Levels of SAT serotype foot and mouth disease viruses in tissues of experimentally infected cattle carcasses and those from the wildlife-livestock interface in the Zambezi Region of Namibia

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Table of Contents

List of Tables

List of Figures

Abbreviations

ALOP	Appropriate Level of Protection
ARC-OVI	Agricultural Research Council Onderstepoort Veterinary Institute
AU-IBAR	African Union InterAfrican Bureau of Animal Resources
BHK	Baby hamster kidney cell line
BSL3	Biosecurity Level 3
CBT	Commodity-based Trade
CCPs	Critical Control Points
CVL	Central Veterinary Laboratory, Windhoek
DAFF	Department of Agriculture, Forestry and Fisheries
DB	Deboned Beef
DVS	Directorate of Veterinary Services
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme-linked Immunosorbent Assay
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
FS	Food Safety
GFRA	Global FMD Research Alliance
HACCP	Hazard Analysis Critical Control Point system
KAZA TFCA	Kavango Zambezi Transfrontier Conservation Area

KMQS	Katima Mulilo Quarantine Station
LPBE	Liquid-phase blocking ELISA
MCA	Millennium Challenge Account
MCA-N	Millennium Challenge Account Namibia
MCC	Millennium Challenge Corporation
Meatco	Meat Corporation of Namibia
NCA	Northern Communal Areas
NSP	non-structural (viral) protein
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PD_{50}	Protective dose 50
pH	Negative log of hydrogen ion concentration
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SADC	Southern African Development Community
SAT	Southern African Territories (serotypes of FMD virus SAT 1; SAT 2; SAT 3)
TADs	Transboundary Animal Diseases
$TCID_{50}$	Tissue culture infective dose; 50% end-point
VIS	Veterinary Inspection Service
VNT	Virus neutralisation test

VP	Variable protein
WAHID	World Animal Health Information Database of the OIE

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Abstract

A value chain approach was employed as the basis for sanitary management of both food safety and animal disease risks. This was achieved through the integration of hazard analysis critical control point (HACCP) and commodity-based trade (CBT) methodologies. That approach enabled identification of and management of critical control points (CCPs) to enable an 'appropriate level of protection (ALOP)' to be achieved, i.e. attainment of negligible risk. This work was done as part of a project led by the Meat Board of Namibia and largely funded by the Millennium Challenge Account – Namibia, aimed at improving market access for beef producers the Zambezi Region (ZR) of Namibia, a foot and mouth disease (FMD)-infected zone.

In order to validate the technical basis for the approach, it was necessary to confirm that matured (pH < 6.0), deboned beef from which the lymph nodes and fat had been removed, did not contain detectable quantities of southern African Territories (SAT) serotype FMD viruses even when derived from cattle in the acute stage of infection. This was done by experimental infection of cattle in a bio-secure facility and testing of tissues derived from those cattle immediately after slaughter and exsanguination. Tissues derived from healthy cattle after slaughter at the official abattoir in the ZR were also subsequently tested for viral content. No evidence of infection was found in those animals.

Experimental infection involved intradermolingual inoculation of six cattle with three different FMD SAT virus isolates obtained from outbreaks in cattle in the ZR. Forty-eight hours after infection the animals were euthanized and exsanguinated (i.e. acute phase of infection) and selected tissues collected and processed to determine viral genomic content. For the abattoir survey, 148 cattle were sampled for identification SAT serotype viruses in specific lymph nodes of carcasses derived from cattle slaughtered at the abattoir in the ZR. Sub-mandibular, pre-scapular and popliteal lymph nodes from these cattle were examined based on the results of the prior experimental infection study that showed those lymph nodes to consistently contain detectable viral RNA levels. Conventional and real time PCR techniques were used for detection of FMD virus RNA.

It was shown that immediately after slaughter and exsanguination, but before maturation, SAT serotype virus RNA was undetectable in striated muscle or fat of acutely infected cattle using the methods employed in this study. A weakness of the method employed in this study was the use of a non-optimized protocol for isolating total RNA from fat tissue. However, high levels of virus RNA were present in some lymph nodes; consistently in the three that were sampled routinely at the abattoir in the ZR. Sampling of the 148 cattle derived mainly from high risk areas and slaughtered at the abattoir failed to reveal presence of FMD virus RNA in any animal. The sample size was however too small to attain statistical significance due to cessation of sampling caused by a FMD outbreak in cattle in the ZR. These results confirm and extend previously published findings on the safety of matured beef from which the bones and lymph nodes have been removed even if such beef is derived from locations that are not recognised as free from FMD.

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Manuscripts

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2. G. Fosgate, B. Mutowembwa, M-L. Penrith, G. Thomson & S. Thalwitzer. Quantitative risk assessment methodology to measure FMD risk posed by chilled deboned beef produced in a non-FMD free location. (*GFRA Newsletter)*

Conference presentations and proceedings

1. Paidamwoyo B Mutowembwa, Gavin R Thomson, Susanne Thalwitzer, Mary-Louise Penrith, Livio E Heath. Assessment of levels of foot and mouth disease (FMD) virus in carcass tissues following experimental infection with SAT serotypes of FMD virus and in slaughter cattle from the wildlife-livestock interfaces of Zambezi Region, Namibia. Book of Abstract, Third International One Health Conference 15-18 March 2015, Amsterdam, Netherlands: 11 (Poster).
2. Paidamwoyo B Mutowembwa, Gavin R Thomson, Susanne Thalwitzer, Mary-Louise Penrith, Livio E Heath. Assessment of levels of foot and mouth disease (FMD) virus in carcass tissues following experimental infection with SAT serotypes of FMD virus and in slaughter cattle from the wildlife-livestock interfaces of Zambezi Region, Namibia. Global Foot-and-mouth Disease Research Alliance (GFRA) Scientific workshop, Hanoi, Viet Nam, 20-22 October 2015, 24 (Poster).

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Background

International trade standards set by the World Organisation for Animal Health (OIE) are designed to facilitate safe trade in animals and animal products through preventing spread of animal diseases that can have devastating health and economic consequences. As specified within the World Trade Organisation (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), OIE standards must balance safety considerations against the need to promote trade and avoid discriminatory measures that are not based on scientific evidence (WTO, 2009; WTO, 2012).

Currently, the TAHC hinges safe importation of livestock and livestock commodities on the geographic absence of transboundary animal diseases (TADs), such as foot-and-mouth disease (FMD), from the country of origin (Scott *et al*., 2006; Thomson *et al.,* 2008). There are drawbacks in relying entirely on geographical freedom from disease (Thomson *et al.,* 2013a). It is becoming increasingly difficult to identify where specific infections do or do not occur due to the increasing rate at which infections of humans and animals are appearing in new and unexpected locations (emerging and re-emerging diseases) (Conlan *et al*., 2008; Thomson *et al.,* 2009). Some of these infectious agents that cause TADs are inclined to cause mild disease or asymptomatic infection which are difficult to detect (e.g. the southern African Territories [SAT] serotype viruses in both wildlife and livestock in extensive rangeland systems, Thomson *et al.,* 2009). This demonstrates that the geographic location of TADs is becoming increasingly complex to determine, and if the trend continues, it will lead to disproportionate disruption of international trade in livestock commodities (Scoones *et al.,* 2006; FAO, 2006). This scenario presents difficulties for all countries whatever their state of development. However, poor livestock producers in developing countries will be the most affected (Pica-Ciamarra, 2007; Teweldemedhin and Van Schalkwyk, 2010).

Southern and East Africa have more TADs than any other part of the world because of the abundant wildlife in these regions (Bengis *et al*., 2004). Producers in southern Africa are

particularly affected because the SAT serotypes of FMD viruses evolved in and are endemic to most African buffalo (*Syncerus caffer*) populations (Bengis *et al*., 2004; Thomson and Bastos, 2004; Scoones and Woolmer, 2008; Rweyemamu *et al.,* 2008). Interaction between wildlife and cattle at wildlife/ livestock interfaces has resulted in these viruses being transmitted from wildlife to livestock on many occasions (Thomson *et al*., 2013b). Historically, effective control of FMD and other TADs in southern Africa has focused on separation of wildlife and livestock (Thomson *et al*., 2013b). This has involved the establishment of large FMD-free zones that enabled access to international beef markets over the last 30 years or more (Thomson *et al*., 2013b). However, only about 15% of cattle in mainland southern Africa are currently located in these FMD-free zones (Vosloo and Thomson, 2017). This means that the vast majority of livestock producers are excluded from high-value markets.

In order to advance the cause of conservation and also after recognising the global comparative advantage when it comes to wildlife-based tourism, governments of southern African countries began investing in transfrontier conservation areas (TFCAs) (Osofsky, *et al*., 2005; Barnes, 2013; Thomson *et al*., 2013b). The extent and intensity of livestock-wildlife interaction in large parts of southern Africa is poised to increase due to the multiple land use approach that characterizes TFCAs. This will potentially increase conflict between livestock interests based on expansion of livestock production and access to markets on the one hand, and wildlife conservation on the other hand (Osofsky *et al*. 2005; Barnes, 2013; Thomson *et al*., 2013b). The main reason for this is that the TFCA concept promotes free movement of wildlife over large geographic areas. In contrast, the present approach to the control of TADs is to prevent movement of susceptible animals between areas where contagious TADs occur and areas where they do not, largely through extensive veterinary cordon fencing, and to trade restriction in commodities derived from animals in these infected areas. Livestock agriculture and the wildlife conservation sector are both vital for balanced rural development in southern and East Africa, and animal health policy is therefore key in resolving inter-sectoral land-use conflict (Osofsky, 2005; Thomson *et al*., 2013a).

The Food and Agriculture Organization of the United Nations (FAO) developed a pathway for the progressive control of FMD based on eliminating FMD infection in livestock from countries or parts of countries – usually referred to as zones – in which FMD is endemic (OIE/FAO, 2011).

This initiative is supported by the OIE as a tool to assist in the management of FMD in endemic situations. However, implementation of this pathway, as it stands, is problematic in that the PCP-FMD is based on Eurasian serotype FMD as it occurs in other parts of the world. SAT viruses have a significantly different epidemiology, including specific maintenance of these viruses by wildlife as well as the extent of antigenic variation (Thomson et al., 2015). Within the SAT viruses, there are at least eight topotypes within SAT1, 14 in SAT2, and six within SAT3 (Vosloo et al., 2005). According to Thomson *et al.,* 2015, FMD SAT serotypes are less amenable to regional elimination than the Eurasian serotypes. This difference between Eurasian and SAT lineages has in the past been ascribed mainly to the fact that SAT serotype viruses appear to have evolved in African buffalo and can be maintained independently of other cloven-hoofed species (Thomson and Bastos, 2004; Thomson et al., 2013a). This situation is not considered by the PCP-FMD and so in some respects the PCP-FMD is inappropriate to southern Africa, more so because of developments associated with establishment of TFCAs. Southern Africa has abundant buffalo populations that maintain and transmit FMD SAT serotypes, with other species like impala also being capable of spreading the infection to domestic animals. Because of the continuous interaction between wildlife and livestock at the interfaces, the implementation of the pathway cannot be fully achieved in some countries (Perry *et al.,* 2005; Thomson *et al*., 2015).

There is growing recognition that a different approach is needed to facilitate regional trade in livestock products derived from places where FMD cannot be eliminated due to the presence of wildlife. Three alternatives are recognised for managing FMD risk associated with beef trade: (1) processing of beef so that any virus possibly present is inactivated (i.e. destroyed), (2) application of 'compartmentalisation' and (3) management of FMD risk along value chains where the concept of commodity-based trade (CBT) can be integrated with the prerequisites and principles of hazard analysis critical control point (HACCP). These options could also be used in combination (Thomson *et al*., 2004, 2009; Scoones *et al*., 2010; Rich and Perry, 2011; Brückner, 2011; Thomson *et al*., 2013a).

This project focused on risk mitigation along the beef value chain in the ZR of Namibia (Thomson et al., 2013b). This approach enables effective integration of food safety and animal disease risk management of beef. It additionally ameliorates adverse environmental and associated socio-

economic consequences of current sanitary standards based on the geographic distribution of FMD (Thomson *et al*., 2013b). This is particularly the situation where vast veterinary cordon fencing systems are depended upon to separate livestock and wildlife as is the case in much of southern Africa (Thomson *et al*., 2013a). Application of the value chain approach needed confirmation that SAT serotype viruses do not occur in matured deboned beef or in fat associated with such beef even if the beef was derived from animals inadvertently slaughtered in the acute phase of FMD infection.

A value chain approach would thus be particularly beneficial to least developed countries of southern Africa where it would lessen incongruence between endeavors to grow and market animals and the need to monitor the subcontinent's wildlife heritage (Thomson *et al*., 2013a). It was therefore important to prove scientifically that for beef, international standards can be set which will ensure safe trade even though FMD is endemic to wildlife in the region concerned (Thomson *et al*., 2008; Thomson *et al*., 2013a). It is important to note that currently, the OIE standard relevant for FMD infected zones with control measures in place is Article 8.8.22 of the Terrestrial Animal Health Code (TAHC). Prior to 2015, when the project was conducted, the OIE TAHC Article 8.7.25 was in use. The fundamental difference between the two articles is clause 1.d, (TAHC 2014) which required a 10 km FMD-free radius around producer establishments. This prevented the standard from being fully applied in the ZR because of the movement of wildlife that cannot be controlled or even accurately monitored. To add on to that, it is also impossible to prove that no wild animal was infected with FMD virus. Clause 1.c of the current version of Article 8.8.22 provides additional flexibility by authorizing quarantine as a risk mitigation mechanism. Article 8.8.22 came into existence since May 2015 (TAHC, 2015), and is being used in the ZR.

1.2 Literature Review

1.2.1 FMD Aetiology

FMD is a vesicular disease caused by RNA viruses belonging to the genus *Aphthovirus* in the *Picornaviridae* family (Arzt *et al*., 2011a). FMD viruses occur as seven serotypes: O, A, C, SAT1, SAT2, SAT3, and Asia1. Based on genome analysis, serotypes O, A, C and Asia 1 constitute a clear evolutionary lineage distinguishable from the other lineage comprising serotypes SAT-1, 2 and 3 (Robson et al., 1977; Palmenberg, 1989; Tully and Fares, 2008; Yoon et al., 2011). There is negligible immunological cross-protection between serotypes, and within each serotype, there are many intra-typic variants, some of which cross-protect ineffectively (Thomson and Bastos, 2004; Knowles, 2008; Ding *et al.,* 2013).

Six of the seven FMD serotypes (i.e. all except Asia-1) are endemic to sub-Saharan Africa (Sangula et al., 2011). Although type C appears to have spontaneously disappeared worldwide, recent serological evidence indicates that serotype C may be present in East Africa (Rufael et al., 2008; Ayebazibwe et al., 2010; Tekleghiorghis et al., 2014). This however needs to be confirmed by virus isolation.

1.2.2 Host range

FMDV affects animals of the suborder *Ruminantia* and family *Suidae* as well as Bactrian camels (*Camelus bactrianus*) (TAHC, 2016). Important livestock hosts include cattle, pigs, sheep, goats, water buffalo and yaks. African buffalo (*Syncerus caffer*) are important maintenance hosts for SAT serotype FMDV in Africa. They may carry FMDV for 5 years, and virus may persist in a herd for 24 years or longer (Condy *et al.,* 1985; Thomson *et.al,* 2003). FMDV has also been reported in at least 70 species of wild (or captive wild) artiodactyls (Weaver *et al.,* 2013; Callis, 1996). Available evidence is that the putative progenitor of FMD viruses infected African buffalo about 1000 years ago. That progenitor gave rise to the SATs and the Eurasian serotypes less than 500 years ago (Knowles, 2013).

1.2.3 International FMD standards on trade in beef products

The SPS Agreement encourages WTO members to base their regulations on the health and safety standards developed by the three-relevant international standard setting bodies, namely the Codex

Alimentarius Commission (for food safety), the International Plant Protection Convention (for plant health) and the World Organisation for Animal Health (for animal diseases and zoonoses). WTO members who impose more stringent requirements must be able to justify those measures scientifically (WTO, 2009).

The WTO mandated the OIE to provide international standards for trade in livestock commodities influenced by animal diseases. The standards for terrestrial animals are published in the Terrestrial Animal Health Code (TAHC) that is available on the OIE website (www.oie.int). A comprehensive chapter on each listed disease, including FMD, is provided in the TAHC.

International human-food safety standards are defined by the *Codex Alimentarius Code of Hygienic Practice for Meat* (Codex), a product of the Codex Alimentarius Commission (www.codexalimentarius.org/standards/en/). The World Health Organisation (WHO) and Food and Agricultural Organisation (FAO) are the United Nations (UN) organisations jointly responsible for food safety guidelines, codes of practice and standards.

Article 3 of the SPS Agreement stipulates that sanitary provisions adopted by member countries of the WTO should be based on existing international standards, guidelines and recommendations. Article 5 points out that where there is no international standard or where equivalence needs to be demonstrated, effective management of SPS hazards should be based on risk analysis, conducted in accordance with international guidelines. 'The principle of equivalence' is covered by Article 4 of the SPS Agreement. This article explains that as long as the exporting country can objectively demonstrate that its system provides the same level of risk mitigation as the published international standard, it should be accepted by importing WTO member countries. Chapter 5.3 of the OIE's TAHC covers these provisions as they relate to diseases of terrestrial animals (OIE, 2016).

Although the risks associated with food safety and animal diseases are generically similar, the Codex Alimentarius Commission and the OIE recommend different methodologies to manage these risks (Thomson et al., 2013b). The Codex Alimentarius Commission uses Hazard Analysis Critical Control Point (HACCP), which, among other provisions, requires identification of critical control points (CCP's) in food production and preparation (FAO, 2006). On the other hand, the

OIE's TAHC traditionally adopts the concept that risk associated with trade in animal commodities is mitigated by ensuring that infections capable of causing the TADs of concern are not present in the locality of production. This geographic approach with respect to the TAHC is progressively being supplemented by the introduction of standards that are not entirely geographic, for instance, compartmentalization and processing of products.

1.2.4 Geographic trade standards for beef trade influenced by FMD

Currently, the TAHC (2016) has four options for beef export where a geographic approach is adopted, i.e. creation of FMD-free counties or zones:

- Country free of FMD without vaccination (TAHC Article 8.8.2);
- Country free of FMD with vaccination (TAHC Article 8.8.3);
- Zone free of FMD without vaccination (TAHC Article 8.8.4);
- Zone free of FMD with vaccination (TAHC Article 8.8.5).

These four options require the competent veterinary authority of the exporting country to apply to the OIE for recognition of a free zone & the application needs to be accompanied by a completed dossier that can be downloaded from the OIE website (TAHC Articles 8.8.22 and 8.8.23).

One of the major drawbacks for livestock enterprises based at locations within zones recognised by the OIE as free from FMD is that if evidence of FMD infection is detected in clinically ill or healthy animals within that zone, the status of that zone will be suspended for at least 3 - 18 months (Article 8.8.4 of the TAHC); unless the country concerned establishes a 'containment zone' in accordance with Article 8.8.6. In that case trade from the previously FMD-free zone (which then maintains its free status) may continue. The variation in the period of suspension is determined by the type of FMD freedom recognised by the OIE and the control measures applied to eliminate the occurrence of FMD. Interruption of business for such prolonged periods presents major operational problems for commercial beef enterprises.

