for onward spread of this lineage to neighbouring countries where serotype O is not present.

2 | MATERIALS AND METHODS

This report describes new FMD viral sequence data for samples collected from Namibia (2021) and Zambia (2018–21).

FMD cases in Namibia were detected in Zambezi Region of Namibia during June–July 2021. The initial report (on 1 June 2021) involved suspect FMD cases in cattle herd of 144 animals (in Katima Rural Constituency (geo-coordinates: −17.7816, 24.3347) where 100 (69.4%) of the animals showed clinical signs of FMD including lameness, salivation and frothing of the mouth, blisters in the mouth and hooves. Further suspicion of FMD was subsequently raised in cattle (on 13 July 2021) at Nfooma located in Katima Mulilo Urban (geo-coordinates: −17.5000, 24.2666) where a total of 70 out of 87 (80.5%) animals exhibited clinical signs indicative of FMD. Vesicular epithelium samples ($n = 7$) collected on 15 July 2021 from Nfooma were submitted to the OIE Sub-Saharan Africa Regional Reference Laboratory (SSARRL) for FMD (Botswana Vaccine Institute, Gaborone, Botswana) where FMDV diagnosis was carried out as previously described (Teye et al., 2019). The samples were thawed at room temperature, and then blotted dry on absorbent paper. Briefly, a 10 % suspension was prepared by grinding 1 g of the sample in a sterile mortar and pestle with a small volume of tissue culture medium (10% Minimum Essential Medium (MEM 10×), 10% lactalbumin hydrolysate, 4.5% sodium bicarbonate, 1% calf serum, 0.2% penicillin and sterile distilled water). The suspension was clarified by centrifugation at 2000 *g* for 10 min at 4°C, and 500 μl of the suspension was inoculated onto primary lamb kidney cell cultures in 25 cm^2 culture flasks and incubated for 1 h at 37°C. Fresh cell culture medium (15 ml) was then added and incubated at 37°C and monitored for cytopathic effect (CPE) for 48 h. Samples were considered negative for virus isolation if no CPE was observed after the third passage. An in-house sandwich ELISA using serotype-specific rabbit and guinea pig antisera for FMDV serotypes O, A, SAT 1, SAT 2 and SAT 3 (Pirbright Institute, UK; Ferris & Dawson, 1988) was used to detect FMDV antigens and to define the serotype of the CPE positive samples. FMDV sequences encoding VP1 were generated for serotype O positive samples using a published RT-PCR amplification and sequencing method (Knowles et al., 2016). Briefly, the RT-PCR amplification used the following primers: O-1C244F 5′-GCA GCA AAA CAC ATG TCA AAC ACC TT-3′, and EUR−2B52R_5′-GAC ATG TCC TCC TGC ATC TGG TTG AT-3′ and cycle-sequencing adopted O-1C244F and NK72 5′-GAA GGG CCC AGG GTT GGA CTC-3′.

The 1D nucleotide sequences encoding VP1 (633 nucleotides) were compared with other related serotype O viruses collected from the region. In addition to published sequences, the comparative data included unpublished sequences recently obtained at the FAO World Reference Laboratory for FMD (WRLFMD, Pirbright, UK) for 33 samples received from Zambia. Collection locations and dates for these samples are outlined in Table 1. At the WRLFMD, field isolates were generated in bovine thyroid primary cell cultures and typed using Ag-ELISA (Ferris & Dawson, 1988). VP1 encoding regions were amplified using two separate RT-PCR reactions with primers O-1C244F and EUR-2B52R as previously described (Banda et al., 2021; Knowles et al., 2016), or with FMD-3161F and FMD-4303R (Dill et al., 2017). Cycle sequencing adopted the same primers in separate reactions (Knowles et al., 2016). New sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers OM259982–OM260015 (Table 1).

The complete VP1 encoding nucleotide sequences were aligned using BioEdit 7.0.5.3 (Hall, 1999) and Clustal W 1.83 (Thompson et al., 1994). To construct a maximum likelihood (ML) phylogeny (Nei & Kumar, 2000), the data set was tested for 24 common nucleotide

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TABLE 1 Sample details of O/EA-2 viruses collected recently from Zambia and Namibia (2018–2021)

