Assessing Swine Meat Juice for the Detection of Foot-and-Mouth Disease Virus by Real-Time Reverse Transcription Polymerase Chain Reaction, Enzyme-Linked Immunosorbant Assays, and pen-side Lateral Flow Immunoassays

by

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ABSTRACT

Many infectious diseases concern agricultural sectors globally. Foot-and-Mouth Disease (FMD) is one of the most contagious diseases that causes severe infections in cloven-hoofed animals. Animals that become infected by Foot-and-Mouth Disease Virus (FMDV) should be slaughtered to prevent spread. Countries that endure endemic outbreaks or are free of the disease contribute large amounts of funding to combat and/or prevent FMD. FMD is diagnosed using sample types such as vesicular fluid, oropharyngeal swabs, lesions swabs, or serum. Situations may be encountered where these samples are not available. In these cases such as illegal importation or absence of the animal carcass, meat juice (MJ) can be alternatively used to test for the presence of FMDV. This was assessed using the *biceps femoris* from experimentally infected pigs. MJ, collected after freeze-thaw cycling, was found viable for the detection of FMDV directly by Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR), Lateral Flow Immunoassay (LFI), sanger sequencing, and transfection. FMDV detection in RNA extracted from MJ was found as late as 21 days post infection (DPI) compared to 7 DPI in serum. LFI was able to detect FMDV antigen in MJ from 1-9 DPI. Isolation of FMDV from MJ was not possible, but was circumvented using transfection as an alternate recovery system. Indirect detection of FMDV was also possible through antibody detection by solid phase competitive enzyme-linked immunosorbent assay. MJ was found to have antibodies to the structural proteins (SP) of FMDV starting at 6 or 7 DPI, mirroring what is found in serum. Detection of antibodies to the non-structural proteins of FMDV did not give meaningful results. These results demonstrate that MJ is a capable sample type that can be used to detect FMDV RNA, antigen, and antibodies to SP.

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PREFACE

Some components of this thesis have been presented at the 2019 Canadian Animal Health Laboratory Network conference in St. Hyacinthe, Quebec, Canada on May 28. This thesis was put together in manuscript format, using two manuscripts – one of which has been published in Pathogens while the other is to be published in the Canadian Journal of Veterinary Research. The manuscripts are as follows:

Manuscript 1. Yeo S., Yang M., Nyachoti M., Rauh R., Callahan J.D., Nfon C. Detection of Foot-and-Mouth Disease Virus in Swine Meat Juice. Pathogens. 2020. 9(6):424.

Manuscript 2[1]. Yeo S., Yang M., Nyachoti M., Nfon C. Detection of Antibodies to Structural Proteins of Foot-and-Mouth Disease Virus in Swine Meat Juice. Can. J. Vet. Res. 2021.

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CONTRIBUTIONS OF AUTHORS

The contributions toward the first manuscript are as follows: Study conception and design: C. Nfon, M. Yang; data collection: S. Yeo; analysis and interpretation of results: S. Yeo, C. Nfon; draft manuscript preparation: S. Yeo; supply of reagents to conduct study: Tetracore Inc., M. Yang; supervision: C. Nfon, M. Nyachoti; results and the final version of the manuscript were reviewed and approved by all other authors: S. Yeo, M. Yang, M. Nyachoti, R. Rauh, J.D. Callahan, C. Nfon.

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LIST OF ABBREVIATIONS

A – Adenine

Ag ELISA – Antigen Detection ELISA

AUD – Animal Use Document

BHK-21 cells – Baby Hamster Kidney 21 cells

C – Cytosine

cDNA – Complementary DNA

CPE – Cytopathic Effect

CSFV – Classical Swine Fever Virus

Ct – Cycle Threshold

DAS – Double Antibody Sandwich

ddNTPs – Di-Deoxynucelotide Triphosphates

DPI – Days Post Infection

ELISA – Enzyme Linked Immunosorbent Assay

EuFMD – European Commission for the Control of FMD

FMD – Foot and Mouth Disease

FMDV – Foot and Mouth Disease Virus

G – Guanine

gRNA – Genomic RNA

IRES – Internal Ribosome Entry Site

JEV – Japanese Encephalitis Virus

kb – Kilo Bases

L protein – Leader Protein

LFI – Lateral Flow Immunoassay

LPBE – Liquid Phase Blocking ELISA

mAbs – Monoclonal Antibodies

MJ – Meat Juice

NCFAD – National Centre for Foreign Animal Diseases

NSP ELISA – Non-structural Protein ELISA

NSPs – Non-Structural Proteins

OD – Optical Density

OIE – World Organization for Animal Health

OPD – O-Phenylenediamine Dihydrochloride

ORF – Open Reading Frame

OS – Oral Swabs

PI – Percent Inhibition

PRRSV – Porcine Reproductive and Respiratory Syndrome Virus

Q – Quencher

R – Fluorophore

RNA – Ribonucleic Acid

ROC – Receiver Operating Characteristic

rRT-PCR – Real-time Reverse Transcription Polymerase Chain Reaction

SAT – South African Territories

Ser – Serum

SPCE – Solid Phase Competitive ELISA

SPs – Structural Proteins

ssRNA – Single Stranded RNA

T – Thymine

TBE – Tris, Boric Acid, EDTA

TCID50 – Tissue Culture Infectious Dose 50

TMB – Tetramethylbenzidine

UTM – Universal Transport Media

UTR – Untranslated Region

VI – Virus Isolation

VNT – Virus Neutralization Test

1. LITERATURE REVIEW

1.1. Foot-and-Mouth Disease Virus

1.1.1. History

Foot-and-Mouth Disease (FMD) is a highly contagious disease caused by Foot-and-Mouth Disease Virus (FMDV) that affects wild and domestic cloven-hoofed animals (Bachanek-Bankowska et al., 2018; Muroga et al., 2013; Stendfeldt, 2016). FMD has existed for centuries with records dating back as far as the sixteenth century (S. M. B. Jamal, G.J., 2013; Mahy, 2005). One of the earliest known records has been traced back to an Italian monk who described a disease infecting cattle that caused decreased appetite and vesicles in both the oral cavity and on their feet (S. M. B. Jamal, G.J., 2013). This description exactly matches the most notable clinical signs that are observed today in diseased animals: oral vesicular lesions and hoof blistering. These characteristic clinical signs have given FMD its name.