1.2.5 Non-geographic trade standards for FMD

The OIE TAHC has a recognition process for geographically based trade standards but there is no independent recognition provided by the OIE on official recognition processes for non-geographic approaches to risk management; it just provides measures that need to be complied with in order to meet the overall standard (Thomson *et al*., 2013a). This situation therefore makes it incumbent

upon the exporter to persuade the importer as well as the competent authority of the importing country that the applicable international standard has been met. Conventionally that takes place by certification provided by the competent authority of the exporting country (Thomson *et al*., 2013a).

1.2.6 Processing products to inactivate FMDV

OIE accepts processes such as canning and thorough cooking during which a core temperature of 70°C or higher is maintained for a minimum of 30 minutes, or curing by drying, and salting as effective in destroying FMD virus in meat (TAHC Article 8.8.31). Such meat and meat products subjected to these treatments pose negligible risk of FMD transmission and can be exported regardless of the FMD status of the area of origin.

1.2.7 Use of Compartments in management of FMD

Compartments comprise one or more establishments managing animal health risks using an integrated biosecurity system. Compartments may consist of a single farm, a group of farms, or one or more farms as well as relevant service providers such as feed and/or animal suppliers. The concept has been most successfully applied to intensive farming systems as found in the pig and poultry industries, where a high level of control over the animals and their environment is possible. Compartments could also be used under extensive livestock production system, albeit with some practical difficulties (Thomson *et al*., 2013a).

The major obstacle to using compartmentalisation for managing FMD in endemic locations is that according to Clause 2.d of TAHC Article 8.8.4 (TAHC, 2016) vaccination is expressly prohibited, effectively prohibiting the introduction of vaccinated animals into FMD free compartments - no cattle vaccinated within the last 12 months may be introduced into such a compartment. In southern Africa where both SAT viruses and buffalo are prevalent, exclusion of FMD vaccination in compartments potentially increases rather than decreases the risk of FMD outbreaks hence compartmentalisation for FMD is likely to be ineffective in southern Africa (Thomson *et al*., 2013a). Furthermore, TAHC Articles 8.8.4 and 8.8.7 (TAHC, 2016) states that if FMD were to be detected in a compartment, that compartment would be precluded from international trade for a period of at least 12 months. This can potentially lead to collapse of the business enterprise because they are rarely able to withstand the financial consequences of such lengthy interruption of their operations (Thomson *et al*., 2013a).

The OIE does not provide a recognition mechanism for FMD-free compartments. The rationale is that for FMD-free compartments the competent veterinary authorities of the importing and exporting countries would need to agree with the beef enterprises' management on the biosecurity plan for the compartment using the TAHC as a guide. This effectively means that export of beef derived from the compartments could only take place following bilateral agreement between the competent authorities of the importing and exporting countries.

1.2.8 FMD management along value chains

An FAO guide (FAO, 2011) provides the foundation for implementation of management of TADs, including FMD, along value chains. Thomson *et al*, 2013 showed that not only can FMD and other animal disease trade risks be managed along value chains, but that food safety risk management can also be incorporated into risk management along the value chain. Value chains are the composite of the people and organisations involved in converting raw material into a ready-for-sale product; each step along the chain contributes value to the product.

Provisions of Article 8.8.22 (see below) in the TAHC detail recommendations for the importation of fresh meat derived from cattle located in FMD infected countries or zones with an official control programme for FMD, involving compulsory vaccination of cattle. This Article, although not referred to as such, could be considered a value chain approach. The provisions of this Article are as follows:

For fresh meat of cattle and water buffalo *(Bubalus bubalis)* (excluding feet, head and viscera) Veterinary Authorities should require the presentation of an international veterinary certificate attesting that the entire consignment of meat:

1. comes from animals which:
    a. have remained, for at least three months prior to slaughter, in a zone of the exporting country where cattle and water buffaloes are regularly vaccinated against FMD and where an official control programme is in operation;

b. have been vaccinated at least twice with the last vaccination not more than six months, unless protective immunity has been demonstrated for more than six months, and not less than one month prior to slaughter;
c. were kept for the past 30 days in an establishment, and that FMD has not occurred within a 10 kilometer radius of the establishment during that period, or the establishment is a quarantine station;
d. have been transported, in a vehicle which was cleansed and disinfected before the cattle and water buffaloes were loaded, directly from the establishment of origin or quarantine station to the approved slaughterhouse/abattoir without coming into contact with other animals which do not fulfil the required conditions for export;
e. have been slaughtered in an approved slaughterhouse/abattoir:
    i. which is officially designated for export;
    ii. in which no FMD has been detected during the period between the last disinfection carried out before slaughter and the shipment for export has been dispatched;
f. have been subjected to ante- and post-mortem inspections within 24 hours before and after slaughter with no evidence of FMD;

2. comes from deboned carcasses:
    a. from which the major lymphatic nodes have been removed;

which, prior to deboning, have been submitted to maturation at a temperature greater than + 2°C for a minimum period of 24 hours following slaughter and in which the pH value was less than 6.0 when tested in the middle of both the longissimus dorsi muscle.

Incorporation of HACCP principles and prerequisites into Article 8.8.22 would improve its practicality (Thomson *et al*., 2013b). Critical control points (CCPs) as applied in the HACCP system focus on the risk management and monitoring of food safety risks at defined points along the value chain. The location of CCPs along the value chain will vary with the product as well as the production process. It has been shown that CCPs can also be used to manage animal disease risks, which enables integration of the management of food safety and animal disease hazards (Thomson *et al*., 2013b).

Steps and principles of the HACCP system (Thomson *et al*, 2013b)

Step 1	Assemble the HACCP team
Step 2	Describe the product
Step 3	Identify the intended use of the product
Step 4	Construct the flow diagram
Step 5	On-site confirmation of the flow diagram
Principle 1	List all potential hazards associated with each step of the flow diagram, conduct hazard analysis & consider control measures to manage the identified hazards
Principle 2	Establish critical control points (CCPs)
Principle 3	Establish critical limits for each CCP
Principle 4	Establish a monitoring system for each CCP
Principle 5	Establish corrective actions for each CCP
Principle 6	Establish verification procedures for each CCP
Principle 7	Establish overall documentation & recording system

Important to the HACCP system is auditing by the competent authorities or bodies they appoint to do the job for them. Risk analysis (either qualitative or quantitative) is essential to prove to the competent authority of the importing country that the proposed sanitary system is safe and reliable.

1.2.9 International beef trade in relation to FMD dissemination

About 54% of primary outbreaks of FMD that occurred in England between 1954 and 1967 were attributed to imported meat, bones and meat wrappers (Beynon, 1968). All these outbreaks occurred before the introduction of requirements for deboning and maturation of carcasses imported from South America as well as introduction of the ban on all swill feeding to UK pigs. In a review of the origins of FMD outbreaks in Europe in the 20 years prior to 2008, only a single case that occurred in Albania in 1996 was attributed to beef importation (Valarcher *et al*., 2008).

In this particular case, bone-in meat was imported even though the import permit on the beef consignment stated that it was deboned.

More than 100,000 tons of boneless beef were imported into the European Community in 1989 from South America and Southern Africa without evidence that this prompted any FMD outbreaks (Blajan and Callis 1991). FMD risk mitigation procedures that have been in use in South America for more than 30 years have added to the advancement of a safe and highly technical and specialized beef exporting industry (Blajan and Callis 1991).

Historically, exports from Argentina to the UK between 1963 and 1995 were derived from steers from the "Pampa region", particularly from its central fattening areas. The Pampa region was considered as a Secondary FMD Endemic zone, and at one time, had the largest number of annual FMD outbreaks in Argentina (Dillon, 2009). Therefore, it is probable that some deboned beef imported into the UK was derived from FMDV-infected animals without evidence of FMD outbreaks having occurred in the UK as a result (Dillon, 2009).

Between 1963 and 1995 small scale exports to Europe additionally occurred from southern Africa (Botswana, Namibia, Zimbabwe and South Africa). These southern African countries utilized comparable standards of isolating FMD-endemic from free zones by fencing and movement restrictions, biannual vaccination of cattle in areas adjacent to infected African buffalo and active clinical surveillance (Thomson, 2008). However, the exports to Europe from the southern African region were (apart from Zimbabwe where beef exports were via a bilateral agreement with the EU) only permitted from OIE-recognized FMD-free zones. A further precautionary measure was that deboned beef from southern Africa could not be imported into Europe until a period of three weeks after the source animals were slaughtered had lapsed, allowing time for recognition of any recent FMD outbreaks that could negatively affect the safety of the commodity. Since the time to ship to Europe exceeds three weeks, the precaution fitted well with the export process. It is important to note that the EU requirement for deboning of beef from southern Africa is additional to the provisions of the TAHC Article 8.8.2 dealing with importation from FMD-free zones where vaccination is not practiced.

1.2.10 Distribution of FMDV in acute phases of infection and virus survival in tissues of infected slaughter cattle

According to reviews by Arzt *et al*., (2011a & b), the epithelia of the pharynx are the tissues most consistently infected during the pre-viraemic phase of FMD; it is therefore likely that these are the primary replication sites in ruminants. Studies by Juleff *et al*., (2008; 2012) showed that FMDV genome could be detected consistently in germinal centres within the dorsal soft palate, pharyngeal tonsil, palatine tonsil, lateral retropharyngeal lymph nodes and mandibular lymph nodes at 38 days post- contact infection. The FMDV genome in these tissues was restricted to the germinal centres (Juleff *et al*., 2008; 2012). Another finding was the presence of viral RNA in the absence of detectable non-structural viral proteins (Juleff *et al*., 2012), indicating the absence of viral replication (De Diego *et al*., 1997; Juleff *et al*., 2008; Juleff *et al*., 2012). The localisation of viral RNA and capsid conformational epitopes, in the absence of non-structural proteins, supports the hypothesis that FMD viral particles or immune complexes are maintained in light zones of germinal centres in a non-replicative state (Juleff *et al*., 2008; 2012).

The outcome of the studies by Juleff *et al*., (2008; 2012) are important for understanding both the mechanism of viral persistence and the ability of FMDV infection to stimulate long-lasting antibody responses. Follicular dendritic cells are known to be non-endocytic cells that are capable of capturing and retaining antigen in the form of immune complexes for long periods of time (Haberman and Shlomchik 2003). Retention of immune complexed FMDV particles within lymphoid tissue presents a possible source of the infectious material detected by pharyngeal sampling of infected cattle; either through direct harvesting of germinal centres in lymphoid tissue or sampling of secondary cells (for instance macrophages, dendritic cells or B-cells) able to support low level virus replication cycles in the presence of high levels of neutralising antibodies (Rigden *et al*., 2002). Macrophages, dendritic cells or B-cells may also act as a source of infectious material capable of seeding other remote sites for ongoing low-level viral replication in the pharynx (Juleff *et al*., 2012; Arzt *et al*., 2011a; Stenfeldt, *et al*., 2012; 2014; Pacheco *et al.,* 2015).

There is a clear difference between very young animals and older ones when it comes to the consequences of myocardial infarction (Alexandersen *et al*., 2002). In the former case, it often results in acute heart failure (tiger heart disease) before vesicles develop, but not in older animals (Alexandersen *et al*., 2002). In myocardium and mammary tissue, viral replication takes place in

myocytes and secretory epithelial cells of the alveoli respectively, thereby creating clear microscopic lesions (Platt, 1960).

Development of characteristic vesicular lesions in FMD is dependent on persistent local irritation or friction (Platt, 1960). This clarifies why the mouth, feet and teats are sites that are more inclined to develop vesicular lesions and why pigs often develop lesions on the dorsum of the snout, i.e. because of snuffling. Similarly, warthogs, which regularly stoop on their carpal joints while feeding, tend to develop carpal joint vesicular lesions (Platt, 1960).

Epithelial lesions at secondary replication sites (oral mucosa, skin of the horn-hoof junction & skin of the teats) are initiated by infection of single cells in the stratum spinosum (Woodbury, 1995). Following infection of these cells, vesicles develop either by lysis of cells swollen as a result of ballooning degeneration and the release of intracellular fluid, or by the formation of areas of focal intercellular oedema. The vesicles then coalesce, rupture or, more rarely, the fluid seeps away resulting in desiccation of the lesion (Woodbury, 1995).

Virus persistence in animal products after slaughter depends upon many variables such as the tissues involved, breed of animal, serotypes and isolates of FMDV, stage of infection and changes in pH that take place in different organs and tissues under different conditions (Arzt *et al*., 2010). There has been considerable work on FMDV survival in cattle carcasses, collected and stored so as to mimic beef abattoir slaughtering processes but none of the in-depth studies has involved serotypes Asia 1 or any of the South African Territory (SAT) serotypes (Paton, *et al*., 2010). In the majority of cases, cattle were not vaccinated against FMD, and were slaughtered a few days after direct inoculation with FMDV representing a worst-case scenario for peak FMDV contamination (Paton, *et al*., 2010). A list of the studies is shown in Table 1.1.

Table 1.1. Studies of FMDV survival in striated muscle, lymph nodes and bone marrow

FMDV serotype	FMDV status of tissues			Reference
	Striated muscle	Lymph nodes	Bone marrow	
O	Negative	Positive	Not evaluated	Henderson and Brooksby, 1948
	Negative	Positive	Positive	Savi *et al.,* 1961
	Negative	Positive	Positive	Wisniewski, 1963
	Negative	Positive	Not evaluated	Gailiunas *et al.,* 1969
	Negative	Positive	Not evaluated	Burrows *et al.,* 1981
	Negative	Positive	Not evaluated	Garcia Vidal *et al.,* 1982
	Negative	Positive	Positive	CEC, 1986
	Negative	Positive	Positive	Lasta *et al.,* 1992
	Negative	Positive	Not evaluated	Gomes *et al.,* 1994
A	Negative	Positive	Not evaluated	Henderson and Brooksby, 1948
	Positive	Positive	Positive	Cottral *et al.,* 1960
	Positive	Positive	Positive	Cox *et al.,* 1961
	Negative	Positive	Positive	Savi *et al.,* 1961
	Negative	Positive	Not evaluated	Gailiunas *et al.,* 1969
	Negative	Positive	Not evaluated	Burrows *et al.,* 1981
	Negative	Positive	Not evaluated	Garcia Vidal *et al.,* 1982
	Negative	Positive	Positive	Lasta *et al.,* 1992
C	Negative	Positive	Not evaluated	Gailiunas *et al.,* 1969
	Negative	Positive	Positive	Lasta *et al.,* 1992

The studies described in Table 1.1 conclude or imply that FMDV does not survive in striated muscle of cattle. Gomes *et al.*, 1994; Cox *et al.,* 1961; and Cottral *et al.,* 1961 however found that acutely infected slaughter cattle had detectable levels of FMDV in striated muscle before and after maturation in the *Latissimus dorsi* and *Semitendinosus* muscles, that did not reach a pH of <6.0 during maturation. The acidification of skeletal muscle that takes place during maturation of the

carcass, if done correctly, is sufficient to inactivate FMDV in this tissue, even when cattle are slaughtered at the height of viraemia. Since the required level of acidification cannot be assumed during the maturation process (due, for example, to stress of animals, starvation, intercurrent disease and other factors leading to incomplete anaerobic glycolysis in the muscle), measuring of the carcass pH can be utilised to guarantee that it has occurred. This is the basis for the current prerequisites concerning maturation and pH assessment of beef carcasses (EEC, 1986; OIE, 2008). There are measures that can be implemented to promote effective post-mortem maturation of the beef, with consequent lowering of the pH of striated muscle to lower than pH 6.0 such as adequate feeding of the animals and resting them before slaughter (Henderson and Brooksby, 1948).

In contrast, other tissues that may harbour FMDV but that do not undergo acidification (these tissues do not have adequate glycogen levels for anaerobic glycolysis to develop the degree of acidity that is present in the muscle tissue) and in these tissues the virus can survive the maturation process and subsequent low temperature carcass storage. These include blood, heads, feet, viscera, bone marrow and major lymph nodes, all of which can be removed to a considerable degree during the processing of the carcass. Fragments of bones are likely to remain in improperly prepared cuts but FMDV in bone tissues occurs in the bone marrow rather than the bone itself. There are, however, no available data that quantify fragments of bone or lymph node that remain in specified beef cuts (USDA, 2002).

1.2.11 Abattoir Procedures and Post Mortem Changes

For the purposes of this dissertation it was assumed that deboned beef consists of muscle tissue and associated fascia, fat, nerves, and small vessels after the process of deboning. Processed products are beef products that undergo processing beyond chilling and freezing - treatments include heating, curing and dehydration.

The slaughter process for export trade involves transportation of cattle to an export accredited abattoir, conducting *ante-mortem* veterinary inspection of each animal in the lairage to confirm absence of clinical disease, stunning and complete exsanguination, evisceration, post mortem veterinary inspection for absence of gross signs of disease, carcass chilling, and deboning (CODEX, 2005; Dagg, et al., 2006; Caporale et al., 2001). It is important that the different stages

involved in the slaughter process are performed correctly, as they are critical in the removal of FMDV infection and thereby ultimately reduce contamination of the final products (CODEX, 2005; Dagg, et al., 2006). If these different stages, or at least some are incorporated into CCPs, certification is rendered more reliable (Thomson *et al*., 2013a).

After death of cattle, anaerobic glycolysis ensues in the muscle tissue resulting in the production of lactic acid. This causes a fall in pH to 5.6- 5.7 (Foegeding *et al*., 1996). In export certified abattoirs, pH is measured in each latissimus dorsi muscle just before the carcass is split in quarters and consequently before entering the deboning room. pH estimation is conducted according to a specified protocol (i.e. electronically measured, with daily calibration of instruments and proper registration chart/notebook) and under the audit of the designated body under the supervision of the official veterinary service (Lister, *et al*. 1981; EU, 2004). Carcasses with a pH reading equal or higher than 6.0 need to be identified, placed in a separate cooling facility, and not exported (Lister *et al*., 1981; IPCVA, 2008).

It is generally accepted that FMDV is totally inactivated under acidic conditions at pH 6.0 or below within 24 hours at temperatures >2ºC (Pharo, 2002). About 60-80 mmol of lactic acid per kg of muscle tissue is needed for muscle pH to drop from 7.0 to about 5.5. (Puolanne, et al., 2002). Striated muscle tissue contains approximately 1% glycogen, which in turn generates 1.0 to 1.1% lactic acid (Ockerman, 1996). For each 1% lactic acid formed the pH will be lowered by approximately 1.8 pH units (Ockerman, 1996). Factors such as species, type of muscle in an animal, genetic variability between animals, administration of drugs that affect metabolism, starvation, stress, post mortem temperature and electrical stimulation during exsanguination influence both the rate of pH fall as well as the ultimate pH achieved (Anon, 1986; Ockerman, 1996; Foegeding *et al*., 1996). If animals are exposed to preslaughter stress such as fear, excessive temperatures, exercise and starvation and intercurrent disease, glycogen may be depleted leading to reduced muscle tissue acidification and improved survival conditions for FMDV (Lister, et al. 1981). It is therefore crucial to adhere to good animal handling and transportation practices in order to obtain deboned beef with an ultimate pH value of 5.8 or lower after maturation (EU, 2002).

1.2.12 Risk associated with FMDV survival in animal products

The major factors determining the risk associated with FMDV survival in animal products include: quantity of surviving FMDV in animal products, quantity of FMDV required to establish infection by different routes of exposure in different domestic animal species, the amount of the material inhaled or ingested and the number of animals exposed (Sellers, 1971; Sutmoller and Vose, 1997). This makes it cumbersome to establish a threshold level of FMDV contamination of a commodity, below which it could be viewed a negligible risk (Paton, 2010).