Virus name	GenBank number	Date collected	Sampling location	Province/region	Country
O/ZAM/1/2018	MZ486060	24/03/2018	Chisamba	Central	Zambia
O/ZAM/2/2018	MZ486061	24/03/2018	Chisamba	Central	Zambia
O/ZAM/3/2018	MZ486062	02/04/2018	Chisamba	Central	Zambia
O/ZAM/1/2019	MZ486065	18/01/2019	Chisamba	Central	Zambia
O/ZAM/2/2019	MZ486066	11/02/2019	Ufwenuka, Monze	Southern	Zambia
O/ZAM/3/2019	MZ486067	11/02/2019	Ufwenuka, Monze	Southern	Zambia
O/ZAM/4/2019	MZ486068	11/03/2019	Magoye, Mazabuka	Southern	Zambia
O/ZAM/7/2019	MZ486069	30/03/2019	Kasako, Mazabuka	Southern	Zambia
O/ZAM/8/2019	MZ486070	30/03/2019	Kasako, Mazabuka	Southern	Zambia
O/ZAM/15/2019	OM259982[b]	31/05/2019	Namwala	Southern	Zambia
O/ZAM/16/2019	OM259983[b]	31/05/2019	Namwala	Southern	Zambia
O/ZAM/18/2019	OM259984[b]	05/06/2019	Monze	Southern	Zambia
O/ZAM07/2019[a]	MZ486072	28/06/2019	Musenga, Chingola	Copperbelt	Zambia
O/ZAM/20/2019	OM259985[b]	28/06/2019	Makeni	Lusaka	Zambia
O/ZAM/21/2019	OM259986[b]	28/06/2019	Makeni	Lusaka	Zambia
O/ZAM/24/2019	OM259987[b]	17/07/2019	Kafue	Lusaka	Zambia
O/ZAM/26/2019	OM259988[b]	17/07/2019	Chibombo	Central	Zambia
O/ZAM/27/2019	OM259989[b]	17/07/2019	Chibombo	Central	Zambia
O/ZAM/28/2019	OM259990[b]	07/08/2019	Chikankata	Southern	Zambia
O/ZAM/54/2019	OM259991[b]	01/09/2019	Chikankata	Southern	Zambia
O/ZAM/55/2019	OM259992[b]	06/09/2019	Kabwe	Central	Zambia
O/ZAM/56/2019	OM259993[b]	06/09/2019	Kabwe	Central	Zambia
O/ZAM/57/2019	OM259994[b]	01/10/2019	Mpande	Northern	Zambia
O/ZAM/58/2019	OM259995[b]	01/10/2019	Sinazongwe	Southern	Zambia
O/ZAM/60/2019	OM259996[b]	17/10/2019	Kasempa	North-western	Zambia
O/ZAM/62/2019	OM259997[b]	01/12/2019	Itezi tezi	Central	Zambia
O/ZAM/1/2020	OM259998[b]	05/02/2020	Chilanga	Lusaka	Zambia
O/ZAM/2/2020	OM259999[b]	05/02/2020	Chilanga	Lusaka	Zambia
O/ZAM/3/2020	OM260000[b]	16/03/2020	Namwala	Southern	Zambia
O/ZAM/4/2020	OM260001[b]	16/03/2020	Namwala	Southern	Zambia
O/ZAM/6/2020	OM260002[b]	15/05/2020	Kalomo	Southern	Zambia
O/ZAM/7/2020	OM260003[b]	15/05/2020	Kalomo	Southern	Zambia
O/ZAM/8/2020	OM260004[b]	10/08/2020	Kazungula	Southern	Zambia
O/ZAM/9/2020	OM260005[b]	10/08/2020	Kazungula	Southern	Zambia
O/ZAM/10/2020	OM260006[b]	10/08/2020	Kazungula	Southern	Zambia
O/ZAM/11/2020	OM260007[b]	18/08/2020	Makeni	Lusaka	Zambia
O/ZAM/12/2020	OM260008[b]	18/08/2020	Makeni	Lusaka	Zambia
O/ZAM/14/2020	OM260009[b]	28/10/2020	Nkeyema	Western	Zambia
O/ZAM/15/2020	OM260010[b]	28/10/2020	Nkeyema	Western	Zambia
O/ZAM/16/2020	OM260011[b]	28/10/2020	Nkeyema	Western	Zambia
O/ZAM/7/2021	OM260012[b]	18/03/2021	Kalomo	Southern	Zambia
O/ZAM/9/2021	OM260013[b]	08/05/2021	Mwandi	Western	Zambia
O/ZAM/11/2021	OM260014[b]	12/05/2021	Mwandi	Western	Zambia
O/NAM10/2021[a]		15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia
O/NAM11/2021[a]		15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia
O/NAM13/2021[a]	OM260015[b]	15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia
O/NAM14/2021[a]		15/07/2021	Nfooma, Katima Mulilo	Zambezi	Namibia

NB: All samples were collected from cattle except ZAM/1/2020, ZAM/2/2020 and ZAM/18/2019 which were from pigs.
[a]Not a WRLFMD isolate number.
[b]New sequence data presented in this report.

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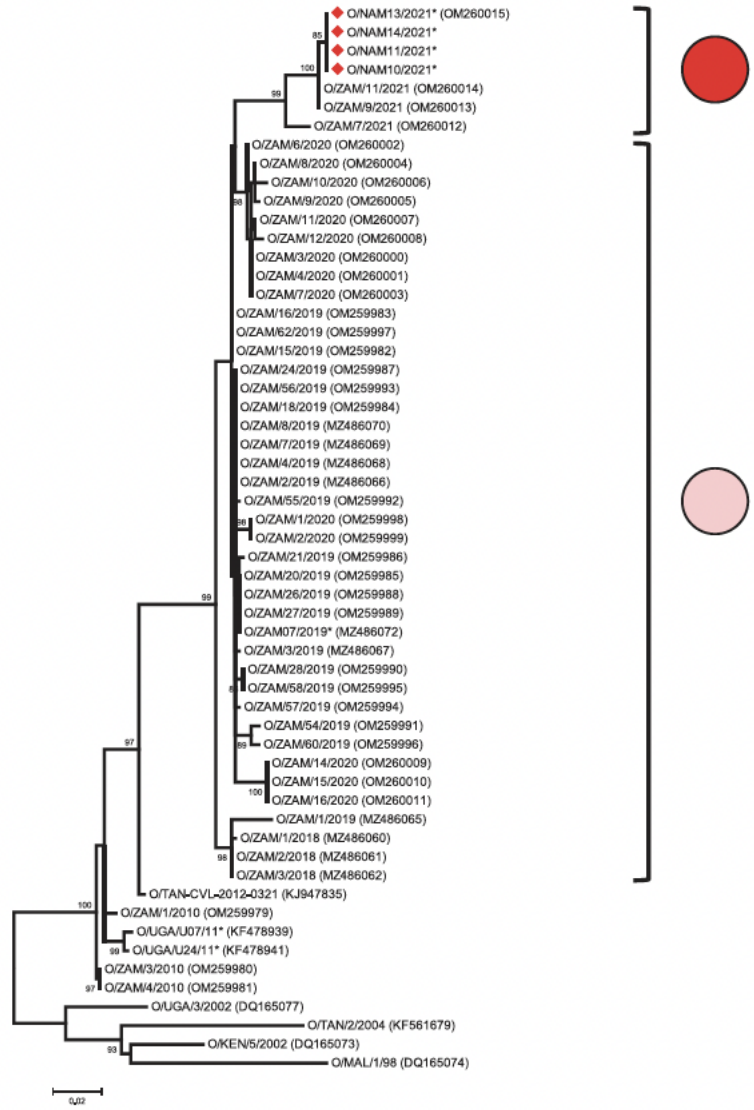