1.1.2. Importance in Agricultural and Peripheral Industries

At the turn of the 21st century, meat consumption began to rapidly increase among developing nations (Delgado, 2003). Animal rearing has also significantly increased to satiate demand for meat products (Zhang, 2018). Producers have turned to increasing animal numbers on farms to increase supply and turn larger profits. This became a concern that continues to this day as modernization moves forward at an unprecedented pace. Furthermore, the surge in livestock farming poses greater risks for infectious diseases. FMDV remains a risk among the majority of the world and is considered one of the most contagious viruses known (Grubman et al., 2004; Mansley et al., 2003). Travel has become commonplace in modern day society and brings with it constant fear of global outbreaks. This has been most notably observed with the FMD outbreaks in 2001 in the UK and 2010 in Japan (Hayama et al., 2017; Haydon et al., 2004). Outbreaks of FMD can cause significant financial hardship in countries that rely on production of meat

and meat products (Mahy, 2005). Outbreaks of FMD in FMD-free countries trigger immediate culling of animals and result in significant income loss and increased meat prices (Knight-Jones et al., 2013). The UK outbreak of FMD in 2001 is estimated to have cost £8 billion total directly and indirectly (Haydon et al., 2004). The 2010 outbreak of FMD in Japan affected the world-renowned Miyazaki Prefecture that produces highly sought after wagyu beef (Harada et al., 2015; Hayama et al., 2017). It is estimated to have cost the country C$109 billion from culling of cattle and pigs (Hayama et al., 2017). In the same year, an outbreak in South Korea resulted in the culling of approximately 3.5 million animals – 3.3 million of those being pigs (Park et al., 2013). Following this, vaccination was employed in the later stages of the outbreak to mitigate losses and prevent further spread of FMD in South Korea (Park et al., 2013). Aside from the loss of animals and the immediate economic losses, countries where FMD is present are also unable to trade animals or meat with other countries. When this occurs, FMD-free countries that may rely on purchasing animals and meat from other countries are impacted. Ultimately, food security would be affected in FMD-free countries while countries combatting FMD outbreaks would see significant losses in gross domestic product. Indirectly, elimination of FMDV may also improve poverty rates in endemic countries while also improving quality of life at the individual level (P. Kitching et al., 2007).

FMD outbreaks also affect peripheral industries such as travel, veterinary services, and food in countries both combatting and those preventing outbreaks. Travel restrictions are often imposed to prevent further spread of foreign animal diseases (Fèvre et al., 2006). Enforcement is also imposed on international travellers returning to Canada or the United States of America that have visited farms in other countries (Canadian Food Inspection Agency, 2017; United States Department of Agriculture, 2021). Increased operational costs due to maintaining travel restrictions, disinfection of equipment between farms and abattoirs, and increased utilization of veterinary or lab testing services all contribute to the expense of managing an outbreak of FMD.

1.1.3. Causative Agent

1.1.3.1. Virus classification

FMD is caused by FMDV of the *apthovirus* genus in the *picornaviridae* family (Gao et al., 2016; Habiela et al., 2014; S. M. B. Jamal, G.J., 2013; Mahy, 2005). FMDV is divided into seven different serotypes: A, O, C, Southern African Territories (SAT) 1-3, and Asia 1. Among these seven serotypes, type O was discovered in France, type A and C in Germany, SAT 1-3 in the South African Territories, and Asia 1 in Pakistan (S. M. B. Jamal, G.J., 2013; Mahy, 2005; Tully et al., 2008). Within each serotype, there are subtypes that differ slightly from each other in VP1 genetic sequence (El Nahas et al., 2020; N. J. Knowles et al., 2003; A. R. Samuel et al., 2001). The *picornaviridae* family includes other well known species of viruses such as poliovirus (genus *enterovirus*), rhinovirus (genus *enterovirus*), and hepatitis A virus (genus *hepatovirus*) (Yin-Murphy, 1996). All members of the *picornaviridae* family have a positive sense single-stranded RNA (+ssRNA) genome contained in a non-enveloped icosahedral capsid roughly 22 to 30 nm in size (Mahy, 2005; Yin-Murphy, 1996). The icosahedral protein capsid with five-, three-, and two-fold symmetry is composed of 60 copies each of VP1, VP2, VP3, and VP4 (Gao et al., 2016; Goodwin et al., 2009; Liu et al., 2015; Puckette et al., 2018). Encased in the icosahedral capsid is a +ssRNA genome that is about 8-8.5 kilo bases (kb) genome consisting of three major components that each have a unique function – the 5' untranslated region (UTR), the coding region which contains structural and non-structural protein codes, and the 3' UTR (Gao et al., 2016).

1.1.3.2. 5' UTR

The 5' end of the genome is covalently linked to VPg which is a protein encoded by the 3B gene of FMDV and is important in the process of uridylylation – a key process to produce a free 3' end for replication of the FMDV genome (Figure 1.1) (Gao et al., 2016; Mahy, 2005; Juan M. Pacheco et al., 2003). Following the VPg protein is a complex group of secondary RNA structures called the 5' UTR and is approximately 1300 bases long (Figure 1.1) (Gao et al., 2016; S. M. B. Jamal, G.J., 2013; Mahy, 2005). The 5' UTR

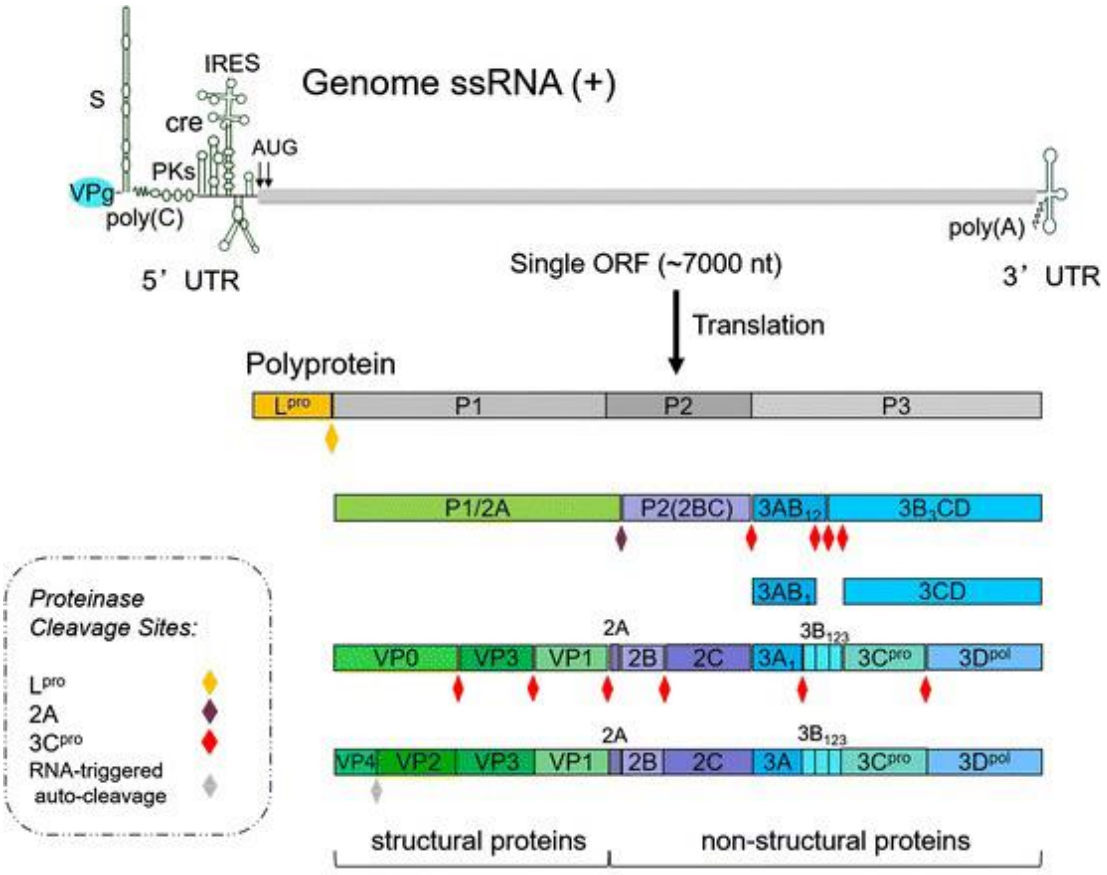
functions in both genome replication and cap-independent translation (Kloc et al., 2017; Mahy, 2005). The end region of the 5' UTR is the internal ribosome entry site (IRES) that is approximately 450 bp long (Figure 1.1) (Gao et al., 2016; Mahy, 2005; Martinez-Salas et al., 2017). This site forms complex secondary structures composed of hairpin loops containing binding sites that associate with host Eukaryotic translation Initiation Factors to carry out translation of the FMDV genome in host cells (Fernández-Miragall et al., 2003; Gao et al., 2016; Mahy, 2005; Martinez-Salas et al., 2017). Each domain of the IRES element work together to recruit host protein complexes to initiate translation in the absence of a 5' cap (Mahy, 2005).