The most likely way by which contaminated meat products could start an outbreak of FMD is ingestion of contaminated animal products by pigs (Donaldson, 1997). Pigs are omnivorous, and are the only FMDV susceptible species that naturally consume meat (Donaldson, 1997). The minimum oral dose of FMDV to infect pigs has been estimated at around 10^5 tissue culture infectious doses (Sellers, 1971; Donaldson, 1997). However, literature on how readily pigs become infected by ingestion of FMDV contaminated carcass material is limited and of all the experiments conducted to date, there appear to have been no studies in which material equating to deboned beef as a commodity has been fed to pigs (Paton, 2010). There are also other means that have been presumed to result in infection of ruminants; for instance, some infections of cattle in the UK in 1967 were attributed to exposure to personnel who had been handling imported meat contaminated by FMDV (Paton, 2010). Ruminants are most likely infected by sniffing contaminated materials rather than by eating them due to their higher susceptibility to inhalation of FMDV (Sellers, 1971).

Based on the daily feed intake of a pig, Sellers (1971) concluded that at a FMDV concentration of less than 10 ID_{50} /g, the amount of product needed to be ingested by a pig to establish infection would exceed its daily feed intake. However, the relationship between FMDV concentration, volume and total infective dose is poorly understood (Sellers, 1971). Non-homogeneous commodity contamination further complicates the understanding of the total effective dose needed for infection (Sellers, 1971). For example, if a small fragment of bone within a large amount of meat had a virus concentration above 10 ID_{50} /g, there might still be insufficient virus in total to infect pigs through ingestion. It has been demonstrated that FMDV contaminated bone marrow was infectious to pigs only if crushed bones were incorporated into the feed (Sellers, 1971). It was

presumed that crushed bones facilitated infection through causing oral abrasions (Sellers, 1971; Anonymous, 1927). This suggests that animals with pre-existing oral lesions might also be more susceptible to infection by FMDV (Sellers, 1971; Anonymous, 1927).

Further risk mitigation of infecting animals from FMDV contaminated, imported animal products can be achieved by practices within an importing country such as vaccination against FMD or prohibitions on swill feeding of pigs.

1.2.13 FMD situation in southern Africa

The epidemiology of FMD in southern and eastern Africa is influenced by two different patterns; a cycle involving wildlife and another that is independent of wildlife but maintained within cattle. In the wildlife cycle, SAT 1, 2 & 3 serotypes of FMD viruses are maintained within African buffalo (*Syncerus caffer*) populations, the natural maintenance hosts of FMD viruses (Thomson and Bastos 2004). These buffalo provide a potential source of infection for other wildlife and domestic livestock. The independent maintenance of SAT serotypes by wildlife presents a serious complication for the control of FMD in parts of Africa where these virus serotypes and abundant wildlife occur together (Thomson *et al*., 2003a, 2013a; Weaver *et al*., 2013; Maree *et al*., 2014). The evidence is that SAT viruses are in fact African buffalo viruses that periodically spill over into other wild and domestic cloven-hoofed species in their close vicinity. Another compounding factor is that SAT serotypes have a wider array of antigenic variants than other FMD virus serotypes, rendering vaccination against FMD caused by SAT serotypes less effective than is the case for FMD elsewhere in the world (Maree *et al*., 2015).

Cattle may become persistently infected (carrier status) but there is no evidence to suggest that persistently infected cattle are able to transmit the infection to susceptible animals with which they come in close contact (Alexandersen, *et al.,* 2002; Vosloo, *et al*., 2002). Wildlife outside Africa has not, so far, been shown to be able to maintain FMD viruses (Weaver *et al*., 2013). In fact, the only wildlife species that has been shown to be capable of long-term maintenance of FMDVs is African buffalo, and this is only with SAT viruses (Weaver *et al*., 2013; Rweyemamu, *et al*., 2008b).

Currently, South Africa, Botswana and Namibia have recognition from the World Organisation for Animal Health (OIE) for zones being "free from FMD without vaccination". For more than three decades, some countries in the Southern African Development Community (SADC) region (Botswana, Namibia, South Africa and Zimbabwe) have used vaccination and fencing as well as other zoo-sanitary measures to prevent FMDVs entering these FMD-free zones (Thomson, G.R. and Penrith, ML., 2011). Vaccination has been used predominantly to control FMD in areas not recognised as free from FMD. These methods were effective until around the early 2000s (Thomson *et al*, 2013a). Thereafter the FMD situation in the SADC Region started deteriorating significantly. This has included cross-border movements of SAT viruses identified by genome sequencing (Brito et al., 2014; Thomson *et al*, 2013a).

There are three significant factors for this heightened FMD activity and the unexplained transboundary spread. Firstly, there has been a decline in the efficacy of vaccines used against SAT serotypes of FMD virus in the SADC Region (Thomson *et al*, 2013a). There is also a general decline in standards of control on the part of country Veterinary Services, especially vaccination programmes that do not administer vaccines to the numbers of animals and at the frequency required to generate adequate levels of herd immunity (Thomson *et al*, 2013a). The other significant factor is an increasing number of wildlife and cattle in endemic areas, such as the Zambezi Region (ZR) in Namibia and Ngamiland in Botswana, resulting in increased wildlife/livestock interaction (Thomson *et al*, 2013a; Cassidy *et al*., 2013). This prompted proposals for development of a regional strategy for progressive management of FMD in sub-Saharan Africa (Rweyemamu, *et al*., 2008; Domenech, 2011).

1.3 The significance of the study

The study was designed to create a system based on laboratory testing of sera and lymph nodes obtained from slaughter cattle to minimise the risk of carcasses infected with FMD virus entering the export value chain.

In order to validate the technical basis for the value-chain management approach and incorporating critical control points (including critical limits), background information on the distribution and levels of SAT viruses present in the tissues of infected animals needed to be established,

particularly for some tissue/organs that have so far not been adequately evaluated, i.e. the scientific data-base is inadequate (Paton *et al.,* 2010). The distribution of virus in the tissues under study was intended to provide background data essential for demonstrating the effectiveness of the value chain approach adopted. The presence or absence of virus in the tissues was intended to provide data for use in a quantitative risk assessment (Fosgate et al., in process) and thereby evaluation of the effectiveness of risk management measures applied in the ZR (Thomson *et al.,* 2013b).

1.4 Aims and objectives

Objectives of the study were to:

- Confirm findings from previous studies that, even in acutely infected animals, SAT viruses do not occur in detectable amounts in striated muscle before maturation;
- Quantify the levels of SAT viruses as measured by PCR (polymerase chain reaction – both conventional and real-time) that occur in body fat of slaughtered animals. Until now there were no data to indicate whether FMD viruses can be detected in body fat or not;
- Quantify the levels of SAT viruses as measured by PCR (conventional and real-time) that occur in lymph nodes of cattle in the acute stages of infection. The original protocol envisaged measurement of virus levels using virus isolation in cell cultures as well as PCR. However, virus isolation in cell cultures was not performed due to technical problems.
- Identify the lymph nodes in which SAT viruses can be detected most consistently in cattle in the acute stage of experimental infection;
- Design of an abattoir-based sampling strategy for identification of infected carcasses;
- Use of PCR and serology to develop a system through which carcasses derived from animals sub-clinically infected with FMD virus can be identified.

2 ASSESSMENT OF LEVELS OF FMDV IN CARCASS TISSUES FOLLOWING EXPERIMENTAL INFECTION WITH SAT SEROTYPES OF FMD VIRUS

2.1 Introduction

To better understand the virulence and tissue tropism in cattle of representative isolates of SAT1 and SAT2 serotypes obtained from cattle in the ZR of Namibia, 6 cattle were experimental infected in the ARC- OVI (TADP) bio-containment facility.

Levels of viral RNA in a range of tissues were determined using rt-PCR and quantitative RT-PCR assays as described previously (Alexandersen *et al*., 2001, 2002; Oleksiewicz *et al*., 2001; Reid *et al*., 2001, 2002, 2003, 2004; Moonen, 2003; Oem *et al*., 2005; Niedbalski and Kesy., 2010).

The information gathered from this experiment was necessary to identify lymph nodes that were most consistently infected; this information was used to design an abattoir-based sampling strategy for identification of carcasses derived from sub-clinically infected cattle slaughtered at Katima Mulilo abattoir through a combination of PCR and serology. That system was then used to test cattle routinely slaughtered in the ZR for presence of FMDVs.

2.2 Materials and Methods:

2.2.1 Ethical consideration and study approval:

The experimental infections were conducted in line with the animal welfare and ethics guidelines as stipulated by the ARC- OVI and University of Pretoria Animal Ethics Committees. The study was approved by the OVI animal ethics committee (*Approval number AEC 20.11*) (Appendix A), and the University of Pretoria Research Committee and the University of Pretoria Animal Ethics Committee (*Approval number V056-13*) (Appendices B and C).

Department of Agriculture, Forestry and Fisheries provided permission for this work in accordance with section 20 of the Animal Diseases Act (Act No. 35 of 1984). The reference number for the study is 12/11/1/1a (Appendix D).

FMDV infected cattle usually develop clinical signs that include a rise in body temperature, vesicles, erosions and ulceration in the mouth and at the skin-hoof junction on the feet, lethargy and inappetance (Thomson and Bastos, 2004). The study was designed to prevent animals from exhibiting exceptionally severe signs of disease such as severe lameness or hoof shedding, and extreme difficulties in eating. A NSPCA welfare score sheet shown in Appendix E was used to define the severity of result disease in the cattle.

2.2.2 Facilities for the experimental study

This study was conducted at the Agricultural Research Council–Onderstepoort Veterinary Institute (Transboundary Animal Diseases), ARC–OVI (TADs), an animal disease research laboratory with BSL-3 containment.

Each group of cattle (n=2) was housed in a self-contained stable with a minimum floor surface of 25.23 m^2. Different stables were used to house cattle infected with different isolates. The three pairs of cattle were infected at different times to minimize opportunity for cross contamination. The cattle were able to move freely within the confines of the stable. Rubber mats were supplied for bedding. Animals were fed a balanced commercial ration of high-roughage pellets (hay not suitable due to its impact on the facility's effluent sterilization system).

Two stable workers also maintained physical contact with the animals (including grooming) on a daily basis to get them used to being handled and reduce anxiety. The stables and food troughs were inspected daily to ensure they were clean. Water was provided *ad libitum* by the automated watering system.

The environment within the stables was controlled at relative humidity of ±35% and temperature of ±23°C. The pressure was maintained at approximately -40 Pascal, light was ±600 lux (automatically switched on around 6am and automatically switched off around 6pm).

2.2.3 Virus inoculums

The viruses used for intradermolingual inoculation in the experimental infection study are shown in Table 2. 1:

Table 2.1: SAT virus isolates used to infect cattle experimentally by intradermolingual inoculation.

Virus	Passage level	Dosage	Animal identity
SAT1 (Bot 1/06/1)	PK1; IBRS2/5	10^4 $TCID_{50}$ in a volume of 0.2 ml	1059; 1065
SAT2 (Nam 1/07/2)	PK1; IBRS2/4	10^4 $TCID_{50}$ in a volume of 0.2 ml	1017; 1071
SAT2 (Nam 3/10/2)	PK1; IBRS2/4	10^4 $TCID_{50}$ in a volume of 0.2 ml	1036; 1052

PK; pig kidney cells, IBRS: Instituto Biologico renal suino cells

These virus isolates were obtained from clinical material that was submitted to ARC–OVI (TADs) Diagnostics Section from outbreaks in the Zambezi Region of Namibia. The materials were stored in the ARC–OVI (TADs) virus bank.

2.2.4 Experimental animals

The cattle subject to experimental infection were approximately 10-month-old Nguni steers weighing about 200 kg each and sourced from the experimental farm animal unit of ARC – Onderstepoort Veterinary Institute. All animals were identified by ear tags in the left and right ears, with a unique number for each animal.

2.2.5 Experimental design

While the cattle were still on the Institute farm, 10 ml blood in red capped vacutainer® tubes was collected from ten randomly selected cattle. The blood was allowed to clot at ambient temperature and clotted blood samples were then centrifuged at 1450g for 10 minutes. Sera from each of the ten blood samples were screened at ARC-OVI (TADs) Diagnostics Laboratory to confirm absence of antibodies against FMD virus using Liquid Phase Blocking Enzyme Linked Immunosorbent Assay (LPB ELISA) and Non-structural protein ELISA (NSP ELISA) as described below. All animals used for experimental infection were serologically negative.

On day -7 post-infection, six cattle were transported from the experimental farm to the high containment animal facility. The cattle were allocated stables in pairs and allowed to habituate to the new environment for 7 days as stipulated in the ARC-OVI AEC approval letter.

2.2.6 Infection of cattle with FMD viruses

On day 0, each pair of cattle was sedated using 2% Xylazine; 0.67ml/100 kg intramuscularly into the neck muscles. Blood was collected from the external jugular vein into BD Vacutainer® tubes with clot activators and gel for serum separation from the packed cells. Two aliquots were collected, for testing purposes in the ARC –OVI (TADs) diagnostic laboratory.

The cattle were infected using the intradermolingual route into two sites on the dorsal surface of the tongue. The animals were then observed for two hours to check for any adverse reactions to the challenge.

All animals were under clinical observation every morning and afternoon from while in the bio-containment unit until euthanasia. The presence or absence of the following clinical signs was recorded by the responsible veterinarian in all cases:

- elevated temperature;
- inappetance to complete anorexia;
- nasal discharge;
- hypersalivation;
- lameness;
- recumbency;
- vesicles on tongue, mouth and feet

Clinical observations were recorded on a Clinical Signs Form.

2.2.7 Euthanasia and sample collection

Forty-eight hours after intradermolingual inoculation (period representing acute FMDv infection), the animals were euthanised by deep sedation with 2% Xylazine (0.67 ml/100 kg intramuscularly into the neck region) followed by stunning using a penetrating captive bolt pistol and then decapitation to ensure complete exsanguination. Using sterile instruments and taking care to ensure no contamination with other tissues (including blood and adventitious fascia), the following tissues were then collected within two hours of slaughter: Blood in EDTA and without coagulant

(2 x 5 ml), muscle tissue (*M*. psoas and *M*. longissimus dorsi); perirenal, mesenteric, intra-muscular and subcutaneous fat; bronchial, prescapular, submandibular, popliteal, ileac, mesenteric, parotid and retropharyngeal (medial and lateral) lymph nodes; spleen and red bone marrow from the tibia and radial bones. Tissue samples were stored at –80±10°C immediately after collection. Carcasses of cattle that were euthanised were, after *post mortem* examination and sampling, incinerated at TADs in accordance with relevant guidelines and codes of practice as well as statutory requirements.

2.2.8 Serological testing for FMDV specific antibodies

2.2.8.1 Liquid phase blocking ELISA (FMD antibody detection)

The LPBE, one of the OIE recommended ELISA methods for the detection of antibodies against FMDVs (OIE, 2012a), was used routinely in this study (Roeder and Le Blanc Smith, 1987; Hamblin, *et al*., 1986a; Hamblin, *et al*., 1986b; Hamblin, *et al*., 1987; Ferris and Dawson, 1988; Robiolo, *et al*., 1995; Smitsaart, *et al*., 1998; van Maanen and Terpstra, 1989; Paton *et al.,* 2006).

The test was based upon specific blocking of liquid phase FMD antigen by antibodies in the test serum sample. Rabbit anti-serum was raised to serotype-specific FMDV, absorbed onto microplates and used to capture the FMD type-specific antigen. FMD antigen was incubated in liquid phase with serial dilutions of serum and allowed to react with the specific FMD antigen and then the serum/antigen mixture was transferred to the coated plates. FMD antigen in the serum sample results in the formation of immune complexes and consequently reduces the amount of free antigen trapped by the immobilized rabbit antiserum. Guinea pig serum raised to the same serotype as the capture antibody was then added to the plates. Fewer guinea-pig anti-FMDV detection antibodies reacts in the next incubation step. This was followed by the addition of species-specific horseradish peroxidase conjugate. Colour developed after the addition of substrate/chromogen solution. If antibodies were present in the serum they would block the antigen/virus binding to the guinea pig antibodies, which resulted in little or no colour development. If colour development occured, it indicated that FMDV serotype-specific antibodies were absent in the test sample. If the end-point log titre of a sample was greater than or equal to 1.6 that was interpreted as positive for antibodies against FMDV. If a sample end-point dilution

was less than or equal to log 1.5, the sample was regarded as negative (no antibodies against FMDV present in a sample). Please refer to Appendix F for the protocol that was used for testing.

2.2.8.2 NSP ELISA Non-structural protein assays (FMD antibody detection)

The pre-bleed sera collected from the six experimental study cattle were also tested for NSP antibodies using the NSP Priocheck® FMDV ELISA. The NSP Priocheck® FMDV ELISA is a blocking ELISA which can be used to screen for recent infection with all serotypes of FMD virus (De Diego *et al*., 1997; Sørensen *et al*., 1998).

The test plates were coated with 3ABC specific monoclonal antibodies (mAb), followed by incubation with antigen (3ABC protein). Consequently, test plates of the kit contained FMDV non-structural antigen captured by the coated mAb.

Test samples were dispersed to the 96 well plate. After an incubation period, the plates were washed and the conjugate added. FMDV non-structural specific antibodies, directed against the non-structural proteins that were present in the test sample bound to the 3ABC proteins and blocked the binding of the monoclonal antibodies-horse radish peroxidase (mAb-HRPO). After incubation, the plates were washed and the chromogen (TMB) substrate was dispensed. After incubation, the colour development was stopped. Colour development measured optically at a wavelength of 450nm showed the presence of antibodies directed against FMDV. A percentage inhibition of less than 50% is interpreted as absence of antibodies against the non-structural protein of FMDV in the test sample. A percentage inhibition greater than 50% is interpreted as positive, that is, antibodies against the non-structural protein of FMDV are present in the test sample. Please refer to Appendix G for the protocol that was used for testing.

2.2.8.3 FMDV RNA detection

The viral RNA in the tissue samples was detected using conventional RT-PCR and a two-step real time RT-PCR assay.

Viral RNA was extracted from processed tissue supernatant using a modification (Bastos, 1998) of the guanldinium-silica based method. The samples were stored at -80C until utilised. Please refer to Appendix 5.8 for detailed procedures.

Complementary DNA synthesis from the RNA template was achieved using methods described by Bastos (1998). The RNA template was reverse transcribed with SuperSeript III^{rM} (Life Technologies) using with a modified olio-dT (CCATGGCGGCCGCTTFTTTTTTTTTTTT (polySAT DT) that annealed to the 3' UTR/polyA tail.

The conventional RT-PCR assays were performed using the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties (2012). Carrier RNA reconstituted in Buffer AVE was added to freshly prepared Buffer AVL. An amount of 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into each sample in the 1.5 ml micro centrifuge tube followed by mixing by pulse-vortexing. The mixture was incubated at room temperature (15–25°C) for 10 min. An amount of 560 µl of ethanol (96–100%) was added to the sample. The solution (630 µl) was then carefully applied to the QIAamp Mini column (in a 2 ml collection tube) and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube, and the waste was discarded. The centrifugation was done twice after which 500 µl of Buffer AW1was added. The samples were again centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube (provided), and the waste discarded. Buffer AW2 (500 µl) was added to the QIAamp Mini column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The QIAamp Mini column was placed in a clean 1.5 ml micro centrifuge tube and the old collection tube containing the filtrate was discarded. An amount of 60 µl Buffer AVE equilibrated to room temperature was added to the QIAamp Mini column and incubated at room temperature for 1 minute. The samples were then centrifuged at 6000 x g (8000 rpm) for 1 minute and further examined using agarose gel electrophoresis. This procedure will be carried out in the dark room. A 1.5% agarose gel was prepared. About 5 µl was loaded in the big well tray and 12 µl in a small well tray, followed by negative control and lastly the positive control. An amount of 5 µl 100 bp DNA ladder was loaded of in the 1st well and well following the positive control. The gel was placed in a tank and run the gel at 120 volts for 15 - 30 minutes. The gel was examined and

photographed under the UV transilluminator. The presence of a discrete band in the test sample PCR product that co-migrates with the PCR product of the positive control indicated positive results. The absence of bands in the PCR products of the tested samples indicated that samples are negative.