FIGURE 1 A midpoint-rooted maximum likelihood tree highlighting the monophyletic relationship of O/EA-2 FMD viruses collected from Zambia and Namibia during 2018–2021. Red diamonds highlight FMDV sequences from Namibia and coloured dots represent data for samples that were sequenced during 2021 (●) and 2018–2020 (○). Bootstrap values of 70% and above are shown

substitution models using MEGA 7 (Kumar et al., 2016). The model with the lowest Bayesian information criterion (BIC) score (Tamura 3-parameter with gamma distribution; Tamura, 1992) was chosen to construct the tree. The robustness of the tree topology was assessed with 1000 bootstrap pseudo-replicates.

3 | RESULTS AND DISCUSSION

Testing at BVI, Botswana, yielded FMDV positive results for five out of seven of the Namibian samples using virus isolation which were all characterized as serotype O using antigen ELISA. The

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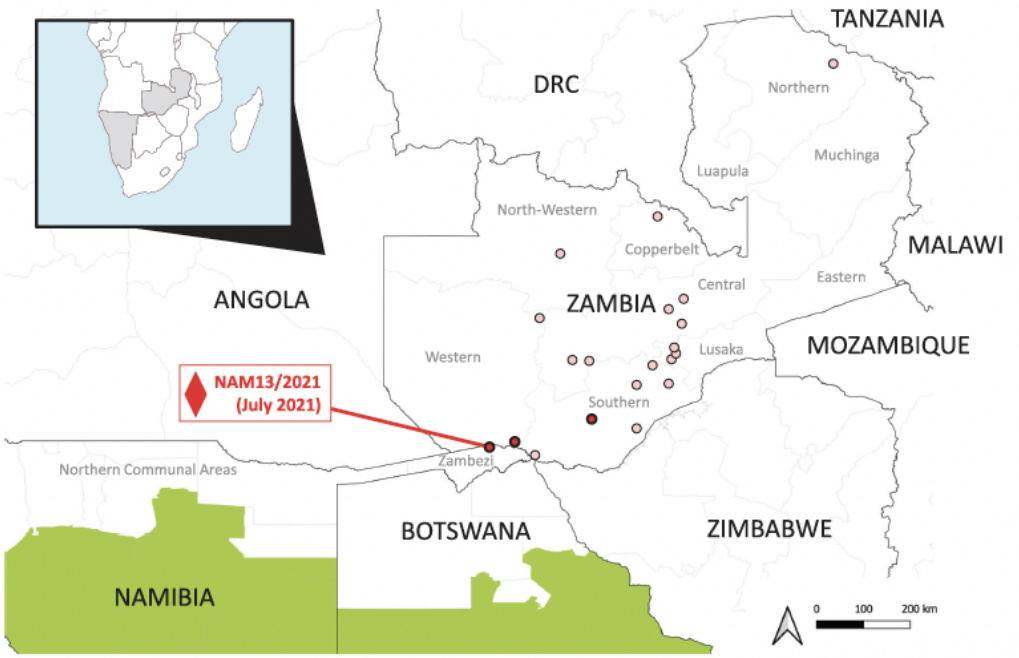


FIGURE 2 FMD outbreaks caused by the O/EA-2 topotype that have occurred in Southern African countries since 2018. Locations represent outbreaks from which FMD viruses were sequenced during 2021 (●) and 2018–2020 (○), where (♦) denotes the location of the FMD report in Zambezi Region, Namibia. NB: All FMD outbreaks due to the O/EA-2 topotype that have been detected in Zambia since 2018 are plotted on the map, although multiple samples and sequences have been collected at some of the locations. Namibia and Botswana maintain FMD-free zones (without vaccination) which are highlighted in green

sequences encoding VP1 of four selected virus isolates from Namibia (O/NAM10/2021, O/NAM11/2021, O/NAM13/2021 and O/NAM14/2021) were identical to each other and were categorized as belonging to the EA-2 topotype within serotype O. This analysis showed that the Namibian sequences shared closest nucleotide identity (99.5%) to two FMDV isolates that had been collected from the Western Province of Zambia during 2021 (O/ZAM/9/2021 and O/ZAM/11/2021; Figure 1).

The O/EA-2 topotype originates from East Africa (FMD endemic Pool 4; Paton et al., 2009) where it has been detected in countries such as Burundi, Democratic Republic of the Congo, Kenya, Tanzania and Uganda (Kasanga et al., 2015; Kerfua et al., 2019; Namatovu et al., 2015; Wekesa et al., 2015). The O/EA-2 topotype has also previously appeared transiently into the northern parts of some Southern African countries such as Malawi where an isolate collected in 1998 shared a close genetic relationship to FMD viruses from Tanzania (Kasanga et al., 2015). The first confirmed serotype O outbreak in Zambia was in Mbala District of Northern Province in 1976 (Perry & Hedger, 1984). Prior to 2018, serotype O outbreaks in Zambia were restricted to the Northern Province where they were recorded on three occasions (1982, 2000 and 2010). Sequence data for VP1 encoding sequences for O/EA-2 viruses collected in 2010 close to the border with Tanzania are shown in Figure 1 (O/ZAM1/2010, O/ZAM/3/2010 and O/ZAM/4/2010; Banda et al., 2021).