1.1.3.3. The Coding Region

The FMDV genome has a single open reading frame (ORF) containing two initiation sites on the 5' end to produce slightly different primary polyproteins (Gao et al., 2016; Mahy, 2005; Yuan et al., 2020). The ORF is grouped into four main sections: the first being the Leader (L) protein sequence, followed by P1-2A, P2, and P3 precursor proteins (Figure 1.1) (S. M. B. Jamal, G.J., 2013; Mahy, 2005). Translation of this ORF results in a single polyprotein that is cleaved into various functional components (S. M. B. Jamal, G.J., 2013). The alternative initiation site results in the translation of two different L proteins or Leader proteases (L^{pro}): Lab and Lb (S. M. B. Jamal, G.J., 2013; Mahy, 2005; Yuan et al., 2020). The start codon (AUG1) for Lab is upstream from the start codon (AUG2) for Lb (Mahy, 2005). These variants remain functionally similar despite a sequence difference of 84 nucleotides (S. M. B. Jamal, G.J., 2013; Yuan et al., 2020). L^{pro} exhibits crucial proteolytic activity to cleave the translated primary polyprotein at the L^{pro}-P1 junction (Mahy, 2005; Yuan et al., 2020).

Figure 1.1 Visual representation of the FMDV +ssRNA genome showing the VPg protein, the 5' UTR and all of the secondary structures formed within this region, the coding region, and the 3'UTR with the poly A tract. Translation of the coding region is shown as the polyprotein with the L^{pro} enzyme attached. Cleavage sites by proteases or auto-cleavage are indicated with a diamond and the respective colour. Structural proteins

are encoded on the 5’ end of the genome while the non-structural proteins are encoded on the 3’ end of the genome.

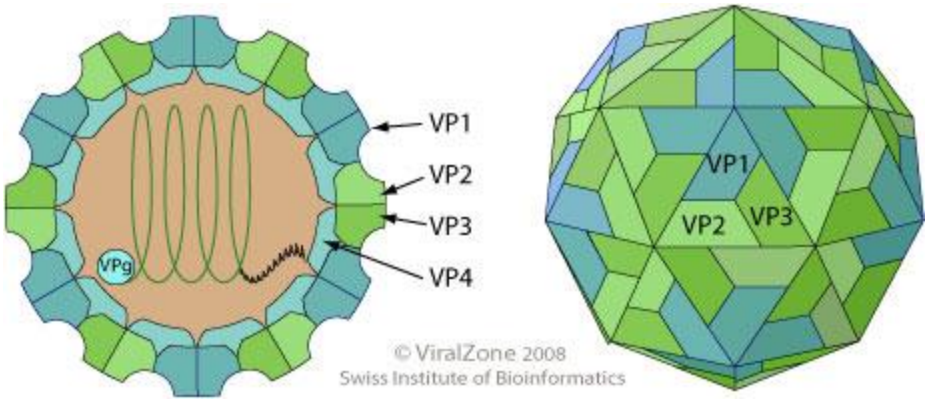


Note: Adapted from “Biological function of Foot-and-mouth disease virus non-structural proteins and non-coding elements”, Gao et al., 2016, *Virology Journal*, 13(1): p. 107. Licensed under CC BY 1.0.

P1-2A consists of 1AB (VP0), 1C (VP3), 1D (VP1), and 2A. This complex is cleaved at its C-terminus by 2A to release the structural protein precursor P1 from the primary polyprotein (Figure 1.1) (Mahy, 2005). This is followed by removal of the 2A component and separation of the 3 precursor capsid proteins VP0, VP3, and VP1 by $3C^{pro}$ (Figure 1.1) (Gao et al., 2016; S. M. B. Jamal, G.J., 2013; Mahy, 2005). VP0 is further auto-cleaved into the final capsid protein components VP4 and VP2 (Figure 1.2) (Gao et al., 2016; S. M. B. Jamal, G.J., 2013; Mahy, 2005). VP4 remains associated with VP2 and is an important factor for virus assembly (Goodwin et al., 2009; Strauss et al., 2015). This

last cleavage step occurs only during the encapsidation step and is triggered by assembly of the pro-virion (RNA encapsidated in structural proteins VP0, VP2, and VP1) (S. M. B. Jamal, G.J., 2013; Mahy, 2005).

Figure 1.2 Structure of the icosahedral FMDV capsid. Capsid is composed of 60 proteins each of VP0, VP1, and VP3. VP0 is cleaved into VP2 and VP4 during the encapsidation step when genomic +ssRNA is inserted into the provirion to form the mature virion.



Note: Reprinted from ViralZone, Swiss Institute of Bioinformatics, Retrieved June 9, 2021, from https://viralzone.expasy.org/resources/Picornaviridae_virion.jpg. Copyright © ViralZone, 2008. Licensed under CC BY-SA 4.0.

The P2 (2BC) and P3 (3ABCD) precursor proteins are split by an essential protease within the P3 element known as $3C^{pro}$ (Figure 1.1) (Gao et al., 2016; S. M. B. Jamal, G.J., 2013; Mahy, 2005). P2 is first separated from P3, and is then split into 2B and 2C (Gao et al., 2016; S. M. B. Jamal, G.J., 2013; Mahy, 2005). The 2B protein is a viroporin that contains two predicted hydrophobic domains allowing it to integrate into the phospholipid bilayer of host endoplasmic reticulum or Golgi apparatus (Gladue et al., 2018). The 2B viroporin acts as a transmembrane protein and increases membrane permeability of the host cell (Gao et al., 2016; Gladue et al., 2018; Mahy, 2005). P3 is split into 3A, 3 unique copies of 3B (or VPg), $3C^{pro}$, and $3D^{pol}$ (Figure 1.1) (S. M. B. Jamal, G.J., 2013; Mahy, 2005). The $3C^{pro}$ is an vital protease that cleaves the FMDV polyprotein at junctions while the $3D^{pol}$ is an RNA-dependent RNA polymerase

responsible for replication (Figure 1.1) (Gao et al., 2016; S. M. B. Jamal, G.J., 2013; Mahy, 2005). Intermediate cleavage structures of P3 exist and vary in longevity based on function (Gao et al., 2016). The three unique copies of 3B allow the formation of the different intermediates 3ABC, 3BCD, 3AB, and 3CD (Gao et al., 2016).