The real time RT-PCR assays were performed using the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by Office International des Epizooties (2012). For a single 50µl volume reaction, 7µl of cDNA was added to the 43µl of master mix containing the following; 4 µl each of 10pmol primer; forward ACTGGGTTTTACAAACCTGTGA and reverse GCGAGTCCTGCCACGGA (Callahan *et al*., 2002); 2µl of TaqMan® probe TCCITTGCACGCCGTGGGAC (Callahan *et al.*, 2002); 25µl 2x TaqMan universal PCR mix (Applied Biosystems) and 8µl nuclease-free water. The PCR amplification was done in a thermal eyeler of model sds 7500 (Applied Biosystems) using the following program; 1 cycle of 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds and 60°C for I minute. The threshold cycle (Ct) values that determined weather a sample was positlve or negative were determmed by in-house protocols at the ARC-OVI (TADs) laboratory. Positive controls had a Ct value below 40 whereas the strong positive controls had a Ct value below 20. The qPCR results were transformed to copies of RNA per ml by generation of a standard curve with different dilutions of the cDNA sample. All other reactions constituents were kept exactly the same as the reaction conditions. The cDNA concentrations were then plotted against the Ct time.

2.3 Results

2.3.1 Serology:

All the six animals had undetectable antibody levels to all three SAT serotypes (Titres <1.6 were considered negative for SAT serotype FMD). None of the cattle had detectable levels of non-structural proteins upon testing using NSP ELISA.

2.3.2 Clinical signs and gross lesions

There was an initial increase in body temperature to above 39.5°C within 24 hours following inoculation in all the six animals used in the study. The temperature rose further, in some cases beyond 40^0C as shown in Figure 2.1 below.

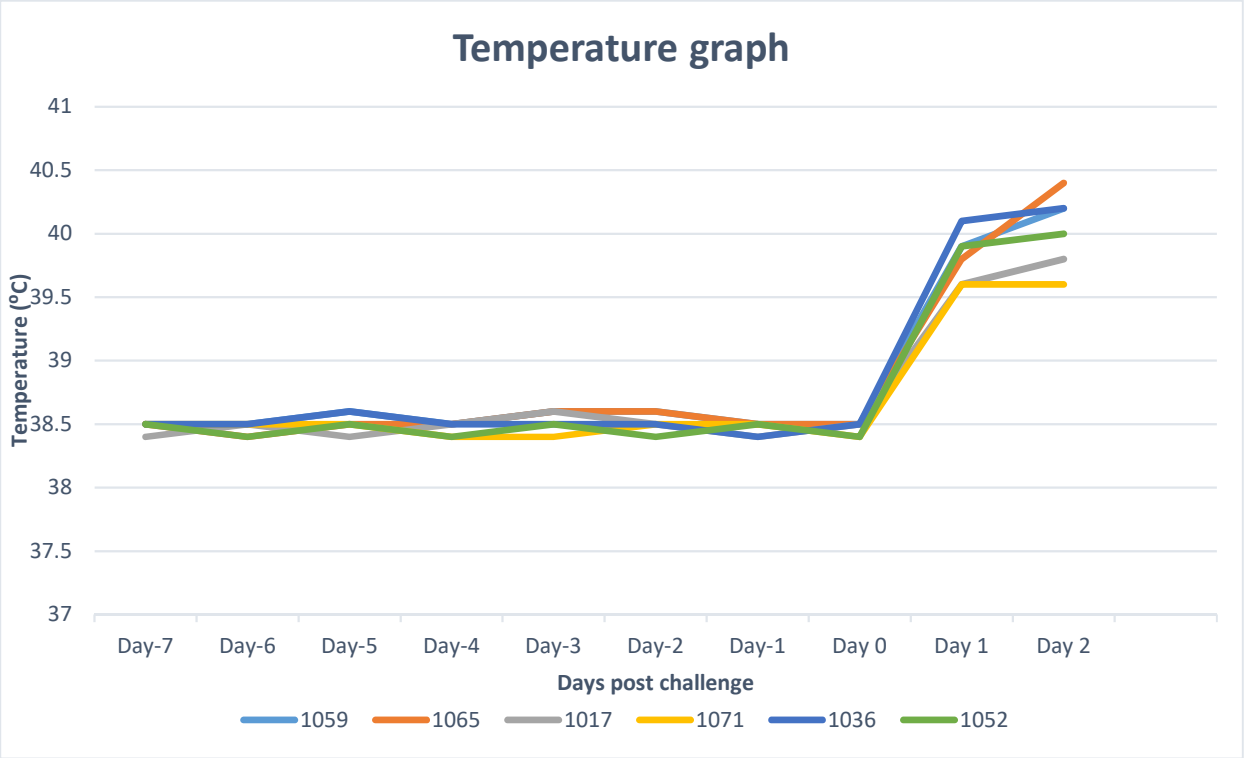

Figure 2.1: Rectal temperatures (^{0}C) of the six cattle from -7 days pre-infection to 2 days post infection. Temperatures $\geq$ 39.5 ^{0}C were considered as fever.

Moderate to severe tongue erosions with a diameter of ±20 to 30 mm were observed in all inoculated cattle at 24 hours post challenge. These lesions were only noticed on, and around the site of inoculation – there were no secondary lesions on the gums and muzzle. At 48 hours after inoculation, these erosions remained the same in size. At 24 hours post challenge, secondary lesions were noticed in at most one hoof in all the animals under observation. By 48 hours post inoculation, the hoof lesions had generalised to at least two feet for all animals as shown in tables 2.2, 2.3 and 2.4 below.

Table 2.2: FMD SAT 1 (Bot 1/06/1) clinical scores of animals at 0, 24 and 48 hours post infection

	1059			1065		
	0hpi	24hpi	48hpi	0hpi	24hpi	48hpi
Tongue (inoculation site)	0	1	2	0	1	1
RF hoof	0	0	0	0	1	1
LF hoof	0	1	2	0	1	1
RH hoof	0	0	0	0	0	0
LH hoof	0	0	1	0	0	0

hpi- hours post infection; RF- right fore; LF- left fore; RH- right hind; LH- left hind; 0- no lesion; 1- erosions; 2 severe erosions

Table 2.3: FMD SAT 2 (Nam 1/07/2) clinical scores of animals at 0, 24 and 48 hours post infection

	1017			1071		
	0hpi	24hpi	48hpi	0hpi	24hpi	48hpi
Tongue (inoculation site)	0	1	1	0	1	2
RF hoof	0	0	0	0	0	1
LF hoof	0	0	1	0	0	1
RH hoof	0	0	0	0	1	1
LH hoof	0	1	2	0	0	0

hpi- hours post infection; RF- right fore; LF- left fore; RH- right hind; LH- left hind; 0- no lesion; 1- erosions; 2 severe erosions

Table 2.4: FMD SAT 2 (Nam 3/10/2) clinical scores of animals at 0, 24 and 48 hours post infection

	1036			1052		
	0hpi	24hpi	48hpi	0hpi	24hpi	48hpi
Tongue (inoculation site)	0	1	2	0	1	1
RF hoof	0	0	0	0	0	1
LF hoof	0	0	1	0	0	0
RH hoof	0	1	1	0	0	1
LH hoof	0	0	1	0	0	0

hpi- hours post infection; RF- right fore; LF- left fore; RH- right hind; LH- left hind; 0- no lesion; 1- erosions; 2 severe erosions

2.3.3 FMD Viral RNA quantification

Quantitative real time rt- PCR results are shown in table 2.5 below.

Table 2.5 Summary of virus detection by conventional- PCR and real-time (quantitative) PCR (qPCR) in tissues/organs tested in 2 cattle experimentally infected with SAT 1 (Bot 1/06/1)

Virus	Animal number	Tissue/organ	rt-PCR	Real time rt-PCR Ct Values	qPCR (log_{10} copies per ml)
SAT 1 (Bot 1/06/1)	1065	*M. longissimus dorsi*	-	-	-
		M. psoas	-	-	-
		Retropharyngeal. ln – medial	+	32.39	3.5
		Retropharyngeal. ln – lateral	+	33.67	3.2
		Submandibular. ln	+	29.67	4.1
		Parotid ln	-	33.67	3.2
		Bronchial ln	+/-	-	-
		Prescapular ln	+	35.78	2.7
		Mesenteric ln	+	34.35	3.3
		Popliteal ln	+	34.23	3.3
		Spleen	+	29.32	4.2
		Perirenal fat	-	-	-
		Mesenteric fat	-	-	-
		Intra-muscular fat	-	-	-
		Subcutaneous fat	-	-	-
		Bone marrow	-	-	-
		Coronary band epithelia	+	24.36	4.9
	1059	*M. longissimus dorsi*	-	-	-
		M. psoas	-	-	-
		Retropharyngeal. ln – medial	+	-	-
		Retropharyngeal. ln – lateral	+	32.70	3.4
		Submandibular. ln	+	34.60	3.0
		Parotid ln	+	-	-
		Bronchial ln	-	-	-
		Prescapular ln	+	36.00	2.7
		Mesenteric ln	+	35.70	2.7
		Popliteal ln	+	31.30	3.8
		Spleen	-	-	-
		Perirenal fat	-	-	-
		Mesenteric fat	-	-	-
		Intra-muscular fat	-	-	-
		Subcutaneous fat	-	-	-
		Bone marrow	-	-	-
		Coronary band epithelia	+	28.20	4.3

Table 2.6: Summary of virus detection by conventional- PCR and real-time (quantitative) PCR (qPCR) in tissues/organs tested in 2 cattle experimentally infected with SAT 2 (Nam 1/07/2)

Virus	Animal number	Tissue/organ	rt-PCR	Real time rt-PCR Ct Values	qPCR (log_{10} copies per ml)
SAT 2 (Nam 1/07/2)	1071	*M. longissimus dorsi*	-	-	-
		M. psoas	-	-	-
		Retropharyngeal. ln – medial	-	-	-
		Retropharyngeal. ln – lateral	+	36.57	2.5
		Submandibular. ln	+	34.75	2.9
		Parotid ln	+	35.31	4.8
		Bronchial ln	-	-	-
		Prescapular ln	+	35.25	2.8
		Mesenteric ln	-	-	-
		Popliteal ln	+	34.20	3.0
		Spleen	+	36.15	2.6
		Perirenal fat	-	-	-
		Mesenteric fat	-	-	-
		Intra-muscular fat	-	-	-
		Subcutaneous fat	-	-	-
		Bone marrow	-	-	-
		Coronary band epithelia	+	29.24	4.3
	1017	*M. longissimus dorsi*	-	-	-
		M. psoas	-	-	-
		Retropharyngeal. ln – medial	+	34.69	3.0
		Retropharyngeal. ln – lateral	-	-	-
		Submandibular. ln	+	28.96	4.3
		Parotid ln	-	-	-
		Bronchial ln	-	-	-
		Prescapular ln	+	38.75	2.0
		Mesenteric ln	+	32.65	3.4
		Popliteal ln	+	32.42	3.4
		Spleen	+	36.58	2.1
		Perirenal fat	-	-	-
		Mesenteric fat	-	-	-
		Intra-muscular fat	-	-	-
		Subcutaneous fat	-	-	-
		Bone marrow	-	-	-
		Coronary band epithelia	+	26.84	4.8

Table 2.7: Summary of virus detection by conventional- PCR and real-time (quantitative) PCR (qPCR) in tissues/organs tested in 2 cattle experimentally infected with SAT 2 (Nam 3/10/2)

Virus	Animal number	Tissue/organ	rt-PCR	Real time rt-PCR Ct Values	qPCR (log_{10} copies per ml)
SAT 2 (Nam 3/10/2)	1036	*M. longissimus dorsi*	-	-	-
		M. psoas	-	-	-
		Retropharyngeal. ln – medial	+	29.10	4.3
		Retropharyngeal. ln – lateral	+	32.11	3.6
		Submandibular. ln	+	26.91	4.8
		Parotid ln	+	31.42	3.7
		Bronchial ln	+	32.23	3.5
		Prescapular ln	+	28.75	4.6
		Mesenteric ln	+	35.35	2.8
		Popliteal ln	+	30.26	4.0
		Spleen	-	-	-
		Perirenal fat	-	-	-
		Mesenteric fat	-	-	-
		Intra-muscular fat	-	-	-
		Subcutaneous fat	-	-	-
		Bone marrow	+	30.97	3.8
		Coronary band epithelia	+	24.66	4.9
	1052	*M. longissimus dorsi*	-	-	-
		M. psoas	-	-	-
		Retropharyngeal. ln – medial	-	-	-
		Retropharyngeal. ln – lateral	-	-	-
		Submandibular. ln	+	33.64	3.2
		Parotid ln	+	36.51	2.1
		Bronchial ln	-	-	-
		Prescapular ln	+	36.71	2.1
		Mesenteric ln	-	-	-
		Popliteal ln	+	34.30	3.0
		Spleen	+	35.42	2.8
		Perirenal fat	-	-	-
		Mesenteric fat	-	-	-
		Intra-muscular fat	-	-	-
		Subcutaneous fat	-	-	-
		Bone marrow	-	-	-
		Coronary band epithelia	+	26.76	4.8

2.4 Discussion

Typical clinical signs of an acute FMDV infection followed within 48 hours of intradermolingual inoculation. All animals had a fever 24 hours post infection, the fever persisted to 48 hours post infection at which point the animals awere euthanised.

FMD viral RNA was detected in all tissues sampled except fat and striated muscle; however, there were pronounced differences in the levels of viral load among tissues. Some tissues had consistently high viral RNA loads in all six carcasses while some had only detectable RNA levels in a proportion of the carcasses; however, there was no significant differences between serotypes. The tissues that were consistently shown to contain viral RNA were coronary band epithelia, submandibular lymph nodes, popliteal lymph nodes, and prescapular lymph nodes. One out of the six animals had detectable viral RNA in the bone marrow.

The load of viral RNA in hoof epithelia was typically 10 to 1000 times greater than the load of FMDV RNA measured in any other tissue. The presence of high viral RNA levels in foot epithelium indicated successful infection (Prato Murphy *et al*., 1999; Woodbury *et al*., 1995).

The above data, together with previous studies, which reported that lymph nodes were consistently positive for FMDV RNA (Prato Murphy *et al*., 1999; Woodbury *et al*., 1995; Zhang & Kitching, 2000; Zhang & Kitching, 2001), indicated that lymph nodes are a good indicator of the presence of SAT virus RNA in cattle. Whether replication occurs in lymphoid tissue remains unresolved (Juleff et al., 2008; 2012). This experimental study nevertheless provided the technical basis for using submandibular-, popliteal-, and prescapular lymph nodes as reliable sampling sites for demonstrating the presence of infection in acute cases of FMD.

In contrast, SAT viral RNA was not demonstrable in the striated muscle of experimentally infected cattle. This confirms results from previous studies, i.e. that viral RNA is rarely detectable in striated muscle of cattle even in animal slaughtered at the height of viraemia (Henderson and Brooksby, 1948; Anon, 1986).

The tests used did not to detect viral RNA in fat derived from three different sites. However, a deficiency of the method employed in this study was the use of a non-optimized protocol for isolating RNA from fat tissue. RNA extraction is a crucial step for monitoring gene expression; therefore, poorly extracted RNA (which may include degradation or presence of contaminants) can result in misleading results because extraction of high quality RNA with a reasonable yield from animal tissues with high lipid content is difficult, especially from adipose tissue (Méndez *et al.,* 2010). Improvement can be achieved in this respect by using TRI Reagent® (MRC Inc., US) and miRNeasy (Qiagen, Germany), i.e. methods that enable sufficient amounts of high quality RNA free of contaminants to be obtained (Méndez et al., 2010).

These findings support the rationale of the value chain approach to risk management in respect of FMD (Anon, 1986; Thomson *et al*., 2013). The implication is that as long as the lymph nodes and bones are removed from beef there is little risk of SAT viruses being present, i.e. even before maturation and deboning that provides significant addition risk mitigation (Paton *et al*., 2010; Thomson *et al*., 2013b). Follow up tests of the fat tissues using optimised methods (methods that enable sufficient amounts of high-quality RNA to be extracted) will further strengthen this argument.

2.5 Conclusions

The above results provided important information for the abattoir based study (see below) and achieved the set objectives. It was shown that SAT serotype viral RNA could not be detected with the methods employed in this study in body fat from three different sites in the carcasses sampled of six acutely infected cattle, *viz.* subcutaneous, peri-renal and intermuscular fat. However, as explained before, use of a new optimized protocol capable of yielding sufficient amount of high quality total RNA from adipose tissue free of contaminants and suitable for downstream experiments like RT-qPCR needs to be adopted to increase reliability of the results. This needs to be done before it can be safely claimed that fat presents negligible FMD risk. Only then will it be possible to fill this technical gap identified by the OIE with respect to safe trade in deboned beef (Paton *et al.,* 2010).

Confirmation was obtained of earlier findings indicating that FMD virus RNA rarely occurs in detectable quantities in striated muscle of infected cattle, even before maturation (Anon, 1986). Maturation of the beef, involving a decline in pH <6, would further decisively reduce the likelihood of FMD virus being present in beef from which bones and lymph nodes have been removed.

The occurrence of FMD virus in lymph nodes indicated that while most contained significant concentrations of virus, only submandibular, prescapular and popliteal lymph nodes had detectable virus RNA in all samples tested. This, together with quantification of virus RNA levels in lymph nodes (use of real-time [quantitative] PCR – Table 1) enabled the identification of these three lymph nodes as being appropriate for detection of SAT serotype viral RNA in cattle carcasses.

3 ASSESSMENT OF PRESENCE OF FOOT AND MOUTH DISEASE (FMD) VIRUS IN SLAUGHTERED CATTLE AT THE KATIMA MULILO ABATTOIR (ZAMBEZI REGION, NAMIBIA)

3.1 Introduction

The ZR of Namibia, until 2013 known as the Caprivi Region, lies at the centre of the Kavango-Zambezi Transfrontier Conservation Area (KAZA TFCA). Rivers and wetlands inundate its terrain (Namibia Tourism, 2015). This region is home to a large number of free roaming wildlife species susceptible to FMD.

The presence of buffalo in ZR poses a constant risk of transmission of FMDV from buffalo to cattle. Separation of cattle and wildlife populations using fences in the ZR is largely impractical owing to topography (major rivers and wetlands), and in addition, fencing is undesirable from the transfrontier conservation area perspective because it creates barriers to natural migratory routes of wildlife. This effectively means that it is impossible to create an FMD-free zone in the ZR and that livestock and their products from the region will, for the foreseeable future, be seen as a trade threat (Thomson et.al, 2013b). Based on currently available methodology, the only way to eradicate SAT serotypes of FMDV from the Zambezi region would be to eliminate African buffalo from the Region. This is unsustainable from a wildlife conservation perspective (Thomson and Penrith, 2015).

An abattoir, owned by Government of Namibia and managed by the semi-private company, MeatCo, operates intermittently and has in the past been able to apply basic commodity-based trade (CBT) principles in the export of beef to South Africa, i.e. until 2007 when the ZR experienced a prolonged FMD outbreak. This abattoir has now been closed for about a year but only after the Meat Board of Namibia ZR project closed. All of the recorded outbreaks since 2000 are shown in the OIE's World Animal Health Information Database (WAHID) database as being in the east of the ZR (Fig. 3.1). Apart from the 2007-8 FMD outbreak in ZR, which was attributed to illegal cattle movements from Zambia, all the other outbreaks listed in Table 3.1 were attributed to contact between cattle and buffalo (DVS, 2013).