Since 2018, O/EA-2 viruses within a new genetic clade have spread across Zambia to cause outbreaks in Central, Copperbelt, Lusaka, North-Western, Southern and Western Provinces (Figure 2; Banda et al., 2021). The precise transmission pathways for the introduction of O/EA-2 into central, southern and western parts of Zambia are not completely understood, but it has been previously suggested that the closure of an abattoir in Mbala District of the Northern Province may have incentivized stock owners to move animal southwards in search of market access (Banda et al., 2021).

Phylogenetic analyses (Figure 1) highlight the transboundary connectivity between Zambia and Namibia and understanding epidemiological connections that underpin the recent spread of the O/EA-2 topotype in Zambia and Namibia is a priority for new studies. By themselves, these sequence analyses cannot reconstruct the specific epidemiological routes by which O/EA-2 has been introduced into Namibia; however, the Katima Mulilo Bridge which was built in 2004 to carry the Trans-Caprivi-Highway (Walvis Bay-Ndola-Lubumbashi Development Road) across the Zambezi River could provide an obvious pathway. Across the border in Zambia, the proximity to international border crossing points and major roads were identified in a previous

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study as risk factors for FMD outbreaks (Hamoonga et al., 2014). Further work is now required to quantify the risks associated with these transboundary animal movements and to develop controls that reduce the likelihood of further FMD incursions into the region.

The detection of O/EA-2 in the Zambezi Region located in the Caprivi Strip in north-east Namibia represents the first time that serotype O has been detected in the country. This incursion now poses a new threat to the FMD endemic region of Namibia and other countries in the region, such as Angola, Botswana and Zimbabwe. Botswana also maintains an OIE official FMD-free without vaccination zone in the south, but like Namibia has no OIE status for the northern part of the country. The introduction of a new serotype or an antigenically novel variant can pose challenges for regional FMD control initiatives as was recently documented by the rapid spread of the A/ASIA/G-VII lineage in Asia (Bachanek-Bankowska et al., 2018). In FMD endemic regions of Southern Africa, onward transmission of O/EA-2 may go unnoticed due to the significant under-reporting of the disease. In these areas, serotype O-specific immunity is likely to be very low since FMD vaccines, where these are used in Southern Africa countries, do not typically contain a serotype O component.

In summary, these findings highlight a new FMD threat for countries in Southern Africa and motivate field teams and laboratory scientists to increase surveillance within the region to rapidly detect further outbreaks due to this topotype. Now that this serotype has been identified, new resources will be required to ensure that laboratories in the region can correctly identify new clinical cases due to serotype O as well as detect serotype O-specific antibodies in sera collected as part of surveillance activities. Furthermore, FMD vaccines selected for use in the region should now consider these risks, and vaccination campaigns may need to absorb the extra costs of adding a serotype O antigen component to the vaccines that are deployed.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/nuccore, reference numbers OM259982–OM260015.

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7.5. Article 3

vaccines

MDPI

Brief Report

The Immunogenicity of a Foot-and-Mouth Disease Virus Serotype O Vaccine in Commercial and Subsistence Cattle Herds in Zambia

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Abstract: The recent introduction of foot-and-mouth disease (FMD) virus serotype O (O/EA-2 topotype) in Southern Africa has changed the epidemiology of the disease and vaccine requirements of the region. Commercial and subsistence cattle herds in Zambia were vaccinated with an FMD virus serotype O Manisa vaccine according to a double- or single-dose vaccination schedule. Heterologous antibody responses induced by this vaccine against a representative O/EA-2 virus from Zambia were determined. Virus neutralisation tests (VNTs) showed double-dosed cattle had a mean reciprocal log virus neutralisation titre of 2.02 (standard error [SE] = 0.16, n = 9) for commercial herds and 1.65 (SE = 0.17, n = 5) for subsistence herds 56 days after the first vaccination (dpv). Significantly lower mean titres were observed for single-dosed commercial herds (0.90, SE = 0.08, n = 9) and subsistence herds (1.15, SE = 0.18, n = 3) 56 dpv. A comparison of these results and those generated by solid-phase competitive ELISA (SPCE) tests showed a statistically significant positive correlation by Cohen's kappa coefficient. Therefore, SPCE might be used in assessing the immunogenicity of vaccines in place of VNT. Furthermore, for this vaccine and field strain, a vaccination regime employing a two-dose primary course and revaccination after 4–6 months is likely to be appropriate.

Keywords: foot-and-mouth disease; immunogenicity; vaccine; field evaluation

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1. Introduction

Foot-and-mouth disease (FMD) is one of the most economically important infectious diseases of livestock in the Southern African Development Community (SADC) due to its impact on livestock productivity and resulting international trade restrictions on live animals and livestock products. The causative agent, FMD virus (FMDV), is contagious and antigenically diverse, with six currently circulating serotypes that do not cross-protect. This role of virus diversity in the epidemiological dynamics of FMD in Africa has been described [1].

There has been an escalation in FMD outbreaks in the past decade in southern Africa [2] with Zambia and Namibia reporting novel serotype O incursions due to the O/EA-2 topotype [3]. Before 2018, FMD was confined to three FMD high-risk areas of Zambia [4–6], but, in 2018, an FMDV serotype O outbreak was reported in the Chisamba District of Central Zambia [3]. This disease spread to all the provinces in Zambia except the Luapula Province.