1.1.3.4. 3' UTR

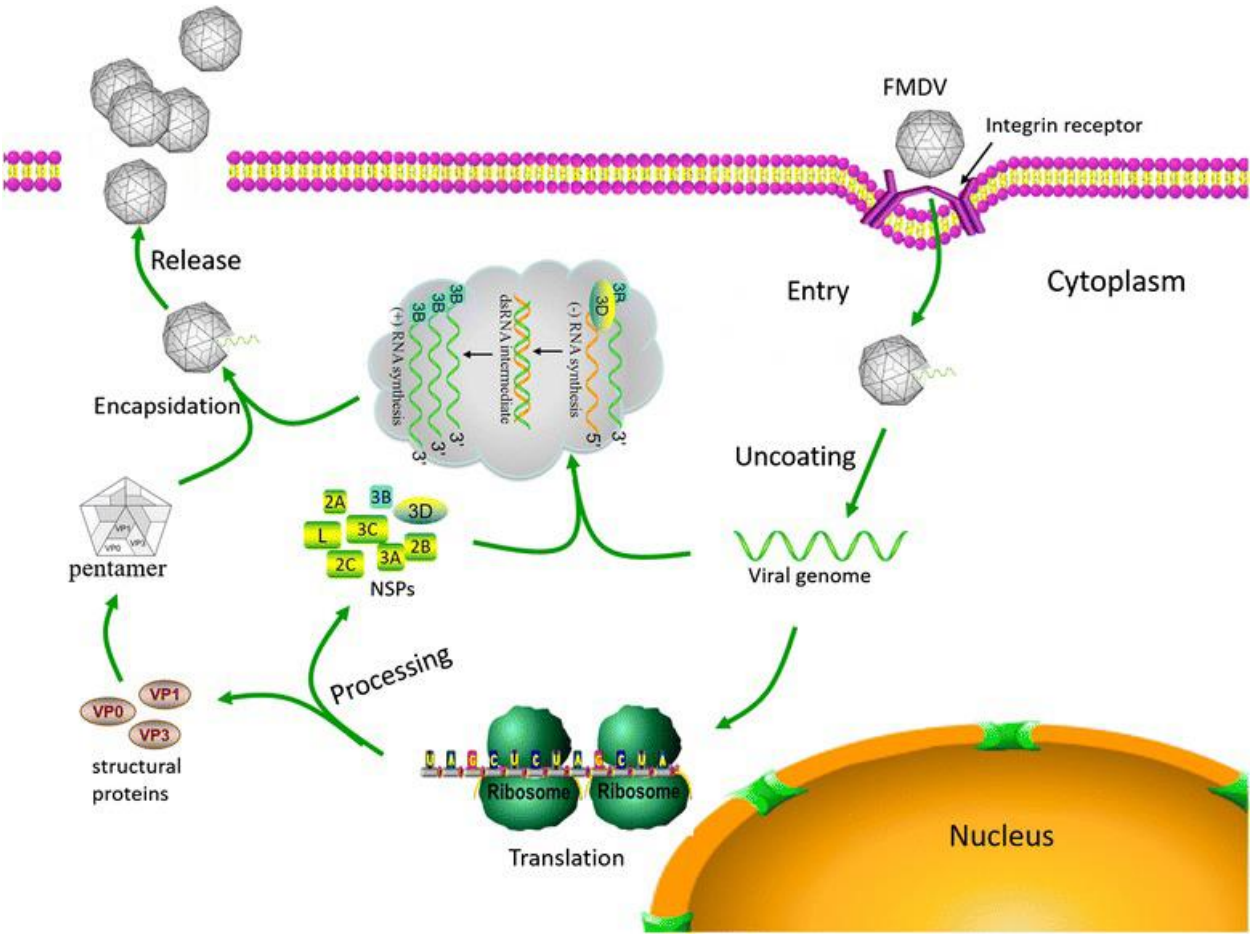
The 3'UTR of the FMDV genome is a stretch of sequence on the 3' end that is essential for virus viability (Han et al., 2015). It is composed of stem loops and a poly-adenosine tract that both contribute to viral replication and infectivity (Figure 1.1) (Han et al., 2015).

1.1.4. FMDV Replication

The exact mechanisms involved with the FMDV life cycle are not fully understood due to the numerous modes of entry utilized by FMDV (Berryman et al., 2005; Curry et al., 1995). It is suspected to be similar to other members of the family *Picornaviridae* such as poliovirus. The most understood method of entry is through binding to various integrin receptors of target cells to start the infection cycle (Gao et al., 2016; Jackson et al., 2000; Kotecha et al., 2017; Mahy, 2005). Of these various receptors, the $\alpha_v\beta_6$ receptor is especially important because of its high levels of expression on epithelial cells, and high binding affinity that FMDV has towards this receptor (Berryman et al., 2005; Gao et al., 2016; Jackson et al., 2000; Kotecha et al., 2017; Mahy, 2005). Binding of FMDV to integrin receptors triggers endocytosis of the virion (Berryman et al., 2005; Gao et al., 2016). This process of autophagy is induced in part by VP2 (Peng et al., 2020). The virion becomes enclosed in a vesicle that is acidified upon fusion with a lysosome (Berryman et al., 2005; Gao et al., 2016; Goodwin et al., 2009). Acidification triggers conformation changes in the capsid structure and subsequent release of the FMDV genome into the host cell (Curry et al., 1995; Gao et al., 2016; Goodwin et al., 2009). How the genome is released into the cytoplasm is still not well understood. The genome is first cleaved by a host enzyme to remove the VPg which is followed by direct

translation with host ribosomes as it is +ssRNA (Grubman et al., 2004). Viral polypeptides produced by translation are further processed into individual viral components (Gao et al., 2016). As the amount of viral proteins increases, the host cell machinery is diverted to virus production (Rodríguez Pulido et al., 2017). Pentamers consisting of VP0, VP1, and VP3 are first assembled to form a capsid (Goodwin et al., 2009). Viral genome replication occurs through the production of negative -ssRNA for use as a template for +ssRNA production (Grubman et al., 2004). This is followed by the addition of genomic RNA (gRNA) into the immature capsid (Figure 1.3) (Goodwin et al., 2009; Han et al., 2015). The 5' end of the gRNA binds covalently to the VPg resulting in the formation of the provirion (Goodwin et al., 2009; Han et al., 2015). Maturation occurs later upon cleavage of VP0 into VP2 and VP4 (Goodwin et al., 2009; Han et al., 2015). New viral progeny is eventually released through lysis of the host cell (Rodríguez-Habibe et al., 2020). The virions will then travel to neighbouring cells or secondary sites within the host and begin the infection cycle again.

Figure 1.3 Viral life cycle of FMDV that starts with binding of infectious virions to integrin receptors on the cell membrane of host cells. Virus is taken into the cell via endocytosis, and release of the viral genome is triggered upon acidification of the endosome. The +ssRNA FMDV genome is directly translated to produce viral proteins. Capsid proteins are assembled into a capsid and a copy of the genomic RNA is inserted into the capsid to form the mature virus that is released to infect other host cells.



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1.1.5. Species affected by FMDV

FMDV preferentially targets both wild and domestic cloven-hoofed animals. Among domestic animals, FMDV targets cattle, pigs, sheep, and goats (Grubman et al., 2004; S. M. B. Jamal, G.J., 2013; Li et al., 2021; Mahy, 2005; OIE, 2013). These are the most concerning target species infected by FMDV in the agricultural industry due to their popularity as a source of meat. There are up to 70 different wild animal targets of FMDV that include wild boars, deer, African buffalo, warthogs, African elephants, giraffes, antelopes, and gazelles (Di Nardo et al., 2015; Mahy, 2005; Alan R. Samuel et al., 2001; Weaver et al., 2013). There are many potential hosts for FMDV that may be infected by

the virus, but not produce any clinical signs or shed new virions (Mansley et al., 2003; Weaver et al., 2013). Some hosts such as the giraffe act as dead end hosts where the virus does not jump from one host to another (Weaver et al., 2013).