Figure 3.1: FMD outbreaks in ZR and surrounding areas from OIE WAHID database, 2007 to 2015

Table 3.1: Timing and size of FMD outbreaks from 2000 to 2015 in the ZR

	FMDV serotype	**Duration of outbreak**	**No of cases**	**Number of villages affected**	**Max days between reports**	**Location of index case**
2000	SAT1	29 days	1	1	0	Kasika
2007	SAT2	396 days	63	24	42	Nankuntwe

2010	SAT1	28 days	11	1	9	Impalila
2011	SAT1	85 days	12	1	13	Masikili
2013	SAT1	24 days	3	3	5	Invilivinzi
2014	Not typed	43 days	9	5	8	Linyanti
2015	SAT2	21 days	7	3	15	Kanono/Shaile

Article 8.7.25 of the OIE's TAHC[1] (2014) recommended measures required to ensure safe importation of fresh meat from FMD infected countries or zones, where there is an official control programme for FMD which involves compulsory systematic vaccination of cattle. In an attempt to demonstrate how HACCP and CBT approaches can be integrated to manage FMD risk associated with beef production in an area where the SAT serotypes of FMD virus are endemic in free-ranging African buffalo (S*yncerus caffer*), a pilot study was conducted in the ZR, Namibia (Millennium Challenge Account, Namibia, undated) over the period 2010-2015.

Management of the FMD risk for this value chain, required by Article 8.7.25 of the OIE's TAHC (2014) and Namibia's Competent Authority, incorporated additional measures that were part of the prerequisite program (Figure 3.2). This resulted in a multiple-barrier system for FMD risk management. Part of the FMD risk management involved a three-week quarantine of all slaughter cattle in a government-owned and managed quarantine facility. All cattle were re-vaccinated against FMD upon entry to the quarantine facility. Physical inspection was conducted on all cattle entering and leaving the quarantine station. This was not a requirement of Article 8.7.25 of the OIE's TAHC (2014). Using this integrated HACCP and CBT approach, sequential barriers against

[1] Article 8.7.25 was used when this project was conducted in the ZR. It was replaced by Article 8.8.22 since May 2015. Article 8.8.22's Clause1(d) TAHC (2016) requires that meat should be derived from animals which 'were kept for the past 30 days in an establishment, and that FMD has not occurred within a 10- kilometer radius of the establishment during this period, or that the cattle underwent pre-slaughter quarantine'. This clause was absent in Article 8.7.25 which prevented its full application in ZR because of the abundant free-roaming wildlife.

the presence of FMD virus at critical stages along the value chain would be created together with appropriate monitoring of critical control points to enable recording, auditing and certification.

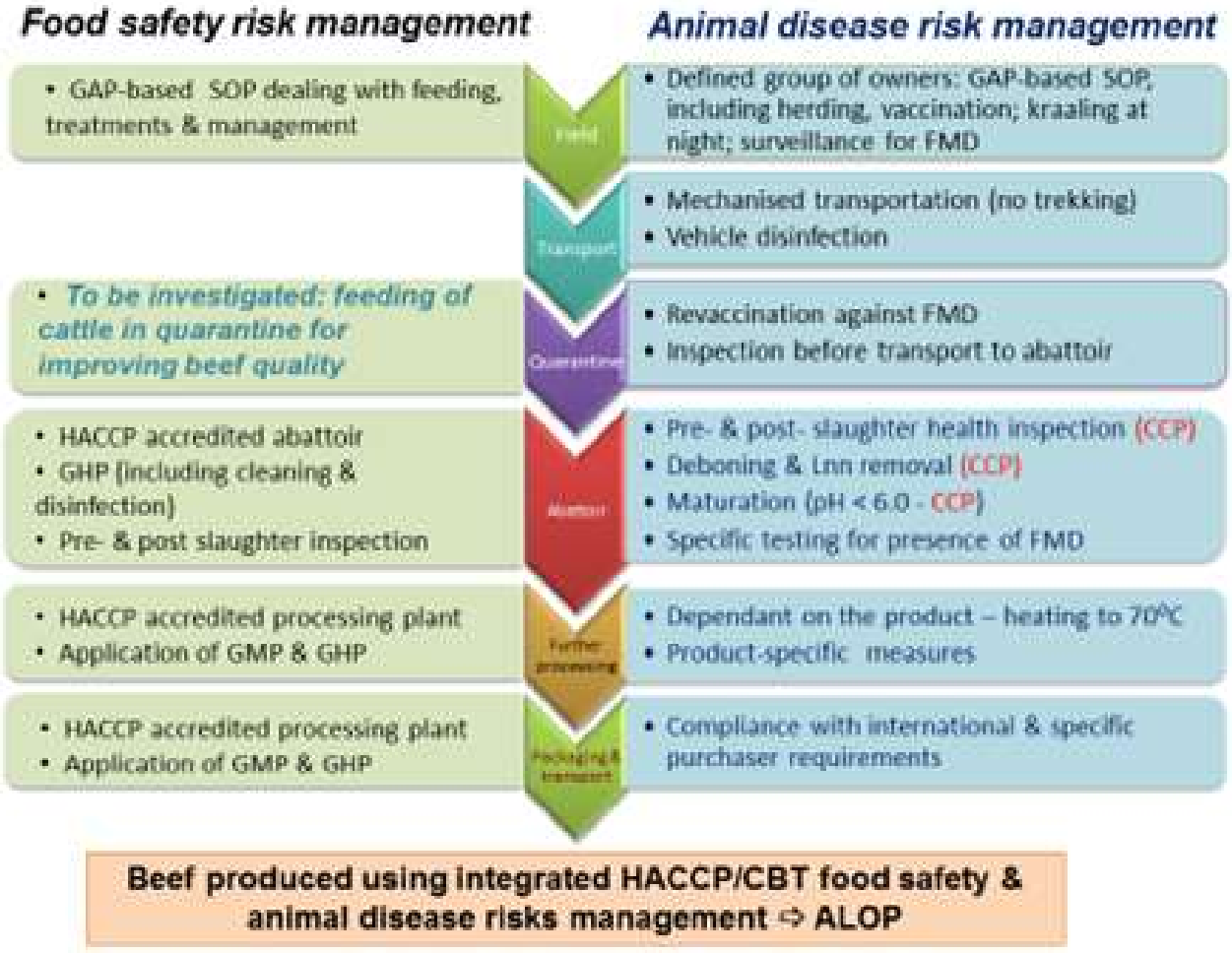

Figure 3.2: The integrated (food safety & FMD) risk management system for deboned beef produced in the Zambezi Region of Namibia (Thomson et al., 2013b)

This project was conducted to show that the risk posed by deboned beef derived from cattle raised in the FMD infected zone of the ZR is negligible, i.e. acceptable/appropriate. This chapter focuses

on providing laboratory evidence on the safety of deboned beef that has been subjected to value chain-risk mitigation. The specific activities of the study were:

(1) Serological monitoring of slaughter cattle to verify their FMDV antibody status;

(2) Screening of selected lymph nodes from slaughtered cattle by quantitative RT- PCR in order to identify FMD viruses possibly present in slaughtered cattle derived from high-risk areas.

3.2 Materials and methods

3.2.1 Research committee approval

The study was approved by the University of Pretoria, Faculty of Veterinary Science Research Committee (*Approval number V056-13*).

3.2.2 Study location

The study was conducted at the Katima Mulilo abattoir located on the outskirts of Katima Mulilo town in the ZR of Namibia. All beef exported from ZR is derived from cattle slaughtered at the Katima Mulilo abattoir. All cattle slaughtered at the abattoir are kept in quarantine for 21 days based on an "all-in, all-out" approach. The quarantine stations used in this study are managed by the Department of Veterinary Services (DVS) to control contagious diseases like FMD and contagious bovine pleuropneumonia (CBPP) by quarantining slaughter cattle.

3.2.3 Study design

3.2.3.1 Sample size justification

The sampling design for identification of SAT virus infection in cattle using a combination of RNA detection (rt-PCR), LPB ELISA and NSP ELISA in high-risk populations where there are no physical (i.e. clinical) signs of infection, was based on the following facts or justifiable assumptions:

1. Two high risk cattle populations in the Zambezi were identified (Sangwali and Lizauli); the risk being determined by regular contact between cattle in the location concerned and resident buffalo.

2. The cattle populations of Sangwali and Lizauli are estimated to be 6 288 and 2 522 respectively, giving a combined total of 8810 head. This is according to the records obtained from Veterinary Office at Katima Mulilo.

All cattle in the Zambezi have reportedly been repeatedly vaccinated against FMD in the last few years and therefore a high sero-prevalence rate (i.e. >70%) was anticipated. This was checked by testing of the sera of cattle using LPB ELISAs. For an animal to be identified as having suffered active FMD infection would require it to be NSP antibody positive.

3. The results of the experimental infection study, conducted at the ARC-Onderstepoort Veterinary Institute (Chapter 2) aimed partly at identifying candidate tissues for collection during the abattoir survey, indicated that in infected animals, of all the tissues tested, three lymph nodes (submandibular, prescapular and popliteal) contained viral-specific RNA in 6/6 cases, i.e. viral RNA present in all the of 6 cases.

4. For the purpose of identifying FMD-positive animals, it was assumed that at least 3 of 4 tests will need to be positive; the four tests being the three PCRs on the lymph nodes mentioned above and NSP serology.

3.2.3.2 Calculation of sample size required

The following values for variables were set in order to perform the calculation that utilized the open-access. FreeCalc2. facility developed by Ausvet (http://epitools.ausvet.com.au/contentphp?page=Freecalc2):

- Population size. 8 810
- Design prevalence. 0.05 (i.e. prevalence of infection set at 5%)

- Test specificity and Test sensitivity[2]

Diagnostic test	Test specificity	Test sensitivity
Diagnostic PCR (RT-PCR)	0.99	0.95
LPBE (SAT1)	1	0.92
LPBE (SAT2)	1	0.91
LPBE (SAT3)	1	0.99
NSP ELISA	0.95	0.87

3.2.3.3 Outcome

Required sample = 59 + 10% to allow for wastage

Cut point = 2

Total sample size = 65

3.2.3.4 Interpretation

If a random sample of 59 cattle was taken from the Sangwali and Lizauli areas, and 2 or fewer reactors are found, the probability that the population is undergoing active infection with a SAT virus at a prevalence of 10% or higher is 0.047 (i.e. very unlikely).

3.2.4 Conclusion of the number of cattle required

Sixty-five cattle needed to be selected from the two high-risk areas (Sangwali and Lizauli) and slaughtered at the Katima Mulilo Abattoir for sample collection. This would allow a statistical assessment of the possibility that FMD was circulating in the populations from which the cattle were derived.

[2] These data were derived from the preliminary validation work conducted at the TADs laboratory

3.2.5 Specimen collection and transportation

Sampling was carried out in two phases. Submandibular, popliteal and prescapular lymph nodes, and sera specimens were collected from 76 cattle during first abattoir sampling. In the follow up phase, 72 sera and prescapular lymph nodes were collected.

3.2.5.1 Blood sampling

Whole blood samples were collected from the jugular vein after stunning and decapitation of the cattle along the slaughter chain. About 10 ml blood in red capped vacutainer® tubes were collected per animal. The blood was allowed to clot at ambient temperature in the laboratory of the abattoir. Clotted blood samples were then centrifuged at 1450 g for 10 minutes. Serum was decanted into sterile cryovials and stored at -20°C until testing. Two aliquots were prepared per sample, one destined for ARC-OVI and another was kept for reference purposes at the Central Veterinary Laboratory (CVL) in Windhoek.

3.2.5.2 Tissue sampling

After complete exsanguination, lymph node tissues were collected using clean instruments and taking care to ensure no contamination with other tissues (including blood and adventitious fascia). The lymph nodes were trimmed using clean forceps and scalpel blades to be less than 0.5 cm in at least one dimension and submerged in 2.5 ml of RNAlater® solution. The RNAlater® samples were kept at 4°C prior to testing. Two aliquots were prepared, one destined for OVI and another to be kept as backup at the CVL in Windhoek.

The sera and tissues collected were packaged according to the Regulations of the IATA and National Road Traffic Act, 1996 (Act No. 93 of 1996) of the Republic of South Africa and transported under the necessary movement permit on ice to the ARC- OVI (TADP) for testing.

3.2.6 Serological testing for FMDV-specific antibodies

A total of 148 sera samples (76 sera from the first sampling, and 72 sera from the second sampling) were tested for FMD-specific antibodies against FMDV structural proteins using LPB ELISA as previously described in section 2.2.8.1. Assays were performed using an in-house developed ELISA for SAT 1, SAT 2 and SAT 3. This test is based upon serotype specific blocking of liquid phase FMD heterologous antigen by antibodies in the test serum sample. Antibody titres were

expressed as the 50% end-point titres and sera with titres ≥1.6 $\log_{10}$ were classified as seropositive. Sera with titres ≥2.0 $\log_{10}$ were classified as highly seropositive, and probably protective. The 148 serum samples were also tested for FMD-specific antibodies against FMDV non-structural proteins using the NSP Priocheck® FMDV ELISA as described previously in section 2.2.8.2. Please refer to Appendix G for the protocol.

3.2.7 FMD Viral RNA quantification

Lymph nodes were tested for presence of FMD viral RNA using diagnostic PCR as described in section 2.2.8.3 (please refer to Appendices H and I). The RNA content was analysed by real time RT-PCR using the Applied Biosystems 7500 Thermocycler as described in section 2.2.8.3 (Callahan *et al*., 2002; Shaw *et al.,* 2007). The threshold cycle (Ct) values that determined weather a sample was positlve or negative were determmed by in-house protocols at the ARC-OVI (TADs) laboratory. Positive controls had a Ct value below 40 whereas the strong positive controls had a Ct value below 20. Please refer to Appendix I for the protocol.

3.3 Results:

3.3.1 Serology:

During the first sampling, more than 90% of animals showed very high (probably protective) titres to LPB ELISA (Tables 3.2, 3.3, 3.4 and Appendix J). About 6% of these animals were positive for antibodies to non- structural proteins (Appendix J). Conversely, the 72 samples from the second phase of the project showed low seropositivity across SAT 1, 2 and 3 (Appendix K). A substantial percentage of these animals were negative on LPB ELISA (22.2%, 45.8% and 38.9% for SAT1, SAT2 and SAT3 respectively). Only 40.3%, 25.0% and 29.2% of the cattle showed high (probably protective) titres to SAT1, SAT2 and SAT3 respectively on LPB ELISA (Tables 3.5, 3.6, 3.7 and Appendix K). Just under 7% of these animals were positive for antibodies to non- structural proteins (Appendix K).

3.3.2 Viral RNA detection:

For the first batch of 76 animals, three lymph nodes (submandibular, prescapular & popliteal) were tested per animal using real-time quantitative PCR. In the second batch of 72 cattle only prescapular lymph nodes were tested.

No viral RNA was detected in any of the lymph nodes of the 148 animals sampled at the Katima abattoir.

Table 3.2: Summary of testing for antibodies to the structural proteins of SAT-1 FMD virus from first phase sampling

SAT 1 Log_{10} Titre	Number	Percentage
<1.6 (negative)	1/76	1.3%
1.6-1.9 (weak positive)	5/76	6.6%
≥2.0 (strong positive)	70/76	92.1%

Table 3.3: Summary of testing for antibodies to the structural proteins of SAT-2 FMD virus from first phase sampling

SAT 2 Log_{10} Titre	Number	Percentage
<1.6 (negative)	0/76	0%
1.6-1.9 (weak positive)	2/76	2.6%
≥2.0 (strong positive)	74/76	97.4%

Table 3.4: Summary of testing for antibodies to the structural proteins of SAT-3 FMD virus from first phase sampling

SAT 3 Log_{10} Titre	Number	Percentage
<1.6 (negative)	1/76	1.3%
1.6-1.9 (weak positive)	5/76	6.6%
≥2.0 (strong positive)	70/76	92.1%

Table 3.5: Summary of testing for antibodies to the structural proteins of SAT-1 FMD virus from second phase sampling

SAT 1 Log_{10} Titre	Number	Percentage
<1.6 (negative)	16/72	22.2%
1.6-1.9 (weak positive)	27/72	37.5%
≥2.0 (strong positive)	29/72	40.3%

Table 3.6: Summary of testing for antibodies to the structural proteins of SAT-2 FMD virus from second phase sampling

SAT 2 Log_{10} Titre	Number	Percentage
<1.6 (negative)	33/72	45.8%
1.6-1.9 (weak positive)	21/72	29.2%
≥2.0 (strong positive)	18/72	25.0%

Table 3.7: Summary of testing for antibodies to the structural proteins of SAT-3 FMD virus from second phase sampling

SAT 3 Log_{10} Titre	Number	Percentage
<1.6 (negative)	28/72	38.9%
1.6-1.9 (weak positive)	23/72	31.9%
≥2.0 (strong positive)	21/72	29.2%

3.4 Discussion

The sampling of carcasses derived from clinically healthy cattle at the Katima Mulilo abattoir (i.e. animals that had passed the standard *ante-mortem* examination) was designed to develop a process to exclude the possibility that beef from infected cattle, whether sub-clinical or not, could reach the market. This was achieved using a combination of RNA detection applied to selected lymph nodes and examination of the sera of slaughtered animals. Serological testing comprised tests for antibodies to non-structural viral proteins (NSP), using a commercial ELISA kit (Prionics Priocheck® FMDV NS- FMDV antibody test kit), and an 'in-house' liquid-phase blocking (LPB) ELISA for detection of antibodies directed at structural viral proteins as previously described

LPB ELISA was performed inorder to measure of the level of herd immunity induced by vaccination. Testing sera using NSP ELISA was intended to identify animals likely to have

undergone natural infection in the recent past. Screening of selected lymph nodes by PCR was used to identify animnals in which FMDV was still present in lymph nodes following infection.

There was deviation from the original target numbers and localities where the project intended to sample. This was because during the period that sampling was scheduled too few cattle were booked for slaughter at the abattoir from that source. Therefore, the Project was forced to sample animals from additional locations where cattle-buffalo contact rates were likely to be lower. The original sampling strategy envisaged obtaining a sample size adequate to detect infection prevalence of 10% but as previously explained, that proved unworkable because the abattoir was closed for much of the time when sampling was planned due to a FMD outbreak in another location. The animals that were sampled did were not vaccinated upon entry to the pre-slaughter quarantine station. Ultimately, a sample of 148 cattle was obtained.

The selection of the three lymph nodes (submandibular, prescapular and popliteal) was based on results of the experimental infection study (Chapter 2). The study indicated that in the six infected cattle, these lymph nodes consistently contained viral-specific RNA, i.e. viral RNA present in all of 6 animals. It is important to note that conducting clinical animal studies in a high containment facility is expensive and difficult to manage if the numbers are high. Therefore, even though the results from the six animals show high probability, they are not statistically valid. For practical reasons the six animals were all that was available; it was therefore deemed that detection of infection in these specific lymph nodes can be accepted as a reliable method for identification of the presence of active infection in individual cattle.

None of the lymph nodes derived from the 148 slaughter cattle tested in the 1st and 2nd sampling contained detectable levels of viral RNA. While the sampling of these animals was not actively randomized, this finding suggests a 95% probability that the prevalence rate of cattle infected with SAT viruses in the Zambezi Region is less than 2.2% (assuming a test sensitivity of 0.9 and specificity of 1[G. Fosgate, personal communication, 2014]).