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A subsequent spillover of this outbreak to the Zambezi Region of Namibia during June–July 2021 was also recorded [3]. Further spread of this lineage into Malawi and northern Mozambique was reported in 2022 [7,8].

Vaccination campaigns together with stringent control measures have been used to eradicate FMD from Europe and most of South America [9,10]; in endemic regions, vaccination, especially with good quality FMD vaccines, is an important means of FMD control and can help prevent losses in stock production and reduce the overall incidence of the disease [11]. Post-vaccination monitoring (PVM) guidelines have been published to advise countries on principles and suggested procedures for monitoring various aspects of FMD vaccination [12]. In Southern Africa, FMD vaccination has targeted livestock mainly for prophylaxis and to prevent the spread of the disease using trivalent vaccines containing Southern African Territories serotypes (SAT-1, SAT-2, and SAT-3) antigens [13]. The introduction of serotype O into the region means that new vaccines are needed to control outbreaks due to this serotype. As there are no empirical data available to show the appropriateness of serotype O vaccines in Zambia, this study was conducted using the principles of the PVM guidelines to assess the immunogenicity of an imported serotype O vaccine in Zambia.

The specific objectives of this study were to evaluate FMDV-specific antibody responses after a single dose and a second booster dose in vaccinated cattle and to compare results obtained by the virus neutralisation test (VNT) and a commercial solid-phase competitive ELISA kit (SPCE).

2. Materials and Methods

2.1. Study Design

Field studies were conducted in commercial cattle (Study A) and in subsistence cattle (Study B) to assess the immunogenicity of an imported serotype O vaccine using either a one- or two-dose primary vaccination protocol. Study A animals were Friesian cattle from a commercial dairy farm in the Momboshi area of the Chisamba District, Zambia, whilst Study B animals were mixed breeds in herds from different subsistence farmers in multiple sublocations in the Rufunsa District, Zambia. There was no previous history of any FMD vaccination in either of these areas, and there was no history of FMD outbreaks in the previous two years. Selected animals were six to twelve months old at vaccination and included both males and females. All animals were individually identified by ear tags to ensure accurate follow-up. Consent was obtained from the farmers to use their animals in the study and owners were advised not to move their animals out of the farms during the study. This project was approved under research ethics committee number REC 061-19 from the University of Pretoria, South Africa.

The protocol followed the Food and Agriculture Organization and World Organisation for Animal Health (FAO-WOAH) PVM guidelines Section 3.3 [12]. In Study A, 69 animals were recruited, including ten unvaccinated controls to act as sentinels for FMDV exposure, whilst in Study B, 55 animals were recruited, including five unvaccinated controls. Sera from 32 cattle were randomly selected and tested for antibodies to FMDV for each of the five time points (0–168 days after the first vaccination; dpv).

Of the vaccinated animals in both studies, ten were given a second dose 28 days post-vaccination (dpv). A second dose is recommended by the vaccine manufacturer and previous research on immunologically naïve animals (i.e., with no previous FMDV exposure or vaccination) [14]. Blood samples were collected in plain Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) at first vaccination (0 dpv), 28 dpv (time of second vaccination), 56 dpv, 112 dpv, and 168 dpv.

Not all samples were tested for the presence of FMDV antibodies because the budget for this study was limited. From Chisamba, samples from nine single-dose and nine double-dose cattle were tested as well as samples from four unvaccinated controls. From Rufunsa, samples from three single-dose, five double-dose, and two unvaccinated control cattle were tested.

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2.2. Vaccine

The animals were vaccinated with a monovalent FMDV serotype O Manisa vaccine of at least three PD_{50}, containing purified inactivated FMDV antigen and aluminium hydroxide with saponin as an adjuvant, obtained from the Botswana Vaccine Institute. This aqueous adjuvanted vaccine is indicated for use in cattle, buffalo, sheep, and goats. Each dose (1 mL) was given according to the recommendations from the vaccine manufacturer. In a primary course of vaccination, two injections three to four weeks apart are recommended, whilst a booster dose is advisable every four to six months depending on the risk and following local practice.

2.3. Serological Testing

All samples were shipped on ice to the Central Veterinary Research Institute (CVRI), Zambia, and, on arrival, sera were separated and then stored at −20 °C until testing. All samples were tested with an FMDV non-structural protein (NSP) ELISA kit (IDEXX, Westbrook, MN, USA) according to the manufacturer's instructions at the CVRI to evaluate if natural exposure to FMDV had occurred during the study period. Antibodies to viral NSPs are considered a reliable indicator of evidence of previous or current viral replication in the host, irrespective of vaccination status [15–17]. Test results for the NSP ELISA are expressed as a percentage positivity relative to the strong positive control [(optical density of test or control wells/optical density of strong positive control) × 100] [18]. Values < 20% are considered negative, values ≥ 20% and <30% are considered suspect, and values ≥ 30% are considered positive.

Samples were also tested using a solid phase competitive ELISA (SPCE) or antibodies specific to FMDV serotype O structural proteins (IZSLER Biotechnology Laboratory, Brescia, Italy). This test detects antibodies elicited by vaccination and natural infection. A percentage inhibition for this test is calculated for each well (100 − [optical density of each test or control value/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and a specific murine monoclonal antibody for the FMDV antigen on the ELISA plate. Using the semi-quantitative method applied in this test, sera are considered positive when there is inhibition ≥ 70% and negative when inhibition is <70% at 1:10 dilution. A second dilution (1:30) of strongly positive sera samples (≥80% inhibition at 1:10 dilution) indicates the level of antibodies with strongly positive sera showing ≥80% inhibition at both 1:10 and 1:30 dilutions.