1.1.6. Transmission

Among animals that shed FMDV, transmission to another host can occur through aerosols, direct contact, indirect contact with contaminated fomites, or through human activity (Belsham, 2021; Mahy, 2005; Mansley et al., 2003; OIE, 2013). Transmission from an infected animal to a non-infected animal can occur through contact with milk, semen, urine, feces, contaminated food or water, saliva, and vesicular fluid (OIE, 2013; Paton et al., 2018; Alan R. Samuel et al., 2001). Certain routes of entry are better than others, and the effectiveness of those routes can vary between animals. Spread of FMD occurs most commonly through animal movement resulting in contact between infectious and non-infectious animals, feeding of contaminated food products especially in pigs, or transfer through humans, contaminated fomites and vehicles (Alexandersen, Zhang, et al., 2003; Mahy, 2005). Ruminants can be infected through inhalation of infectious particles in aerosols, however pigs are somewhat resistant through that route (S. Alexandersen et al., 2002; Mahy, 2005; Paton et al., 2018). Pigs can release large amounts of infectious aerosols that can travel to other nearby ruminants and cause infection (Farsang et al., 2013; Mahy, 2005; Stenfeldt, Diaz-San Segundo, et al., 2016). Pigs have been shown to release as much as $10^{5.6}$ to $10^{8.6}$ tissue culture infectious dose 50 ($TCID_{50}$) of FMDV per day and are the likely source of transmission to cattle and sheep (S. Alexandersen et al., 2002; Mahy, 2005). In ruminants it was found that exposure to only 10 viral particles was sufficient to cause infection, while pigs required a minimum of 10^3 $TCID_{50}$ (S. Alexandersen et al., 2002; Mahy, 2005). Infection can also occur by ingestion of contaminated food or entry through abrasions, but higher doses of virus are needed (Paton et al., 2018). In pigs, ingestion of contaminated materials is the main cause of transmission (Stenfeldt, Diaz-San Segundo, et al., 2016). Domesticated animals are typically kept in close quarters – an environment which allows virus to spread rapidly. Wild animals such as the African buffalo are known to carry the virus for 5 or more years

(B. M. d. Bronsvoort et al., 2016; Di Nardo et al., 2015; Mahy, 2005; Moonen et al., 2000; OIE, 2013). Wild animal movements cannot be controlled, so some herds can maintain FMDV for decades due to constant circulation of the virus between individual animals (Di Nardo et al., 2015; Weaver et al., 2013). Domestic cattle are also known to maintain carrier status for up to 3.5 years (B. M. d. Bronsvoort et al., 2016; Moonen et al., 2000). Long periods of time can allow for re-introduction of FMDV back into a farm and new outbreaks to develop. Persistently infected cattle and buffalo are known to harbour FMDV in nasopharynx epithelia and associated lymphoid tissue (Bertram et al., 2020; Stenfeldt, Eschbaumer, et al., 2016). In laboratory experiments, transmission has been found possible with oropharyngeal fluids from persistently infected cattle (Arzt et al., 2018; Bertram et al., 2020). This fluid, when inserted into the nasopharynx of naïve cattle, was found to result in FMD (Arzt et al., 2018; Bertram et al., 2020). Transmission through direct contact was found only with African buffalo in the persistent phase of an FMD infection (Bertram et al., 2020; Dawe, Flanagan, et al., 1994; Dawe, Sorensen, et al., 1994).

Human activity is one of the most common causes of FMD outbreaks and was the cause of the 2001 and 2010 outbreaks in the UK and Japan respectively (Department of Environment, 2002; Mahy, 2005; Muroga et al., 2012; Muroga et al., 2013). Non-susceptible animals such as dogs may also carry the virus and transmit the disease to target animals (Auty et al., 2019). This could be a risk for farms that utilize dogs to herd or direct animals. People are also prone to carrying FMDV and transmitting it to uninfected animals (Amass et al., 2003; Auty et al., 2019). This can be through clothing, footwear, or more uncommonly through the nasal cavities (Amass et al., 2003; Auty et al., 2019; Wright et al., 2010). The modernization of the world and the constant travel of humans between farms has also increased transmission via fomites. Vehicles, farm equipment, and personal protective equipment can carry FMDV from one area to another if disinfection and cleaning is not utilized (Auty et al., 2019; Muroga et al., 2012; Muroga et al., 2013). Trade of animals and animal products also pose a risk to countries that are free of FMDV. Animals destined for sale could potentially carry FMDV, while meat products such as frozen lymph nodes and bone marrow could harbour the virus for extended periods of time (Mahy, 2005). This was demonstrated by the 2001 FMD

outbreak in the UK which originated from the feeding of untreated food waste to pigs (Gibbens et al., 2001; Mahy, 2005).

1.1.7. Pathogenesis and Clinical Signs

Once FMDV is transmitted to an animal, the virus begins to replicate at the site of infection and/or primary replication sites (Arzt et al., 2011). In swine, this would be the oropharyngeal tonsil epithelia since infection occurs mainly through the ingestion of contaminated food, water, or saliva (Eblé et al., 2006; J. M. Pacheco et al., 2010; Stenfeldt, Diaz-San Segundo, et al., 2016).

In cattle, the primary site of infection is the nasopharyngeal mucosa within the nasopharynx that is later followed by amplification in the lower respiratory tract (Arzt et al., 2010; Arzt et al., 2014; Stenfeldt et al., 2015). Aerosols are the main route of transmission in cattle, therefore it follows logically that the nasopharynx is the primary site of infection (Stenfeldt et al., 2015).

In small ruminants such as sheep and goats, the primary site of infection is similar to what is found in cattle (Onozato et al., 2014; Stenfeldt, Pacheco, et al., 2020). However, studies have shown that early replication of FMDV was also detectable in the oropharynx and laryngopharynx region (Onozato et al., 2014; Stenfeldt, Pacheco, et al., 2020).

There is an incubation phase where virus cannot be detected in infected animals and no clinical signs have developed. This stage typically lasts between 1 to 14 days depending on the route of infection, the viral dose, animal host, or the strain of FMDV used (Arzt et al., 2011; Belsham, 2021; Eblé et al., 2006; OIE, 2013). The clinical phase is the stage at which FMDV replication happens very fast and when clinical signs first develop (Arzt et al., 2011; Eblé et al., 2006). These signs include lesions which develop at both primary and secondary sites such as oropharyngeal region and the hoofs respectively (Stenfeldt, Diaz-San Segundo, et al., 2016). FMDV levels in blood peak during this stage. High levels of virus can also be detected at peripheral sites such as vesicular lesions (Arzt et al., 2011; Eblé et al., 2006). Post-viremia or convalescence is the last stage of FMD. At this stage, viremia drops significantly or is completely cleared at approximately 5-7 days

post infection (DPI) (Arzt et al., 2017). This is also the point where clinical signs may begin to resolve, and lesions are healed at approximately 14 DPI (Stenfeldt, Diaz-San Segundo, et al., 2016). Antibodies to the virus begin circulating around 7 DPI, however the virus can remain persistent as infectious virions can still be found in areas such as the oropharynx in ruminants (Arzt et al., 2011; OIE, 2013). In some tissue types like lymph nodes, remnants of FMDV can be found as late as 72 DPI (Arzt et al., 2011; Paton et al., 2018). Ruminants that have detectable levels of infectious FMDV in esophageal-pharyngeal fluid beyond 28 DPI are considered carrier state animals (Maree et al.). Clinical signs among FMDV infected animals are not universal as some animals such as sheep and goats act as carriers without the development of symptoms (Arzt et al., 2011; Di Nardo et al., 2015; Mansley et al., 2003; Alan R. Samuel et al., 2001). These animals typically have longer infectious periods and shed lower levels of infectious virus (Soren Alexandersen et al., 2002; Farsang et al., 2013; Orsel et al., 2007). Therefore these animals more commonly develop subclinical infections that may be missed or discovered late after the disease has spread (Farsang et al., 2013). In some cases, FMD infections in young pigs, sheep, or goats may result in death by heart failure without the development of any typical clinical signs (Belsham, 2021).