The value chain management approach to this Project (Figure 3.2), required that all cattle that were part of the Project be re-vaccinated against FMD on introduction to a quarantine station, i.e. three weeks prior to slaughter. This was implemented to ensure that all cattle slaughtered would have high levels of vaccinal immunity to the SAT viruses at the time of slaughter even if routine field vaccination was inadequately implemented.

LPBE testing conducted during the first sampling demonstrated that revaccination of cattle on introduction to the quarantine station three weeks before slaughter resulted in high levels of vaccine-induced immunity (all titres > 2.0 $\log_{10}$ were considered potentially protective) at the time of slaughter. The results showed that 97.4% of all the animals developed antibody levels considered to be protective to SAT 2 virus and 92.1% to SAT 1 and 3 (Tables3.2, 3.3 and 3.4). Conversely, it is evident from Tables 3.5, 3.6 and 3.7 that many animals were not vaccinated upon introduction into the quarantine station, i.e. they had undetectable antibody levels to all three SAT serotypes when slaughtered. The tables show that 40.3% of all the animals did not develop antibodies to SAT 1 virus, 25.0% to SAT 2, and 29.2% to SAT 3. These results also show that field vaccination of cattle (i.e. before they entered the QS) was inadequate. As explained above, sampling animals that were not vaccinated upon introduction into the QS was necessary to make up numbers of animals that were not officially part of the project (some of the cattle were not vaccinated routinely upon introduction to the quarantine station).

The results pertaining to NSP tests presented in Appendices J and K require explanation because, at face value, 5.4% prevalence of antibody indicates the likelihood of infection in some sampled animals because administration of purified vaccine does not stimulate significant production of antibody to NSP proteins (Doel, 2003; Parida, 2009). However, three factors indicate that the NSP reactors probably represented false-positive reactions: firstly, the failure of PCR testing to identify viruses in the lymph nodes of the same slaughtered cattle; secondly, most animals slaughtered at the Katima Mulilo abattoir are old animals and for that reason they would almost certainly have been vaccinated in the past with non-purified vaccine. Purified vaccine only became available just over a year prior to sampling. Lastly, a test specificity of 95% creates the expectation of 5% false positive reactions.

4 Summary and Conclusions

The study was designed to test a system based on laboratory testing of sera and lymph nodes obtained from slaughter cattle in an area where endemically infected buffalo are present to minimise the risk of cattle carcasses infected with FMD virus entering the export market.

In order to validate the technical basis for the value-chain management approach and incorporating critical control points (including critical limits), background information on the distribution and levels of SAT viruses present in the tissues of infected animals needed to be established particularly for some tissue/organs that have so far not been adequately evaluated, i.e. the scientific data-base is inadequate (Paton *et al.,* 2010). The distribution of virus in the tissues under study was intended to provide background data essential for demonstrating the effectiveness of the value chain approach adopted.

One of the objectives of this study was to confirm previously published findings that, in acutely infected animals, SAT viruses occur rarely in detectable amounts in striated muscle, even prior to maturation. The study also sought to quantify the levels of SAT viruses as measured by PCR (polymerase chain reaction – both conventional and real-time) that occur in lymph nodes of cattle in the acute stages of infection with SAT viruses, as well as in body fat of slaughtered animals. Until now, there were no data to indicate whether FMD viruses can be detected in body fat or not. The original protocol envisaged measurement of virus levels using virus isolation in cell cultures as well as PCR. However, virus isolation in cell cultures was not performed due to deficiencies in the cell culture system available over the course of this study. The experimental workmoreover intended to identify the lymph nodes in which SAT viruses can be detected most consistently in cattle in the acute stage of experimental infection. These lymph nodes could then be utilised in the design of an abattoir-based sampling strategy for identification of infected carcasses. The second objective of the study was to use PCR and serology to test a system through which carcasses derived from animals, even if only subclinically, can be identified.

Experimental infection of six cattle with three different SAT isolates was used ascertain the distribution of test viruses in carcasses of those animals immediately after slaughter and exsanguination. The results showed that: (1) high copy numbers of viral RNA were present in vesicular epithelium (i.e. in secondary lesions) and lymph nodes of all cattle, (2) viral RNA was not detectable in muscle tissue or body fat.

Unfortunately, levels of viraemia were not determined because the samples were lost. Furthermore, the results of testing of fat tissue cannot be considered reliable because the method employed was not optimized as explained in the section dealing with experimental infection. These omissions will need to be corrected to fill the technical gap with respect to safe trade in deboned beef (Paton *et al.,* 2010).

Confirmation was obtained of earlier findings indicating that FMD virus RNA rarely occurs in detectable quantities in striated muscle of infected cattle, even before maturation (Anon, 1986). Maturation of the beef, involving a decline in pH <6, would further decisively reduce the likelihood of FMD virus being present in beef from which bones and lymph nodes have been removed.

The occurrence of FMD virus in lymph nodes indicated that while most contained significant concentrations of virus, only submandibular, prescapular and popliteal lymph nodes had detectable virus RNA in all tests conducted. This, together with quantification of virus RNA levels in lymph nodes (use of real-time [quantitative] PCR – Table 1) enabled the identification of three lymph nodes (sub-mandibular, pre-scapular and popliteal) as being appropriate for reliable detection of SAT serotype viral RNA in cattle carcasses. The results provided important information for the abattoir-based study.

For the abattoir survey, 148 cattle derived mainly from high risk areas were sampled for identification SAT serotype viruses in specific lymph nodes. Sub-mandibular, pre-scapular and popliteal lymph nodes from these cattle were examined based on the results of the prior experimental infection study.

Serological testing of slaughter animals was also conducted to verify their FMDV antibody levels (LPBE) and likely infection status (NSP ELISA). None of the lymph nodes tested contained detectable levels of viral RNA. The serological testing demonstrated that revaccination of cattle on introduction to the quarantine station three weeks before slaughter resulted in high levels of vaccine-induced immunity (possibly protective) at the time of slaughter. There were a few NSP ELISA positive animals from the sample. This indicated the possibility of infection in some sampled animals at some indeterminant time prior to slaughter or false-positive reactors or animals vaccinated with unpurified vaccines. That issue was therefore not resolved.

It was established that RT-PCR testing of selected lymph nodes (submandibular, prescapular and popliteal) in addition to DIVA serology, is likely to identify the presence of infection were SAT viruses to be circulating in the cattle population. This system could potentially be used to provide an assurance mechanism for the value chain approach. However, for that to be the case a system to ensure the rapid availability of test results would be required.

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5 Appendices

5.1 Appendix A: ARC-Animal Ethics Committee Approval

ARC • LNR
Excellence in Research and Development

APPROVED
AEC 20.11

Onderstepoort Veterinary Institute

Animal Ethics

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

APPROVAL PERIOD: 2011/ 2012

PROJECT NUMBER:	TADP-S-2011/02			
PROJECT TITLE:	Investigation on the distribution levels of FMD viruses in tissues of cattle infected with SAT serotypes			
PROJECT LEADER:	Dr G R Thomson			
DIVISION:	Transboundary Animal Diseases Programme			
CATEGORY:	D			
SPECIES OF ANIMAL:	Bovine			
NUMBER OF ANIMALS:	6			
NOT APPROVED:				
APPROVED:	APPROVED			

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

SIGNATURE: Dr L. Lopez
CHAIRPERSON ANIMAL ETHICS COMMITTEE

DATE: 03-10-2011

12

5.2 Appendix B: University of Pretoria Rescom Appoval

Ref: V056/13

22 January 2014

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(gavin@tadscientific.co.za)

Dear Prof Thomson

PROTOCOL V056/13: DEVELOPING QUALITY CONTROL SYSTEMS THAT WILL MEET THE REQUIREMENTS OF LUCRATIVE MARKETS FOR BEEF PRODUCED IN THE FOOT AND MOUTH DISEASE ENDEMIC ZONES IN THE CAPRIVI REGION OF NAMIBIA – *PB Mutowembwa*

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly take note of the attached document.

Kind regards

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Prof JAW Coetzer, Deputy Dean: Research (koos.coetzer@up.ac.za)
PB Mutowembwa, Researcher (pbmuto@gmail.com / mutowembwap@arc.agric.za)
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5.3 Appendix C: University of Pretoria Animal Ethics Committee Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Investigation on the distribution levels of FMD viruses in tissues of cattle infected with SAT serotypes**
PROJECT NUMBER	**V056-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. PB Mutowembwa**

STUDENT NUMBER (where applicable)	**131 48 487**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Bovine**	
NUMBER OF ANIMALS	**6**	
Approval period to use animals for research/testing purposes		**January – May 2014**
SUPERVISOR	**Prof. G Thompson**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	27 January 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

5.4 Appendix D: DAFF Section 20 Approval

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7502
Fax: 012 319 7470
E-mail: ThaboMo@daff.gov.za
Enquiries: Mr. Thabo Motsisi
Our Ref: 12/11/1/1a
Your Ref No :

Date: 03 November 2011

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

Dr Gavin Thomson

Your fax / memo / letter/ Email dated 26 August 2011 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study ,with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa

Title of research/study: "Investigation on the distribution levels of FMD viruses in tissues of cattle infected with SAT serotypes"
Researcher (s): Dr Gavin Thomson
Institution: ARC-OVI-Trans Boundary Animal Diseases Programme
Your Ref./ Project Number:
Our ref Number: 12/11/1/1a

Kind regards

DIRECTOR: ANIMAL HEALTH

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5.5 Appendix E: NSPCA welfare sheet

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

MILD	MODERATE	SUBSTANTIAL	SUBSTANTIAL PROTOCOL HUMANE END-POINTS
General			
• Separation from others, • Delayed response to stimuli, slower movements, lethargic	• Abnormal posture e.g. head hung, back arched, lethagic	• Ignores human approach • rises and/or moves with great reluctance,	• Prolonged substantial behaviour of more than 3 days • signs of severe pain e.g. vocalization, grinding of teeth, trembling, oblivious to stimuli, • unwilling to rise for more than 4 hours, • unconscious
• Lack of enthusiasm for food	• Anorexia of up to 3 days duration	• Anorexia of more than 3 days duration.	• Anorexia of more than 4 days duration substantial weight loss
• Roughened coat	• Early signs of diarrhoea or other discharges	• Watery or bloody diarrhoea of more than 3 days duration, • heavy discharges from eyes, nose or mouth, • sunken eyes, • staring coat, • evident dehydration of skin, • appearance of pressure sores	• Watery or bloody diarrhoea of 4 days duration, • severe dehydration of 4 days duration, • multiple or large pressure sores unresponsive to therapy
• Body temperature increase	• Fever above 40ºC	• Fever above 40ºC for 4 or more days	
Infectious Agents: ***Foot-and-Mouth Disease***			

• Local surface temperature increase just above hoof • Evidence of vesicular lesion development such as blanching on sites such as limbs, face, mouth, teats or vulva. • Some nasal discharge and salivation	• Lameness • Small vesicles or ulcers at no more then three sites. • Erosive lesions on feet & tongue/snout.	• Unable to bear weight on more than one foot • Large tongue erosions leading to hypersalivation and lip smacking, painful erosions on feet leading to loss of weight bearing, blisters or erosions on teats.	• Persistence of substantial signs beyond 3 days • Severely superinfected lesion that does not respond to therapy within 24 hours and prevents eating or standing, severe under-running or shedding of a hoof.
Interventions			
Normal blood collection Anxiety and resistance during blood collection. Need to administer sedation. (Mild)	• Severe inflammation or infection at site of venepuncture (Moderate)	• Haemorrhage leading to anaemia, dehydration, tachycardia or tachypnoea. 'Shock syndrome' in pigs leading to cyanosis, dyspnoea	• Weakness or inertia due to blood loss. 'Shock syndrome' in pigs from which the pig does not recover when left quietly for 1-2 hours
Physical administration of infectious agent	• Anxiety & resistance during administration. Possible need to administer sedation • Severe inflammation or infection at site of administration		
Probang sampling	• Blood flecks in probing material. Discomfort on sampling (especially small ruminants) • Regurgitation of rumen content with risk of inhalation. • Pronounced haemorrhage into oral cavity/pharynx (extremely rare). Insertion of probing cup into trachea with consequent transient asphyxia.	• Inhalation pneumonia due to rumen contents. Delayed recovery of normal breathing following obstructive asphyxia (unlikely)	• Non-recovery of normal breathing following removal of probing. Pneumonia not responsive to treatment.

Collection of lachrymal fluid, nasal, oral or rectal swabs and/or milk			
Mild discomfort. Flecks of blood on/in the sampled material.	• Pronounced discomfort, haemorrhage or secondary infection at site of sampling.	• Pronounced discomfort, haemorrhage or secondary infection at site of sampling.	• Severe signs that do not respond to treatment within 2 days.
Drug or reagent e.g. corticosteroid, anti-inflammatory, vaccine, other infectious agent combinations, low dose general anaesthesia			
Adverse drug reactions that are mild and are included on the drug datasheet as recognised side effects of the drug or its administration	• Severe drug reactions, whether or not they are included on the drug data sheet.	• Severe drug reactions that compromise animal welfare and that do not respond to palliative treatment within 2 days.	• Severe drug reactions that compromise animal welfare and that persist for more than 2 days despite palliative treatment
Actions			
No action required maintain monitoring	• No direct action required maintain monitoring, discuss signs with named veterinary surgeon. Vet to perform full clinical examination if signs are unexpected in nature or severity.	• Direct action required increase monitoring, inform veterinary surgeon. Vet to perform full clinical examination. • Animal to be segregated where possible. • Placed on straw bedding • Consider oral rehydration treatment. • Ameliorative treatment course initiated if appropriate	• Direct action required inform named veterinary surgeon &/or project licence holder. • Humane termination of affected animal/s by schedule 1 method

5.6 Appendix F: ARC OVI (TADs) Liquid Phase Blocking ELISA SOP

DETECTION OF ANTIBODIES AGAINST THE STRUCTURAL PROTEINS OF FOOT-AND-MOUTH DISEASE VIRUS (FMDV) IN A LIQUID PHASE BLOCKING ELISA (LPBE)

CONTENTS:

1. Aim and field of application

The aim of the LPBE is to test sera of cloven-hoofed animal species for the presence of antibodies against the structural proteins of FMDV. Antibodies directed against one or more types of FMDV can either be identified in a four-fold dilution test (screening test) or a quantitative eightfold dilution test (titration test). The test has a very wide application. Since it is not dependent on anti-species conjugates it offers a single test with which the sera of any animal species can be tested. Serum antibodies are induced against the outer capsid structural proteins following both vaccination (domestic animals) against and infection (both domestic animals and game) with FMDV. The test can be used to establish FMD-free status in animals destined for export from South Africa or otherwise, import into South Africa.

2. Definitions and abbreviations.

FMDV	Foot-and-Mouth Disease Virus
NBS	Normal bovine serum
NRS	Normal rabbit serum
FMD antigen	Inactivated FMD virus used in ELISA
ELISA	Enzyme Linked Immunosorbent Assay
SAT	South African Type
PBS	Phosphate-buffered Saline
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
LPBE	Liquid Phase Blocking ELISA
Ag	Antigen

3. Principle

The test is a LPBE for the detection of FMDV antibodies in serum. The test is based upon specific blocking of liquid phase FMD antigen by antibodies in the test serum sample. Rabbit anti-serum is raised to serotype-specific FMDV, absorbed onto microplates and used to capture the FMD type-specific antigen. FMD antigen is incubated in liquid phase with a serial dilution of serum and allowed to react with the specific FMD antigen and then the serum/antigen mixture is transferred to the coated plates. FMD Ag in the serum sample will result in the formation of immune complexes and consequently reduce the amount of free antigen trapped by the immobilized rabbit antiserum. Guinea pig serum raised to the same serotype as the capture antibody is then added to the plates. Fewer guinea-pig anti-FMDV detection antibodies will react in the next incubation step. This is followed by the addition of species-specific horseradish peroxidase conjugate. Colour develops after the addition of substrate/chromogen solution. If antibodies are present in the serum they will block the antigen/virus binding to the guinea pig antibodies, which will result in little or no colour development. If colour development occurs, it indicates that FMDV serotype-specific antibodies are absent in the test sample.

4. Materials and equipment

4.1 Reagents and preparation

Refer to SOP FMD 17 for all reagents and buffers used in different diagnostic tests.

4.2 Consumables

4.2.1 Pipettes

Finnpipette, single channel pipettes, variable ranges from 20-200 and 200-1000ul

Biopette 12 Channel pipette, variable ranges from 50-300ul

Eppendorf combipette

Tips: blue and yellow tips

Combitips

4.2.2 Glassware/plasticware

A selection of beakers (25-1000ml), flasks (50-1000ml), graduated cylinders (50-1000ml), graduated pipettes (1-10ml), bottles with screw caps 1-2000ml, dilution tubes (2-10ml) and suitable test tube racks.

4.2.3 Reagent Troughs (Reservoirs)

Reagent troughs (reservoirs; AEC Amersham) suitable for simultaneous, multi-channel pipetting of a single reagent.

4.2.4 Microplates

NUNC-Immuno Plate F96 Maxisorp (flat bottom)

NUNC-96 well Tissue culture plates (U shape) carrier plates

4.2.5 Tupperware (or similar plastic) containers

A selection of various sizes of these containers, each with a tight fitting lid.

4.3 Apparatus

4.3.1 Photometer

Microplate reader with an interference filter of 450nm. The photometer must be interfaced with a computer.

4.3.2 Computer (PC)

The reader transmit software, necessary for the transfer of data from the reader to the computer, must be installed on the computer (Saved on a computer disk as back-up).

4.3.3 Orbital Shaker

Orbital shaker (or similar) capable of rotating between 40 – 100rpm.

4.3.4 Plate Washer

Automated Micro-titre plate washer.

4.3.5 Refrigerator

With a range between 2°C - 6°C.

4.3.6 Freezer

Walk in -20ºC ± 2ºC freezer

Ultra freezer -70°C ± 5ºC.

4.3.7 Incubator

Walk-in incubator with a range between 35ºC - 39°C.

4.3.8 pH meter

Cyberscan 500 (or equivalent), with an accuracy of 0.01 pH units.

4.3.9 Balance

Denver (or equivalent) with sensitivity of 0,01g.

4.3.10 Timer

An electronic timer with countdown capabilities and an audible alarm.

5. Method

5.1 Safety and precautions

Sera must be treated as potential zoonotic and infectious material and handled according to standard procedures (refer to SOP GEN 35: Procedure for safe handling of infectious material).

NOTE: Always add sulphuric acid to water - never add water to sulphuric acid.

5.2 Setting the Incubator

Set the incubator at 37°C ± 2ºC.

5.3 Assay procedure

5.3.1 Coating of microtitre plates

Thaw on bench the trapping antibody (1/50 dilution) and dilute 1/2500 in carbonate buffer pH 9.6 just prior to coating of plates. Dispense 100ul of the diluted coating serum to all the wells of a 96-well flat bottom NUNC maxisorp plate using the Labsystems multidrop.

The plates are placed inside a large Tupperware container with tight fitting lid. The plates are incubated overnight at room temperature (18°C – 25°C) followed by a **three cycle** wash with washing buffer using an automatic plate washer. The coated plates are placed in a container with a tight fitting lid and stored at -20ºC ± 2ºC chest freezer (diagnostic section corridor) until required. These plates can be stored for a period of up to one year (Refer SOP FMD 8: Shelf life).