Samples were shipped on dry ice to the FAO World Reference Laboratory for FMD (WRLFMD), Pirbright, UK, for testing by VNT, as described previously [15,19]. A representative Zambian field isolate from the O/EA-2 topotype (O/ZAM/7/2021) was used in the VNT to derive field strain-specific (heterologous) titres. Earlier comparison of the neutralisation of O Manisa and O/ZAM/7/2021 viruses by O Manisa post-vaccination sera had shown an antigenic similarity score (r_1 matching value) of 0.47, where 1.0 is a perfect match and values greater than or equal to 0.3 are considered indicative of an acceptable match for field vaccination [20]. The post-vaccination VNT titres to heterologous viruses associated with post-vaccination cross-protection have been evaluated. Using VNT, an indicator of likely heterologous cross-protection is considered to be a $\log_{10}$ reciprocal titre of 1.5 (1:32) after a single-dose vaccination with serum collected 21 days later [21,22].

2.4. Data Analysis

Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and SPSS Statistics v29 (IBM, Armonk, NY, USA) were used to analyse the data.

3. Results

During the study, one animal died in Study A, and five cattle died or went missing in Study B. The animal that died in Study A belonged to the unvaccinated control group. From Study B, deaths were observed at different time intervals during the study, with one animal dying from each group, i.e., unvaccinated controls, single dosed, and double dosed.

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Their deaths were attributed to East Coast fever, a tick-borne disease which is prevalent in some areas due to poor dipping practices, especially by farmers from Study B. This was supported by pathological signs which included fever, enlarged lymph nodes, anorexia, laboured breathing, corneal opacity, nasal discharge, diarrhoea, and anaemia. The two missing animals could have been sold by the farmers, as in this sector, animals serve as a source of income, food, and social security [23].

Although three cattle tested weakly positive for NSP antibodies at single time points in the study all animals eventually tested negative for NSP antibodies, and there was no evidence of clinical FMD in the study areas.

There were differences in the VNT titres between cattle receiving one and two doses of vaccine (Table S1 and Figure 1). The mean logs of the reciprocal VNT titres 56 dpv were 1.15 (standard error of the mean [SE] = 0.18, n = 3) and 1.65 (SE = 0.17, n = 5) for subsistence cattle receiving 1 and 2 doses of vaccine, respectively, and 0.90 (SE = 0.08, n = 9) and 2.02 (SE = 0.16, n = 9) for commercial cattle receiving 1 and 2 doses of vaccine, respectively. The mean titre obtained from the samples of unvaccinated animals was 1.01 $\log_{10}$ (range 0.6–1.2). Only one subsistence animal (out of three) and no commercial animals (out of nine) that were vaccinated once developed VNT titres by 56 dpv above the threshold value of 1.5 to be considered protective, but three subsistence animals (out of five) and seven commercial animals (out of nine) that were vaccinated twice developed VNT titres by 56 dpv to be considered protective.

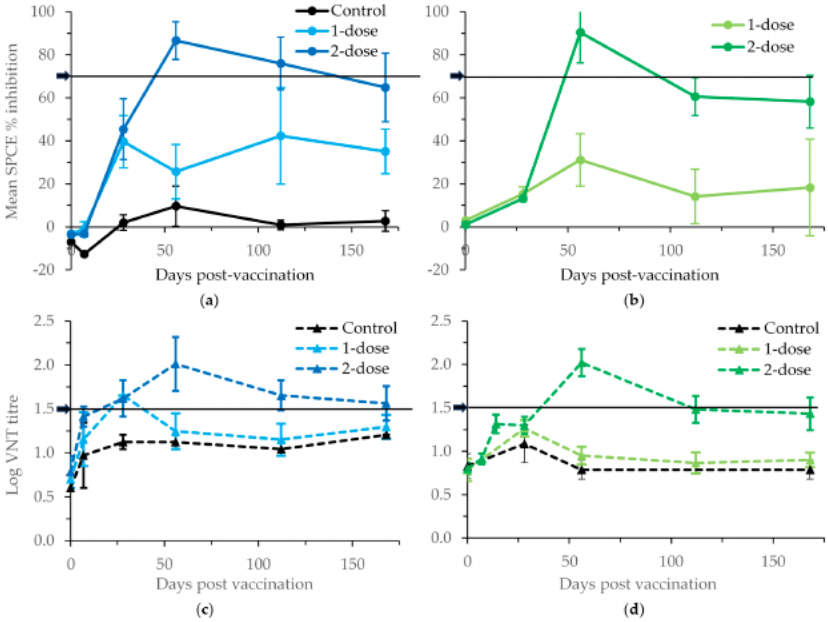


Figure 1. The mean solid-phase competition ELISA (SPCE) % inhibition at 1:10 serum dilution (**a**,**b**) and virus neutralisation test (VNT) titres (**c**,**d**) in subsistence (**a**,**c**) and commercial (**b**,**d**) cattle herds vaccinated against FMDV serotype O at day 0 only (1-dose) or again 28 days after the first dose (2-dose). Control animals were not vaccinated. Values greater than the threshold values indicated on the *y*-axis by arrows are considered protective.

The pattern of structural proteins binding antibody responses largely mirrored those for neutralising antibodies. Peak responses in double-dose cattle were detected 56 days

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after the first vaccination. The mean SPCE percentage inhibitions 56 dpv were 25.7 (SE = 12.6, n = 3) and 86.6 (SE = 8.8, n = 5) for cattle from Rufunsa receiving 1 and 2 doses of vaccine, respectively, and 14.1 (SE = 5.6, n = 9) and 60.6 (SE = 8.6, n = 9) for cattle from Chisamba receiving 1 and 2 doses of vaccine, respectively. None of the animals from Rufunsa (out of three) and only one animal from Chisamba (out of nine) that were vaccinated once developed antibodies by 56 dpv above the threshold value of 70% inhibition to be considered positive, but four animals from Rufunsa (out of five) and all animals from Chisamba (out of nine) that were vaccinated twice developed antibodies by 56 dpv to be considered positive (Table S1, Figure 1). A one-sided independent samples *t*-test where equal variance was assumed showed a significant difference in antibodies 56 dpv between cattle given one and two doses of vaccine (Rufunsa: $p = 0.003$; Chisamba: $p < 0.001$).