Certain serotypes of FMDV also have a higher virulence in specific species (Alan R. Samuel et al., 2001). The most common clinical signs of an FMDV infection include fever, loss of appetite, excessive salivation, bruxism (teeth grinding), mastitis (mammary gland inflammation), hoof lesions or deformations, and most notably vesicle/blister formation on the tongue, oral cavity, and snout (Mahy, 2005; OIE, 2013). Reduced milk production, lameness, and loss of draught power may present as a result of low appetite. In some animals such as cattle and pigs, clinical signs can develop within one day of infection (OIE, 2013). Mortality among animals is low in adults, but can be very high among neonates (Alexandersen, Zhang, et al., 2003; Knight-Jones et al., 2013; Paton et al., 2018; Alan R. Samuel et al., 2001). Clinical signs due to FMDV can vary in severity and presence among different species of animals. For example, excessive salivation due to FMD is more commonly found with dairy cows whereas in pigs this is less common (OIE, 2013). In contrast, foot lesions are more common in pigs and can lead to complete claw detachment (Heinritzi et al., 2001; OIE, 2013). In sheep and goats, FMDV

infections are typically mild and thus introduces difficulties for clinical diagnosis (Alexandersen, Zhang, et al., 2003; Grubman et al., 2004).

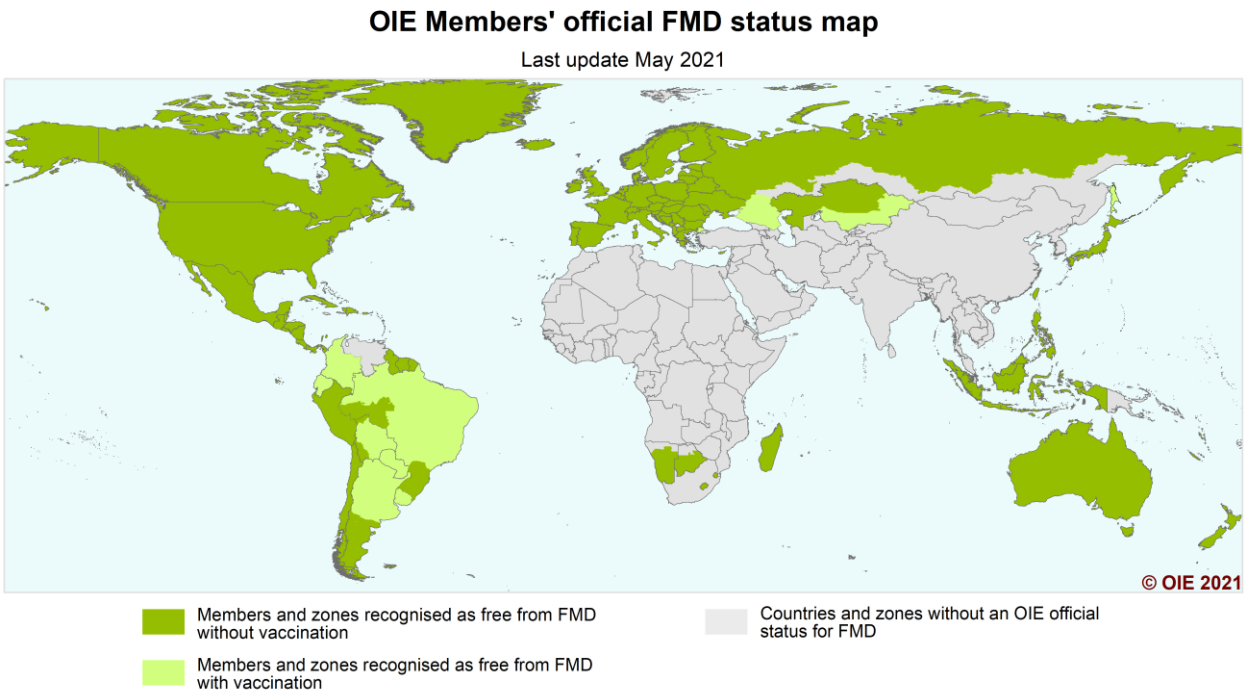
A complicating factor with the management of FMD is that many of the clinical signs are very similar to other viral diseases (Mahy, 2005). These differential diagnoses include Swine Vesicular Disease, Vesicular Stomatitis, Vesicular Exanthema of Swine, Rinderpest, and Bluetongue (Mahy, 2005; OIE, 2013). Each of these diseases produces clinical signs that are similar to FMD (Mahy, 2005). Therefore testing must be done to determine the cause of infection and the course of action in the event of FMD detection.

1.1.8. Epidemiology

1.1.8.1. FMD Pools and the Serotypes within each Pool

As of May 2021, North America, Europe, and Australia remain FMD free while some countries in Africa, Asia, and South America do not have an official status with World Organization for Animal Health (OIE) (Figure 2.1). These regions without an official status typically have a constant circulation of FMDV which keeps the disease endemic (B. M. d. C. Bronsvoort et al., 2004).

Figure 2.1 OIE member official FMD status map showing countries that are designated free of FMD without vaccination (dark green), countries free of FMD with vaccination (light green), and countries that do not have an OIE official status.