5.3.2 Plate layout

	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Pos Ag	Serum Dil
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	CoAg	Neg C	1/20
B	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	CoAg	Neg C	1/40
C	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	CoAg	Neg C	1/80
D	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	CoAg	Neg C	1/160
E	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C++	C+	1/20
F	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C++	C+	1/40
G	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C++	C+	1/80
H	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C++	C+	1/160

Pos Ag	Positive antigen column (50ul)
1 – 12	Column number
A – H	Row number
C++	Strong positive control
C+	Weak positive control
Neg C	Negative Control
CoAg	Control antigen [(Max OD) (Tris-skim milk)]
S1 – S10	Test serum
1/20 – 1/160	Serum dilution

5.3.3 Procedure

5.3.3.1 - DAY 1

Compile a planning sheet for the sera that must be tested [(QF:38) FMD file in serology laboratory].

Reference sera (controls) and test sera are titrated in duplicate wells against a reference antigen in a carrier plate starting with an initial dilution of **1/20.** See SOP FMD 17 No 4.

Step 1: (In tubes)

Dilute by adding **100ul** of the reference sera (controls) and test samples to 1900ul of Tris-skim milk buffer in a **5ml tube**. Label the carrier plate and transfer 100ul of diluted samples and controls **(from the dilutions in the tubes)** to the carrier plates (Row A & E - See plate layout above).

Step 2:

Add 50ul Tris-skim milk to rows B, C, D, F, G & H and columns 1-12. Refer to the plate layout.

Step 3:

Make a twofold dilution series by transferring 50ul from row to row (Rows A-D + E-H) consecutively, discarding 50ul from the last dilution (row D & row H).

Step 4:

Dilute pos antigen, (See SOP FMD 17 for dilution) in Tris-skim milk buffer. Add 50ul of the diluted pos antigen **to all wells.**

Place plates in a container with a tight fitting lid and incubate for 1h at 37ºC ± 2ºC on an orbital shaker.

Step 5:

Thaw the frozen coated plates on the bench. Label it and transfer 50ul of the antigen/serum mixture from the carrier plates to the corresponding wells of the coated ELISA plates.

Place plates in a container with a tight fitting lid and incubate overnight at 4ºC ± 2ºC.

5.3.3.2 - DAY 2:

Step 1:

Following the incubation period, the plates are washed four times with washing buffer using a plate washer.

Tap the inverted plates onto an absorbent surface to remove residual contents.

Step 2:

Dilute the guinea-pig (typing serum) serum **(Refer to SOP FMD 17)** and add 50ul to all wells of all plates.

Plates are placed in the container with tight fitting lid and incubated for 1h at 37ºC ± 2ºC on an orbital shaker followed by washing as described in step 1 of day 2.

Step 3:

Prepare a working dilution as described in 4.1.13 of the conjugate in Tris-skim milk in a volume sufficient for all micro-titre plates (5ml/plate).

Distribute 50ul to all the wells of all the plates with a 12 channel micro-pipette.

Plates are placed in the container with tight fitting lid and incubated for 1h at 37ºC ± 2ºC on an orbital shaker followed by washing as described in step 1 of day 2.

Step 4:

Addition of substrate/chromogen and stopping solution

Prepare the substrate/chromogen solution in a volume sufficient for the number of plates being used (10ml/plate) just before use.

Distribute 100ul substrate to all the wells of the micro-titre plates.

After incubation at room temperature for 15 $\pm$ 5 minutes, the reaction (colour development) is stopped by the addition of 50ul of stopping solution to all the wells of the plates.

Step 5:

Plates are read with the Thermo Multiskan EX at 450nm.

Place an ELISA plate in the reader and initiate the reading sequence.

The Multiskan interfaces with a computer which receives readings from the Multiskan.

The readings are transferred to a excel spreadsheet which contains statistical formulae which perform calculations according to specifications, presenting results in a standard template. The formulae are described in 5.4.

5.4 Calculation of test sera percentage inhibition for LPBE

The serum titre is calculated as that dilution where a 50% reduction of the colour reaction is recorded.

Antibody titres are expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density of the reaction (antigen) control wells (Kärber method).

The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation, the highest and lowest values (alternatively, the mean value can be used after setting suitable tolerance limits to control for inter-well variation).

Calculation of OD_{Ca} (Max OD)

OD_{Ca} = [Median (A11, B11, C11, D11)]

Calculation of C++ (Strong positive control)

OD C++ = Median E11 – H11

Calculation of C+ (Weak positive control)

OD C+ = Median E12 – H12

Calculation of negative control

OD's = Median A12 – D12

Calculation of OD's for each sample dilution

OD sample 1 = Median 1st dilution = (A1-D1) + (A2-D2)/2

OD sample 2 = Median 1st dilution = (A3-D3) + (A4-D4)/2

OD sample 3 = Median 1st dilution = (A5-D5) + (A6-D6)/2

OD sample 4 = Median 1st dilution = (A7-D7) + (A8-D8)/2

Etc.

Calculation of percentage inhibition for each sample dilution

$$\text{Percentage inhibition (PI)} = 100 - \frac{\text{(median OD of the two test serum wells)}}{\text{(median OD of antigen control)}} \times 100$$

The Percentage Inhibition for the four dilutions of each serum (PI1, PI2, PI3, PI4) are used to calculate the serum titre.

NOTE

If % Inhibition < 20% then = 0%

If % Inhibition > 80% then = 100%

Calculation of serum titres

Log TCID 50 XX = [((ΣPI1+ PI2 + PI3 + PI4)/100) – 0.5]*Log serial dilution (2) + Log starting dilution (20)

Serum titre = Antilog of TCID 50

Acceptance of assay

The assay is accepted if the results meet the following criteria:

a. The OD_{Ca} (Max OD) should be between 0.8 and 1.4

b. The PI of the negative control should be <50%

5.5 Interpretation of results

The log titre of a sample ≥ 1.6 = positive – antibodies against FMDV in a sample

The log titre of a sample ≤ 1.5 = negative – no antibodies against FMDV present in a sample

Note: Positive samples or inconclusive results are confirmed by VNT.

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5.6 References

Hamblin, C., Barnett, I.T.R. and Hedger, R.S. (1986) A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I Development. J. Immun. Meth. 93, 115-121

Hamblin, C., Barnett, I.T.R. and Hedger, R.S. (1986) A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. II Application. J. Immun. Meth. 93, 123-129

Karber, G (1931) 50% end-point calculation. Arch. Exp. Pathol. Pharmak., 162, 480-48.

Manual of standards for diagnostic tests and vaccine. Office International des Epizooties. 2012 Chapter 2.1.5 Foot and Mouth Disease.

REVISION HISTORY LOG			
Revision	**Description of Change**	**Reviewed by**	**Effective Date**

5.7 Appendix G: ARC OVI (TADs) Non-Structural Protein ELISA SOP

ONDERSTEPOORT VETERINARY INSTITUTE
TRANSBOUNDARY ANIMAL DISEAS

DETECTION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS (FMDV) NON-STRUCTURAL PROTEINS (NSP) USING A COMMERCIAL PRIOCHECK® FMDV NS ELISA KIT

CONTENTS:

1. Aim and field of applications

The aim of the ELISA is to test sera of cloven-hoofed animal species (cattle, sheep, goats and pigs) for the presence of antibodies against the non-structural proteins of FMDV. The FMD viruses are classified into 7 distinct serotypes which makes diagnosis using conventional serological methods complex. Vaccines consist of (partially) purified structural proteins of the FMD virus and therefore vaccinated animals only elicit antibodies directed against the structural proteins of the virus. However, after infection with FMDV, antibodies against the structural and

non-structural proteins are produced. Therefore an ELISA detecting antibodies against non-structural proteins of FMDV detects not only infected animals but also distinguish between infected and vaccinated animals. The ELISA detects FMDV infected animals independent of the serotype that causes the infection and independent of the fact that the animal is vaccinated or not. Furthermore it can be used to establish FMD free status in animals destined for export from South Africa or otherwise, import into South Africa.

2. Definitions and abbreviations.

FMDV foot-and-mouth disease virus
ELISA Enzyme linked immuno sorbent assay
FMD antigen Inactivated FMD virus used in ELISA
SAT South African type
H_2O_2 Hydrogen peroxide
H_2SO_4 Sulphuric acid
BEI Binary ethyleneimine

3. Principle

The Priocheck® FMDV ELISA is a blocking ELISA. The test plates are coated with 3ABC specific monoclonal antibodies (mAb), followed by incubation with antigen (3ABC protein). Consequently, test plates of the kit contains FMDV non-structural antigen captured by the coated mAb.

Test samples are dispersed to the wells of the plate. After an incubation period, the plates are washed and the conjugate added. FMDV non-structural specific antibodies, directed against the non-structural proteins that may be present in the test sample will bind to the 3ABC proteins and will block the binding of the mAb-HRPO. After incubation, the plate is washed and the chromogen (TMB) substrate is dispensed. After incubation, the color development is stopped. Color development measure optically at a wavelength of 450nm shows the presence of antibodies directed against FMDV. Serum samples are tested in a 1:5 dilution.

4. Materials and equipment

4.1 Reagents (Kit Components)

4.1.1 Component 1 – Test plate

Five test plates in kit in bags which contain a desiccant sachet

4.1.2 Component 2 – Conjugate (30x)

One x 2,5ml vial - Dilute just before use.
Diluted conjugate is not stable, prepare just before use.

4.1.3 Component 3 – Diluent buffer (2x)

60 ml Diluent buffer - Dilute just before use.
Shelf life of the dilution buffer working solution: 24 hours at 5±3ºC.

4.1.4 Component 4 – Additive (lyophilized)
Five vials x 2.5ml lyophilized additive - Reconstitute and dilute just before use.
Shelf life of reconstituted additive: until exp date at -20ºC.

4.1.5 Component 5 – Demineralized water
Two vials x 10ml

4.1.6 Component 6 – Washing fluid (200x)
60ml Washing fluid – Dilute just before use.
Shelf life of washing solution when diluted: One week at 22±3ºC

4.1.7 Component 7 – Positive control
One vial containing 0,6ml positive control, ready to use.

4.1.8 Component 8 – Weak Positive control
One vial containing 0,6ml weak positive control, ready to use.

4.1.9 Component 9 – Negative control
One vial containing 0,6ml negative control, ready to use.

4.1.10 Component 10 – Chromogen (TMB) substrate
One vial containing 60ml chromogen solution, ready to use.

4.1.11 Component 11 – Stop solution
One vial containing 60 ml stop solution, ready to use.

4.1.12 Additional kit contents
Package insert
10 plate sealers

4.2 Consumables

4.2.1 Pipettes
Finnpipette, single channel pipettes, variable ranges from 20-200 and 200-1000ul
Finnpipette digital 12 Channel pipette, variable ranges from 20-200ul
Eppendorf combipette
Tips: blue and yellow tips
Combitips

4.2.2 Glassware/plasticware
A selection of beakers (25-1000ml)
Graduated cylinders (50-1000ml)
Graduated pipettes (1-10ml)

Bottle with screw caps 1-100ml

4.2.3 Reagent Troughs
Reagent troughs (reservoirs; AEC Amersham) suitable for simultaneous, multi-channel pipetting of a single reagent.

4.2.4 Tupperware containers
A selection of various sizes of these containers, each with a tight fitting lid for incubation of sample plates.

4.3 Apparatus

4.3.1 Photometer (Plate reader)
Labsystems, Multiskan EX microplate reader (or similar) with an interference filter of 450nm. The photometer must be interfaced with a PC.

4.3.2 Computer (PC)
The appropriate software (Microsoft office Excel) necessary for the calculation of ELISA results must be installed.

4.3.3 Refrigerator
Any type with a range between 2°C - 8°C

4.3.4 Freezer
-20ºC ± 2ºC freezer

4.3.5 Timer
An electronic timer with countdown capabilities and an audible alarm.

5. Methods

5.1 Solutions to be made in advance

5.1.1 Dilution buffer working solution (2x)
½ in demineralized water (or water of equal quality); e.g. for one test plate prepare 24ml (add 12 ml dilution buffer (2x) to 12 demineralized water).
Can be stored at 5ºC±3ºC for up to 24 hours.

5.1.2 Additive
Equilibrate the vial to 22ºC±3ºC and reconstitute with 2.5ml demineralized water.
Can be stored at -20ºC until expiry date.

5.1.3 ELISA buffer

Dilute reconstituted additive 1/10 in dilution buffer working solution (5.1.1) e.g. for one ELISA plate prepare 2.4ml reconstituted additive to 21.6ml dilution buffer working solution or for 2 strips prepare 4ml (add 0.4ml reconstituted additive to 3.6ml dilution buffer working solution).
Unused ELISA buffer can be stored at 5º±3ºC for up to 24hours.

5.1.4 Conjugate dilution (30x)

Dilute conjugate 1/30 in ELISA buffer e.g. for one plate prepare 12ml [add 400ul conjugate (30x) to 11.6ml ELISA buffer or for 2 strips prepare 2.1ml (add 70ul conjugate (30x) to 3.6ml ELISA buffer)] Prepare just before use.

5.1.5 Washing solution (200x)

Dilute washing solution 1/200 in demineralized water.
Stability of washing solution: 1 week stored at 22º±3ºC.

5.2 Assay procedure

5.2.1 Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	**C ++**	13	21	29	37	45	53	61	69	77	85
B	2	**C ++**	14	22	30	38	46	54	62	70	78	86
C	3	**C +**	15	23	31	39	47	55	63	71	79	87
D	4	**C -**	16	24	32	40	48	56	64	72	80	88
E	5	9	17	25	33	41	49	57	65	73	81	89
F	6	10	18	26	34	42	50	58	66	74	82	90
G	7	11	19	27	35	43	51	59	67	75	83	**C +**
H	8	12	20	28	36	44	52	60	68	76	84	**C -**

C++ Strong positive control (Component 7)
C+ Weak positive control (Component 8)
C- Negative control (Component 9)

5.2.2 DAY 1

Incubation with test serum

- Dispense 80ul ELISA buffer to all wells of the test plate
- Dispense 20ul of control and test sera to wells according to layout
- Cover the test plate using the enclosed lid
- Incubate overnight (16 – 18 hours) at 22º±3ºC

5.2.3 DAY 2

A) Incubation with conjugate

- Empty test plate after the incubation period

- Wash plate 6 times with 200 – 300ul washing buffer and tap the plate firmly after the last wash
- Dispense 100ul of diluted conjugate to all wells (5.1.4)
- Cover the test plate using the enclosed lid
- Incubate 60 minutes at 22º±3ºC

B) Incubation with chromogen (TMB) sustrate

- Empty test plate after the incubation period
- Wash plate 6 times with 200 – 300ul washing buffer and tap the plate firmly after the last wash
- Dispense 100ul of chromogen (TMB) substrate to all wells
- Cover the test plate using the enclosed lid. Incubate 20 minutes at 22º±3ºC
- Add 100ul of stop solution to all wells
- Mix contents of the wells prior to measuring

C) Recording of results

- Measure the optical density (OD) of the wells at 450nm within 15 min after color development has been stopped

D) Calculation of results

- Calculate the mean OD_{450} value of wells D2 + H12 (negative control = OD_{450} max)
- The percentage inhibition (PI) of the controls and the test sera are calculated according to the formula below
- The OD_{450} values of all samples are expressed as PI relative to the OD_{450} max

$$PI = 100 - \left[\frac{OD_{450}\ \text{tets sample}}{OD_{450}\ \text{max}}\right] \times 100$$

5.3 Validation and Interpretation of results

5.3.1 Validation criteria

- OD_{450} max of the negative control must be >1.00
- The mean percentage inhibition of the weak positive control must be >50%
- The mean percentage inhibition of the positive control must be >70%
- **Not meeting any of these criteria is reason to discard the results of that specific test plate**

5.3.2 Interpretation of the percent inhibition

- PI = <50% Negative

Antibodies against the NS protein of FMDV are absent in the test sample

- PI = ≥50%

Antibodies against the NS protein of FMDV are present in the test samples

5.4 Safety Regulations & R&S Statements – See kit insert

5.5 References

Sorensen K.J., Madsen K.G., Madsen E.S., Salt J.S. Nqindi J. & Mackay D.K.J. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of anti-bodies to the non-structural proteins 3D, 3AB and3ABC in ELISA using antigens expressed in baculovirus. Arch Virol (1998) 143: 1461-1476.

RomeyA., Relmy A., Gorna K., Allemandou A., Blaise-Boisseau S., Zientara S., L Bakkali-Kassimi. UMR1161 Virologie (Anses, Inra,Enva) LSA 23 Avenue du Géneéral de Gaulle, 94706 Maisons-Alfort, France. A Dekker, P. Moonen, E.M. v.d. Linde. Comparison of commercial ELISAs for antibodies against FMDV non-structural proteins.

Brocchi E, Bergmann IE, Dekker A, Paton DJ, Sammin DJ, Greiner M, Grazioli S, De Simone F, Yadin H, Haas B, Bulut N, Maliirat V, Neitzert E, Goris N, Parida S, Sorensen K, De Clercq K. Comparative evaluation of six ELISA's for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus.

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Office International des Epizooties. 2012

Priocheck® FMDV NS ELISA Kit Insert.

REVISION HISTORY LOG			
Revision	**Description of Change**	**Reviewed by**	**Effective Date**

5.8 Appendix H: SOP for FMD RNA Extraction

SOP MOL 1 STANDARD OPERATING PROCEDURE FOR THE EXTRACTION OF RNA

PURPOSE

The purpose of this procedure is to extract RNA from whole blood, probangs, tissue culture and clinical samples for molecular diagnostic assays.

SCOPE AND APPLICATION:

The protocol describes the method for RNA extraction from whole blood, clinical samples (spleen, lung, kidney, lymph node, etc) and tissue culture materials submitted for diagnosis of Schmallenberg virus (SBV), Foot-and-mouth disease (FMDV), Classical swine fever (CSF) and Porcine reproductive and respiratory syndrome (PRRS).

DEFINITIONS/ABBREVIATIONS:

SBV	Schmallenberg virus
FMDV	Foot and mouth disease virus
CSF	Classical swine fever
PRRS	Porcine reproductive and respiratory syndrome
RNA	Ribonucleic acid
PC	Positive control
NC	Negative control

HEALTH AND SAFETY:

The entire extraction procedure should be performed with gloves. Make sure the correct size gloves are worn to enable ease with working with Eppendorf tubes. Should the gloves come into contact with a contaminated area, a new pair of gloves should be worn.

Buffers AVL and AW1 contain guanidine thiocyanate and guanide hydrochloride, respectively which can be harmful and an irritant, thus gloves must be worn at all time

PRECAUTIONS:

Gloves must be worn at all times.

Disinfect the working area with 70% ethanol before and after work.

Use chemicals and reagents that are within their shelf lives.

Perform all steps in its designated area e.g. laminar flow or fume hood.

Samples may contain live virus and precautions to prevent virus dissemination should be adhered to.

REAGENTS AND SOLUTIONS:

QiaAmp Viral RNA extraction kit containing:

Lysis buffer Buffer AVL

Wash Buffers: AW1 and AW2

Elution buffer: AVE

Carrier RNA

APPARATUS AND MATERIALS:

100 – 1000 µl micro-pipette and filter tips

20 – 200 µl micro-pipette and filter tips

Sterile Safe-lock 1.5 ml Eppendorf tubes

Vortex mixer

Bench top micro centrifuge

Surgical gloves

PROCEDURE AND ANALYSIS:

RNA isolation using the QiaAmp Viral RNA extraction kit (Qiagen manual)

This procedure must be carried out on the bench in the nucleic acid extraction lab

A. Preparation of Reagents

1. Reconstitute the lyophilized carrier RNA by adding 310 µl Buffer AVE to the tube containing 310 µg lyophilized carrier RNA. Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store at -20ºC. Do not freeze-thaw the aliquots of carrier RNA more than 3 times.
2. Prepare a buffer AVL+ carrier RNA solution as indicated in Table 1 of Qiagen manual. Buffer AVL-carrier RNA should be prepared fresh and is stable at 2-8ºC for up to 48 hours.
   Buffer AVL might develop a precipitate that must be re-dissolved by warming at 80ºC for 5 minutes before use. Do not warm buffer AVL more than 6 times. Do not incubate at 80ºC for more than 5 minutes.
3. Make up buffers AW1 and AW2. Buffers AW1 and AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottles and in Table 2 on page 17 of the handbook.