A Spearman's rank-order correlation determined the relationship between the log of the reciprocal of the virus neutralisation titres and the SPCE % inhibition results (Figure 2). There was a positive correlation between the VNT and SPCE results, which was statistically significant ($\rho = 0.598$ for 1:10 dilution, and $\rho = 0.562$ for 1:30 dilution of SPCE % inhibition results, $p < 0.001$ for both dilutions).

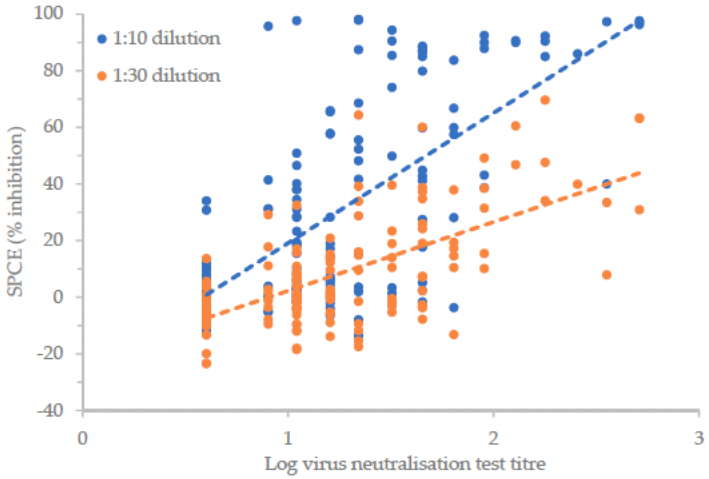


Figure 2. Positive correlation between $\log_{10}$ virus neutralisation titres and 1:10 (blue, $R^2 = 0.446$) and 1:30 (orange, $R^2 = 0.426$) solid-phase competition ELISA (SPCE) % inhibition in cattle vaccinated against FMDV serotype O.

Statistical analysis by Cohen's kappa coefficient showed a moderate agreement [24,25] between the two assays ($\kappa = 0.516$, 95% CI, 0.365 to 0.667, $p < 0.001$) using positive titre thresholds of ≥1:32 for the VNT and ≥70% inhibition for the SPCE.

4. Discussion

In this study, the immunogenicity of an imported FMD vaccine was evaluated to determine its suitability for use in Zambia and other Southern African countries where serotype O has been described as becoming important [26]. The evaluated vaccine is already widely used in sub-Saharan Africa and was an obvious candidate to control the FMDV serotype O outbreaks reported in the Southern African region. FMDV-specific antibody responses were measured after one- or two-dose primary courses of the vaccine. The study also compared results obtained by the VNT and a commercial SPCE kit to evaluate the use of simple-to-use tests to support PVM studies at a population level in settings where high-containment facilities are not available.

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The performance of an FMD vaccine can be assessed in vaccination-challenge studies according to the protocols defined in the WOAH Terrestrial Manual [15]. However, due to several reasons including ethical considerations and costs, in vitro studies are recommended [15]. The relationship between serology and protection in FMD-vaccinated cattle has been previously studied by correlating the antibody titres at the point of challenge with the outcome, i.e., protected or not protected [21,27,28]. A correlation between neutralising antibody titre and protection against homologous challenge is well established [29,30]. The testing and analysis of day-of-challenge sera from vaccination- and challenge-cross-protection studies have long established an association between in vitro neutralising antibody titres to the challenge viruses and in vivo clinical cross-protection [27]. Cross-strain protection between different vaccines and challenge viruses also have similar relationships, although much less data are available from these studies [21,27]. A recommended simple approach to assess cross-protection is to measure the amount of antibodies that vaccinated animals have against the field virus of concern [27]. It is against this background that this study examined day-of-challenge antibody titres to a heterologous challenge virus.

Apart from the three weakly positive samples observed at one time point, neither seroconversion to FMDV NSP nor clinical signs were observed throughout this study, suggesting the FMDV-specific antibody titres detected were vaccine-induced. The observed mean reciprocal log virus neutralisation titre of 2.02 for commercial cattle from Chisamba and 1.65 for subsistence cattle from Rufunsa in second-dosed cattle (Table S1 and Figure 1) imply that the aqueous adjuvanted vaccine used in this study produced neutralisation titres likely to offer protection against the target lineages. However, the observed mean titres from single-dosed cattle from Chisamba (0.90) and Rufunsa (1.15) 56 days after the first vaccination suggest that the vaccine given in this way was not able to reach the recommended protective titres as described [21,22]. This emphasises the value of the second vaccine dose in this context, but its importance is likely to vary according to the potency of the vaccine and its antigenic match to the strain against which protection is required. After the primary course of vaccination, the manufacturer of this vaccine recommends a booster at 4–6 months, which also seems in line with the results obtained here on the duration of the antibody responses found via VNT and ELISA. Apart from these general trends, care must be taken not to overinterpret the findings, as predicting protection from antibody titres cannot be carried out with precision due to several uncontrollable variables [21]. Other studies [30] have also shown that antibodies alone are not responsible for protection but are a correlate of the immune response in the animal.