Note: Reprinted from OIE, World Organization for Animal Health, Retrieved June 9, 2021, from https://www.oie.int/app/uploads/2021/05/fmd-world-eng.png. Copyright © OIE, 2021.
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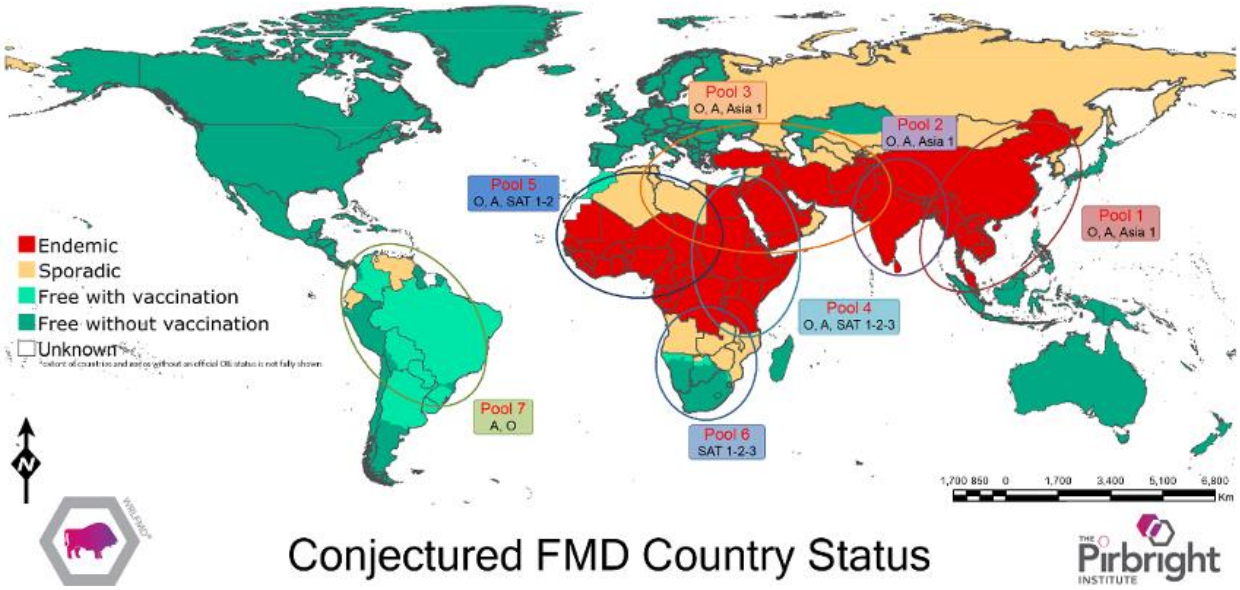
FMD occurrences are regionalized based on serotypes and are divided into 7 unique endemic pools (Brito et al., 2015; King, 2020). Each pool typically has certain serotypes that are more prevalent than others. In general, Serotypes A and O are found in most parts of the world that are endemic except in southern Africa (Habibi-Pirkoohi, 2018).
The first pool is regionalized to East and South-East Asia with serotypes A, ASIA 1, and O in circulation (Brito et al., 2015; King, 2020). Pool 2 is associated with the South Asia region and has serotypes A, ASIA1, and O (Brito et al., 2015; King, 2020). Pool 3 covers Western Eurasia and the Middle East (Brito et al., 2015; King, 2020). Serotypes A, ASIA1, and O are typical with SAT2 being an anomaly in 2017 Oman (King, 2020).

Figure 2.2 Global map showing FMD country status where red indicates endemic, orange indicates sporadic outbreaks, and green indicates FMD free status with or without vaccination (light green with vaccination, darker green without vaccination).

Regionalized FMD pools are also shown where certain serotypes are more prevalent than others. Serotypes O, A, and ASIA1 are typically present in Pools 1-3 (Eastern Asia, Central Asia, and the Middle East). Serotypes O, A, and SAT1-3 can be found in Pool 4 (Northern and Eastern Africa). Serotypes O, A, SAT1, and SAT2 are found in Pool 5 (Western and Central Africa). Serotypes SAT1-3 are found in Pool 6 (Southern Africa). Serotypes A and O are found in Pool 6 (South America).



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https://www.foot-and-mouth.org/terms-conditions

Pool 4 covers North and Eastern African countries (Brito et al., 2015; King, 2020). In this pool serotypes A, O, and SAT2 are present in North Africa while serotypes A, O, and SAT 1-3 are present in Eastern Africa (Brito et al., 2015; King, 2020). Pool 5 covers West and Central Africa with serotypes O, A, SAT1, and SAT2 circulating (Brito et al., 2015; King, 2020). Pool 6 is designated for Southern Africa with SAT 1-3 serotypes present while O and A serotypes are limited to Angola and North Zambia (Brito et al.,

2015; King, 2020). Pool 7 covers Colombia and Venezuela in South America with Serotypes O and A being present (Brito et al., 2015; King, 2020). An eighth pool was assigned to Europe that was later removed in the 1970s due to coordinated control measures put in place by the European Commission for the Control of FMD (EuFMD) to reduce the prevalence of FMD found in that region (Sumption et al., 2012). Currently, no pools exist in North America or Australia as these continents are free of FMD (Figure 2.2). The constant circulation of multiple serotypes in endemic regions brings about the evolution of FMDV and emergence of new FMDV lineages that may not be completely protected against through vaccination (King, 2020).

1.1.9. Prevention and Control of FMD

1.1.9.1. During an Outbreak

In the event of an outbreak in an FMD free country or region, swift and extreme action is usually taken to prevent further spread of FMD and minimize financial losses. Mass culling is employed in cases where there are several animals within a radius of infected animals (Alexandersen, Zhang, et al., 2003; Tildesley et al., 2009). This method of stamping out is necessary to remove any possible hosts that may harbour the disease and greatly amplify infectious virus (Alexandersen, Zhang, et al., 2003; Tildesley et al., 2009). Strict movement control is also put in place to prevent transfer of animals, and to limit human travel (Alexandersen, Zhang, et al., 2003; Fèvre et al., 2006; Kamel et al., 2019). Thorough disinfection of all equipment is employed in outbreak situations to further reduce any risk of fomite transmission (Alexandersen, Zhang, et al., 2003).
On the other hand, many FMD endemic countries do not employ strict FMD control programs (McLachlan et al., 2019). Countries with endemic FMD are likely poverty-stricken and do not have the means or infrastructure in place to control outbreaks. An outbreak may be left to completely run its course without any intervention due to the high cost of control. Culling, which is typically used in response to FMD outbreaks, is not done in these endemic countries because of lacking funds to reimburse farmers. In addition, movement control domestically and internationally is not strictly enforced if at

all. The lack of restrictions allow FMD to spread easily among animals within the country and across borders (Blacksell et al., 2019; Fèvre et al., 2006). Some countries may have policies in place to deter these practices, but enforcement lacks to prevent illegal importation (Chaber et al., 2010; Falk H Fau - Dürr et al., 2013).

Endemic countries are also known to have wild animal populations that cannot be controlled in outbreak situations. The African Buffalo is one such persistent carrier that serves as a source for outbreaks in Southern Africa (Maree et al.). It was found that the majority of buffaloes develop antibodies to FMDV by 2 years of age (Maree et al.). These wild animals come into contact with domesticated cattle and can start outbreaks (Omondi et al., 2018). In some endemic countries like Kenya, many farmers rely on pasture and water that is shared with wildlife (Omondi et al., 2018). This provides opportunities for FMDV to spread among domesticated livestock (Omondi et al., 2018).

1.1.9.2. Vaccination

Vaccination is a control measure that is used to combat outbreaks (Mahapatra et al., 2018). This method of control may be preferred in certain situations to avoid culling of entire farms and when animal welfare is considered (Kamel et al., 2019; P. Kitching et al., 2007; Mahapatra et al., 2018; Park et al., 2013). Vaccines for FMDV are developed using inactivated virus (Antigen) (Mahapatra et al., 2018). Production of antigen for vaccination is difficult and costly especially in endemic regions due to the requirement of higher containment facilities and cold chain delivery (Habibi-Pirkoohi, 2018). Multiple doses are also required for effective immunity as protection in cattle after a single dose is only 50% (P. Kitching et al., 2007). Vaccination must also be continued every 4-6 months since immunity to FMDV wanes as time progresses (OIE, 2019). In addition, the immune response produced from inactivated virus is less potent than if live attenuated FMDV was used (Habiela et al., 2014). Live attenuated virus elicits a much stronger and longer lasting immune response due to active replication that stimulates the innate immune response (Habiela et al., 2014; Zabel et al., 2013).