Things to do before starting with procedure.

1. Remove aliquots of positive and negative controls from freezer
2. Equilibrate samples to room temperature (15-25ºC)
3. Equilibrate buffer AVE to room temperature for elution
4. Check that buffer AW1 and Buffer AW2 have been prepared according to the instruction on page 17. Mark the bottle with a tick (√) to indicate that the buffer is prepared.
5. Add carrier RNA reconstituted in Buffer AVE to Buffer AVL according to instructions on page 15.

B. Procedure

Follow the procedure as described in the QiaAmp Viral RNA Mini Handbook pg 23; Protocol: Purification of Viral RNA (Spin Protocol).

Note that samples are received as 140 µl aliquots (step 2 of procedure) in 1.5 ml microcentrifuge and are ready to use.

1. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into each sample in the 1.5 ml microcentrifuge tube. Mix by pulse-vortexing for 15 s.
2. Continue as in step 3-11 of the procedure on page 24-25 of the handbook.

QUALITY CONTROL:

The TADP laboratory conforms to Good Laboratory Practice. For quality control purpose, the required information is documented on the appropriate form(s).

TADP is aligned to ISO17025 and Dept of Agriculture, Forestry and Fisheries quality standard.

FORMS AND DATA SHEET:

PCR result FORM

REFERENCE

QiaAmp Viral RNA Mini Handbook: Protocol: Purification of Viral RNA (Spin Protocol).

5.9 Appendix I: SOP for cDNA synthesis from RNA

STANDARD OPERATING PROCEDURE FOR THE SYNTHESIS OF cDNA FROM THE ISOLATED RNA

Revision: 0

Date of Review	Reviewed by	Next Review Date

1. PURPOSE

The purpose of this procedure is to synthesise cDNA from the isolated RNA

2. SCOPE AND APPLICATION:

The viral RNA template is reverse transcribed using AMV- reverse transcriptase (RT) (Promega) into cDNA to be used a template for polymerase chain reactions (PCRs)

3. DEFINITIONS/ABBREVIATIONS:

AMV-RT	AMV-reverse transcriptase
DMSO	Dimethylsulfoxide
dNTP	deoxyribonucleotides triphosphates
EDTA	Ethylene-diamine tetracetic acid
HCL	Hydrochloric acid
MM	master mix
PCR	Polymerase Chain Reaction
RNasin	RNA inhibitor
RT-buffer	reverse transcriptase buffer
TE	Tris-EDTA

4. HEALTH AND SAFETY:

DMSO can be harmful, handle with gloves.

5. PRECAUTIONS:

Gloves must be worn at all times

Disinfect the working area with 70% ethanol before and after work.

6. REAGENTS AND SOLUTIONS:

5 x AMV-RT buffer
AMV-RT enzyme
dNTP from 10 mM (stock)
2B primer
DMSO
RNasin inhibitor
70% Ethanol as a disinfectant
1 x TE

7. APPARATUS AND MATERIALS:

2 µl – 20 µl micro-pipette and filter tips
0.5 µl – 10 µl micro-pipette and filter tips
20 µl – 100 µl micro-pipette and filter tips
0.5 ml Eppendorf tubes
Vortex mixer
Bench top micro 5415D Eppendorf centrifuge
GeneAmp PCR amplification system
Surgical gloves

8. PROCEDURE AND ANALYSIS:

cDNA FOR FMD

This procedure should be carried out on the bench in the nuclei acid extraction lab

8.1 Remove the 5 x AMV-RT buffer, dNTPs, primer stock (10 pmol/µl) from the freezer and allow to thaw on bench top. As soon as thawed place on ice.

Make a dilution of RNase Inhibitor (Promega) as follows (depending on sample number). Add 1µl of RNasin (40U/µl) enzyme to:

No. of samples	Volume of 1 x TE (µl)
1	1.5
6	10.5
21	37

Vortex and centrifuge briefly (quick spin) and place on ice

8.2 Prepare a cDNA master mix according to the number of samples to be reverse transcribed as follows:

Reagents	1x MM (µl)	6x MM (µl)	21x MM (µl)

5x AMV-RT buffer	5	30	105
dNTP	3	18	63
2B primer (10 pmol/µl)	0.5	3	10.5
DMSO	0.5	3	10.5
Diluted RNase inhibitor	0.5	3	10.5
dH_20	8	48	168
Diluted RNasin duplicated	0.5	3	10.5

8.3 Vortex and centrifuge (quick spin) the master mix.
8.4 Aliquot 17.5 µl of the cDNA master mix to labeled (cDNA, batch number and date) tubes and place them on ice.
8.5 Add 5 µl of the eluted RNA extract to the correspondingly labeled tubes and vortex.
8.6 Centrifuge (quick spin) and incubate at 70°C for 3 minutes.
Place samples on ice for 2 minutes.

Whilst the tubes are on ice add AMV-RT (10 U/µl; Promega) to the remaining diluted RNase inhibitor solution as follows:

No. of samples	**Volume of AMV-RT enzyme (µl)**
1	1.5
6	9
21	29.5

Mix thoroughly by vortexing and quick spin to settle droplets. Place on ice.
8.7 Add 2 µl of the enzyme/RNase inhibitor mix to each of the cDNA tubes (immediately return the tubes to ice after adding the enzyme, and use a new tip each time).
8.8 Vortex and centrifuge tubes briefly (quick spin) and incubate at 42°C for 1 hour.
8.9 Inactivate the enzyme by incubating at 80°C for 2 minutes.
8.10 Remove, keep at 4°C and proceed to PCR.
8.11 RNA extract and cDNA is stored at -20°C**.**

<u>cDNA FOR CSF/PRRS</u>

Use the GeneAmp kit

	1X	2X	3X	4X	5X	6X	7X	8X	9X
$MgCl_2$	**4**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	36
10x PCR buffer	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	18

water	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	9
dGTP	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	18
dCTP	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	18
dTTP	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	18
dATP	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	18
RNase inhibitor	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	9
MuLV reverse transcriptase	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	9
Random hexamers	1	2	3	4	5	6	7	8	9

Aliquot 18 µl master mix per tube. Add 2 µl CSF/PRRS extracted RNA. Mix by vortexing and short spin to collect and settle droplets. Keep at room temperature for 10 mins, then place in PCR block for CSF cDNA synthesis. Follow prompts on the PCR block to choose the CSF cDNA programme.

Programme for cDNA synthesis on PCR blocks:

- Rt thermal profile:

TEMPERATURE	**TIME**	**CYCLES**
42°C	15 min	1
99°C	5 min	1
5°C	5 min	1
4°C	∞	1

Start the PCR cycler and the cDNA should be ready after 30 minutes.

9. DATA ANALYSIS/CALCULATIONS:

There is no result expected from this procedure, cDNA synthesis will be evidence at the agarose gel stage after PCR procedure.

10. QUALITY CONTROL:

The TADP laboratory conforms to Good Laboratory Practice. For quality control purpose, the required information is documented on the appropriate forms

11. FORMS AND DATA SHEET:

PCR result form

12. REFERENCES:

AMV-RT enzyme (Promega) package insert for FMD

GeneAmp PCR amplification system (Applied Biosystems) package insert for CSF and PRRS

5.10 Appendix J: List of serological responses to the structural proteins of SAT-1, SAT-2 and SAT-3 FMD viruses as well as to non-type specific non-structural viral proteins (phase 1 sampling)

Serological results (SAT structural & NSP ELISAs) [3]					
LAB NO	**ANIMAL ID**	**SAT-1**	**SAT-2**	**SAT-3**	**NSP**
1	EC135994	**>2.2**	**>2.2**	**>2.2**	Neg
2	EC064791	**>2.2**	**>2.2**	**>2.2**	Neg
3	EC064739	**>2.2**	**>2.2**	**>2.2**	Neg
4	EC065586	**>2.2**	**>2.2**	**>2.2**	Neg
5	EC013842	**>2.2**	**>2.2**	**>2.2**	Neg
6	EC013785	**>2.2**	**>2.2**	**>2.2**	Neg
7	EC013758	**>2.2**	**>2.2**	**>2.2**	Neg
8	EC013787	**2.1**	**1.8**	**2.0**	Neg
9	EC013817	<1.3	**1.8**	**1.8**	Neg
10	EC013759	**>2.2**	**2.1**	**2.2**	Neg
11	EC013729	**>2.2**	**>2.2**	**>2.2**	Neg
12	EC013749	**>2.2**	**>2.2**	**>2.2**	Neg
13	EC013829	**>2.2**	**>2.2**	**>2.2**	Neg
14	EC013756	**>2.2**	**>2.2**	**>2.2**	Neg
15	EC014204	**>2.2**	**>2.2**	**>2.2**	Neg
16	EC014804	**>2.2**	**>2.2**	**>2.2**	Neg
17	EC014293	**>2.2**	**>2.2**	**>2.2**	Neg
18	EC014300	**>2.2**	**>2.2**	**>2.2**	Neg
19	EC014180	**2.0**	**>2.2**	**>2.2**	Neg
20	EC014229	**2.1**	**>2.2**	**>2.2**	Neg
21	EC014227	**>2.2**	**>2.2**	**>2.2**	Neg
22	EC014261	**2.2**	**>2.2**	**>2.2**	Neg
23	EC014212	**>2.2**	**>2.2**	**>2.2**	Neg
24	EC014183	**1.7**	**>2.2**	**>2.2**	Neg

25	EC014213	**>2.2**	**>2.2**	**>2.2**	Neg
26	EC014294	**>2.2**	**>2.2**	**>2.2**	Neg
27	EC070709	**2.1**	**>2.2**	**>2.2**	Neg
28	EC070657	**>2.2**	**>2.2**	**>2.2**	Neg
29	EC070705	**>2.2**	**>2.2**	**>2.2**	Neg
30	EC070688	**1.9**	**>2.2**	**2.0**	Neg
31	EC070676	**>2.2**	**>2.2**	**>2.2**	Neg
32	EC070693	**2.0**	**>2.2**	**>2.2**	**w-pos**
33	EC035455	**>2.2**	**>2.2**	**>2.2**	Neg
34	EC035518	**1.9**	**>2.2**	**1.9**	Neg
35	EC152473	**>2.2**	**>2.2**	**>2.2**	Neg
36	EC064797	**1.9**	**>2.2**	**>2.2**	Neg
37	EC064822	**>2.2**	**>2.2**	**>2.2**	Neg
38	EC065159	**2.1**	**>2.2**	**>2.2**	Neg
39	EC064748	**2.1**	**>2.2**	**>2.2**	Neg
40	EC064790	**>2.2**	**>2.2**	**>2.2**	Neg
41	EC064780	**>2.2**	**>2.2**	**>2.2**	Neg
42	EC064689	**>2.2**	**>2.2**	**>2.2**	Neg
43	No Tag 1	**>2.2**	**>2.2**	**>2.2**	Neg
44	No Tag 2	**>2.2**	**>2.2**	**>2.2**	Neg
45	EC064769	**2.0**	**>2.2**	**1.9**	Neg
46	EC089172	**>2.2**	**>2.2**	**>2.2**	Neg
47	EC088101	**>2.2**	**>2.2**	**>2.2**	Neg
48	EC089087	**>2.2**	**>2.2**	**>2.2**	Neg
49	EC089097	**2.1**	**>2.2**	**1.7**	Neg
50	EC089178	**2.1**	**>2.2**	**>2.2**	Neg
51	EC089125	**>2.2**	**>2.2**	**>2.2**	**POS**

[3] Log $_{10}$ reciprocal of the final of the 50% end-point. Titres <1.6=negative; Titres ≥1.6 = significant; ≥2.0 = probably protective;Neg=negative for NSP; Pos= positive for NSP; w-pos= weak positive for NSP

52	EC089123	**>2.2**	**>2.2**	**>2.2**	**POS**
53	EC089130	**>2.2**	**>2.2**	**>2.2**	Neg
54	EC089131	**>2.2**	**>2.2**	**>2.2**	Neg
55	EC089180	**>2.2**	**>2.2**	**>2.2**	Neg
56	EC088826	**>2.2**	**>2.2**	**>2.2**	Neg
57	EC019346	**>2.2**	**>2.2**	<1.3	Neg
58	EC018857	**1.9**	**>2.2**	**>2.2**	Neg
59	EC019347	**>2.2**	**>2.2**	**>2.2**	Neg
60	EC088842	**>2.2**	**>2.2**	**>2.2**	**POS**
61	EC088844	**>2.2**	**>2.2**	**>2.2**	Neg
62	EC088820	**>2.2**	**>2.2**	**>2.2**	Neg
63	EC088840	**>2.2**	**>2.2**	**>2.2**	Neg
64	EC088815	**>2.2**	**>2.2**	**>2.2**	Neg
65	EC088847	**>2.2**	**>2.2**	**>2.2**	Neg
66	EC083824	**>2.2**	**>2.2**	**>2.2**	Neg
67	EC083243	**>2.2**	**>2.2**	**>2.2**	Neg
68	EC087166	**>2.2**	**>2.2**	**>2.2**	Neg
69	EC022573	**>2.2**	**>2.2**	**>2.2**	Neg
70	EC089786	**>2.2**	**>2.2**	**>2.2**	**w-pos**
71	EC022208	**>2.2**	**>2.2**	**>2.2**	Neg
72	EC083318	**>2.2**	**>2.2**	**>2.2**	Neg
73	No Tag	**>2.2**	**>2.2**	**>2.2**	Neg
74	**No Sample				
75	EC087551	**2.0**	**>2.2**	**1.8**	Neg
76	EC087191	**>2.2**	**>2.2**	**2.0**	Neg
77	EC083336	**>2.2**	**>2.2**	**>2.2**	Neg

5.11 Appendix J: List of serological responses to the structural proteins of SAT-1, SAT-2 and SAT-3 FMD viruses as well as to non-type specific non-structural viral proteins (phase 2 sampling)

Serological results (SAT structural & NSP ELISAs)[4]					
LAB NO	**ANIMAL ID**	**SAT-1**	**SAT-2**	**SAT-3**	**NSP**
1	082600	**>2.2**	**>2.2**	**>2.2**	**POSITIVE**
2	084720	**1.8**	1.6	**1.8**	Negative
3	092903	<1.3	<1.3	<1.3	Negative
4	144935	<1.3	<1.3	<1.3	Negative
5	108370	1.5	<1.3	<1.3	Negative
6	095070	**>2.2**	**>2.2**	**>2.2**	Negative
7	EL 068751	1.4	<1.3	1.4	Negative
8	090415	**1.9**	**>2.2**	**1.8**	**POSITIVE**
9	092868	**1.8**	**1.9**	**1.6**	Negative
10	EL 125215	**1.7**	<1.3	**1.6**	Negative
11	EL 049208	**>2.2**	**1.6**	**>2.2**	Negative
12	80010	1.6	1.5	**1.7**	**POSITIVE**
13	136471	**1.6**	<1.3	**1.6**	Negative
14	92842	**>2.2**	**1.6**	**>2.2**	Negative
15	35409	<1.3	<1.3	<1.3	Negative
16	90390	**1.8**	**>2.2**	**1.9**	Negative
17	EL 097880	**>2.2**	1.5	**1.6**	Negative
18	50395	**1.7**	<1.3	1.4	Negative
19	EL 083610	**1.7**	**1.7**	**1.6**	Negative
20	EL 125380	**2.0**	**1.7**	1.6	**POSITIVE**
21	090459	**>2.2**	**>2.2**	**>2.2**	Negative
22	150754	**>2.2**	**1.9**	**>2.2**	Negative
23	090443	**1.8**	**2.1**	**1.9**	Negative

24	EL 092934	**1.8**	**1.7**	**>2.2**	Negative
25	051864	**1.7**	1.4	**1.7**	Negative
26	090474	**>2.2**	**>2.2**	**>2.2**	**POSITIVE**
27	0904090	**1.9**	1.6	**1.9**	Negative
28	051860	1.5	<1.3	1.5	Negative
29	105959	1.4	<1.3	<1.3	Negative
30	EL 049201	**2.1**	**2.1**	**2.0**	Negative
31	146916	**>2.2**	**1.9**	**2.1**	Negative
32	051831	1.5	1.5	**1.6**	Negative
33	155502	**1.6**	<1.3	<1.3	Negative
34	RL 055625	**>2.2**	**>2.2**	**2.0**	Negative
35	EL 147394	**>2.2**	**>2.2**	**2.0**	Negative
36	155523	**1.6**	1.6	1.5	Negative
37	137592	**2.0**	**1.6**	**1.9**	Negative
38	108646	**2.1**	**1.7**	**1.9**	Negative
39	150795	<1.3	<1.3	<1.3	Negative
40	035401	<1.3	<1.3	<1.3	Negative
41	092848	**1.9**	**1.6**	**2.1**	Negative
42	051854	**>2.2**	<1.3	1.4	Negative
43	EL 048118	**>2.2**	**1.8**	**>2.2**	Negative
44	090477	**>2.2**	**>2.2**	**>2.2**	Negative
45	094093	**2.1**	**1.8**	**1.6**	Negative
46	084841	**>2.2**	**>2.2**	**>2.2**	Negative
47	084703	1.6	1.4	<1.3	Negative
48	EL 097808	**>2.2**	1.5	**1.9**	Negative
49	EL 057101	**1.9**	1.5	1.4	Negative
50	090436	**2.0**	**1.7**	**1.9**	Negative

[4] Log $_{10}$ reciprocal of the final of the 50% end-point. Titres <1.6=negative; Titres ≥1.6 = significant; ≥2.0 = probably protective

51	EL 057071	**1.8**	<1.3	<1.3	Negative
52	094101	**2.0**	**1.6**	**1.7**	Negative
53	136440	**>2.2**	**2.0**	**>2.2**	Negative
54	090514	**2.1**	**>2.2**	**2.0**	Negative
55	141025	<1.3	<1.3	<1.3	Negative
56	090513	**>2.2**	**>2.2**	**>2.2**	Negative
57	EL 048123	**>2.2**	**2.1**	**2.1**	Negative
58	140827	**1.8**	<1.3	1.4	Negative
59	092544	**>2.2**	**>2.2**	**>2.2**	Negative
60	051865	**1.8**	<1.3	<1.3	Negative
61	155521	1.6	<1.3	**1.7**	Negative
62	150722	<1.3	1.5	<1.3	Negative
63	EL 125217	<1.3	<1.3	<1.3	Negative
64	094107	**1.7**	**1.8**	**1.7**	Negative
65	EL 048151	**>2.2**	**2.0**	**>2.2**	Negative
66	051869	**1.8**	<1.3	**1.7**	Negative
67	EL 125237	1.5	1.5	1.5	Negative
68	050394	1.5	<1.3	<1.3	Negative
69	EL 125234	1.5	**1.8**	1.4	Negative
70	136402	1.6	<1.3	1.5	Negative
71	90486	**1.8**	**1.6**	1.5	Negative
72	80126	**1.9**	<1.3	**1.7**	Negative