Previous studies have correlated potency test outcomes with pre-challenge antibody titres measured using the VNT and LPBE [29,31]. More recently, commercially available SPCEs have become alternatives to the LPBE for SP serology and use for routine PVM at the population level [12,32]. A limitation of SPCE in having antigens and blocking antibodies which are fixed and usually of unknown antigenic relevance (beyond serotype) has, however, been discussed [32], and this together with antigen instability [33] may account for differences in results compared to VNT. Nevertheless, studies have shown the potential of SPCE in the determination of immunogenicity [34]. The moderate correlation in results between the two test methods (i.e., VNT and SPCE) in this study suggests that the SPCE could be used in assessing the immunogenicity of vaccines where high-level biocontainment facilities are not available to conduct VNT, although the VNT is likely to be superior at predicting cross-protection to specific field strains according to their antigenic match.

5. Conclusions

This study confirms the value of PVM testing and shows that for this vaccine and field strain, a vaccination regime employing a two-dose primary course and revaccination after 4–6 months is likely to be appropriate, as recommended by the vaccine manufacturer. Countries, especially sub-Saharan countries with a diversity of FMDV strains and inconsistent vaccine quality, are encouraged to implement PVM studies that can account for variations in vaccine potency and antigenic match.

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Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines11121818/s1, Table S1: The solid-phase competitive ELISA (SPCE) % inhibition results at 1:10 dilution (results for 1:30 dilution not shown) and the log reciprocal of virus neutralisation test (VNT) titres in subsistence (Rufunsa) and commercial (Chisamba) cattle herds vaccinated with either one (day 0) or two doses (day 0 and 28) of an FMDV serotype O vaccine.

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Data Availability Statement: All datasets produced and analysed for this study are available from the corresponding author upon reasonable request.

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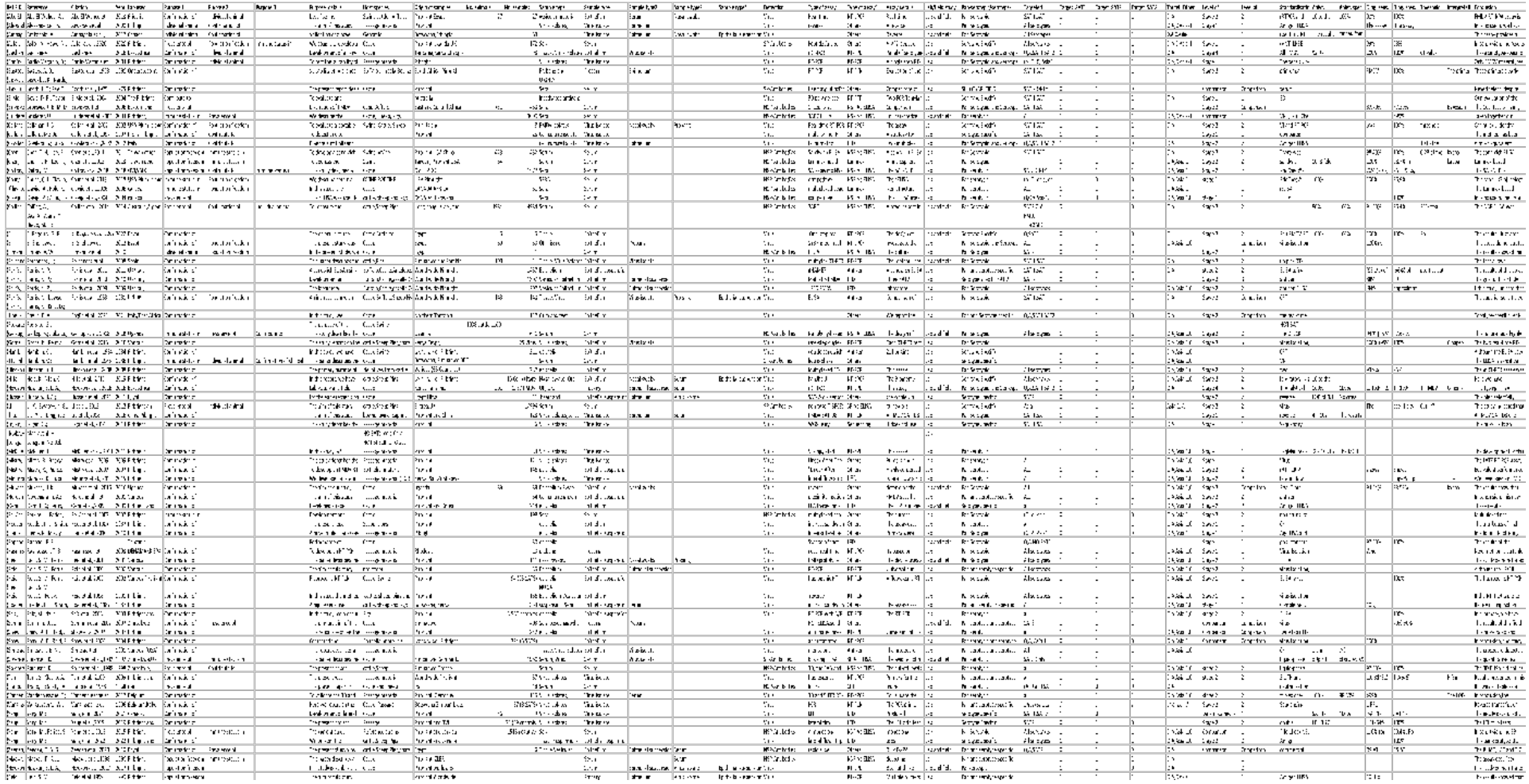
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7.6. Data sheet



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