Vaccination with antigen for one serotype does not provide protection to other serotypes of FMDV due to antigenic differences (Mahapatra et al., 2018). Endemic countries

usually have various FMDV serotypes circulating (P. Kitching et al., 2007). When vaccination is employed to combat outbreaks, differences in subtypes and isolates of circulating virus must be considered since improperly matched vaccines would not confer protection to the vaccinated animals (Ashkani et al., 2016; P. Kitching et al., 2007; R. P. Kitching et al., 2005; Ludi et al., 2014; Mahapatra et al., 2018). Animals may also be infected regardless of vaccination when exposed to higher titers of virus (P. Kitching et al., 2007). Even if the vaccine used is homologous to the virus strain, it may not prevent infections that are subclinical or persistent (Bertram et al., 2020; Moonen et al., 2004; Parthiban et al., 2015; Stenfeldt, Eschbaumer, et al., 2016). This is due to the high rate of mutations found with RNA genomes that allow the evolution of antigenically distinct variants and subsequent immune evasion (Mahapatra et al., 2018). In some cases, vaccines produced using multiple strains can provide the needed protection to animals.

1.1.10. FMDV DIAGNOSIS

1.1.10.1. Clinical Diagnosis

FMD produces clinical signs that can be used to make a presumptive diagnosis. However, these clinical signs are similar to several other diseases as stated in 1.1.7. Therefore, FMD cannot be diagnosed only by clinical signs (Grubman et al., 2004). Laboratory or onsite testing is required to accurately identify the causative agent that is responsible for the development of the clinical signs in animals. Quick diagnosis is also important in situations of suspected cases. Early detection of FMD can result in significant cost savings since animals can be spared from slaughter if proper movement restrictions are put in place. Consequences resulting from late detection can be detrimental as was found with the 2001 outbreak in the UK (Fèvre et al., 2006). In that outbreak, detection of the first case had not happened until several days had passed (Fèvre et al., 2006). It is estimated that 30-79 farms already had cases of FMD once the first case was confirmed (Fèvre et al., 2006). The rapid spread and highly detrimental effects of delayed response highlights the importance of a quick diagnosis.

1.1.10.2. Laboratory Diagnosis

Laboratory analysis is used to confirm the causative agent that is responsible for observed clinical signs in diseased animals. FMD can be diagnosed through direct methods where viral presence can be confirmed, and through indirect methods by the detection of antibodies to the virus (OIE, 2021).

Confirmation of FMDV in sample specimens from suspected animals can be detected for directly by Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR), sequencing, antigen Enzyme Linked Immunosorbent Assay (ELISA), Lateral Flow Immunoassay (LFI), or by Virus Isolation (VI) (OIE, 2021).

Indirect diagnosis employs assays that detect the antibody response produced by a host in response to FMD. Assays that can detect these antibodies are the Non-Structural Protein ELISA (NSP ELISA), Solid Phase Competitive ELISA (SPCE), Liquid Phase Blocking ELISA (LPBE), and Virus Neutralization Test (VNT) (OIE, 2021).

1.1.10.3. Sample Types used for Testing

The sample types recommended by the OIE for laboratory diagnosis of FMD are epithelium tissue from vesicles and/or vesicular fluid (OIE, 2019). These sample types can be collected from unruptured or freshly ruptured vesicles located on the feet, tongue, and buccal mucosa of infected animals (OIE, 2019). In the absence of these sample types or in cases where clinical signs have not yet developed, oesophageal-pharyngeal fluid should be collected by probang or by swabbing (OIE, 2019). Probang samples are harvested from ruminants using a metal cup attached to a shaft that is inserted into the oro-nasopharynx to obtain fluid secretions (Stenfeldt & Arzt, 2020; Stenfeldt et al., 2013). Serum is also collected in these cases for detecting antibody response and viremia . Milk from dairy cattle has also been found useful in detection of FMDV (Armson et al., 2020; Tomasula et al., 2004).

1.1.10.4. Meat Juice as an Alternative

In some cases, the preferred sample types for FMDV detection may not be readily available. These situations may arise when animals have already been slaughtered and processed before the suspicion of FMD, or when only parts of the animal are available. The speed at which the meat production industry moves means animals can be slaughtered and sold before samples can be collected for the detection of suspected FMD. At this stage, typical samples cannot be collected for diagnosis. Therefore, meat juice (MJ) can be an attractive alternative for FMDV detection through testing of meat directly from abattoirs, markets/shops, or international ports of entry.

Meat is comprised of myocytes, mitochondria, and myofibrils arranged in long filaments. While the majority of meat is muscle fiber, small portions of connective and fat tissue are present (Ben-Arye et al., 2019; Listrat et al., 2016). Meat also contains blood vessels and nerves (Ben-Arye et al., 2019; Listrat et al., 2016). FMDV is known to circulate in blood, and therefore can potentially be found in meat. For application as a test sample for diagnosis of FMDV, MJ can be harvested by freeze-thaw cycles to disrupt membrane structure (Wallander et al., 2015). MJ is composed of intracellular and extracellular fluid, blood, and lymph (Gutierrez et al., 2008; Wallander et al., 2015).

Many countries conduct post-mortem inspection of carcasses. FMD surveillance using MJ can be done in addition to routine examination of carcasses with minimal disturbances. MJ can also be collected more swiftly and easily at these stages since animal restraint for the collection of samples is not necessary. Increased safety of technicians and less investment of time and money are additional benefits of MJ collection in comparison to other sample types.

International trade of meat has become commonplace to supply growing demand and brings with it risks of foreign animal diseases. Furthermore, the rise in global travel adds pressure to countries that are free of FMDV because passengers travelling intercontinentally may carry these contaminated meat or meat products and introduce FMD unintentionally. In these cases, carcasses may be completely absent and thus collection of more conventional samples is not possible.

The presence of FMDV in meat can be ruled out by testing MJ. MJ has found success as a sample type for the diagnosis of various bacteria and viruses while also being utilized for measuring biomarkers as indicators of animal health. Reverse Transcription PCR (RT-PCR) assays and ELISAs have been proven to detect Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in MJ from animals that are confirmed positive for PRRSV (Strutzberg-Minder, 2007). Measurable differences in Haptoglobin and C-reactive protein levels in animals infected with PRRSV were detected using MJ from experimentally infected pigs (Gutierrez et al., 2008; Strutzberg-Minder, 2007). Many groups have also shown that MJ can be used as an alternative substrate by ELISAs to indirectly detect other pathogens or parasites such as Hepatitis E Virus (Wacheck et al., 2012), Classical Swine Fever Virus (CSFV) (Kaden et al., 2009; Lohse et al., 2011), African
Swine Fever Virus (McKillen et al., 2010), Japanese Encephalitis Virus (JEV) (Yonemitsu et al., 2019), Porcine Circovirus Type 2 (Fabisiak et al., 2013), Aujeszky's Disease Virus (Panyasing et al., 2018), *Toxoplasma gondii* (Berger-Schoch et al., 2011), *Salmonella enterica* (Mousing et al., 1997), and *Trichinella spiralis* (Beck et al., 2005). MJ has not yet been examined as a sample type for detection of FMDV.

In countries with wild animal populations harbouring FMDV, MJ can be collected from animals that are found dead for determining viral prevalence. In the field, MJ is advantageous because serum requires the use of specialized equipment to separate clotting factors within blood (Yonemitsu et al., 2019). Meat only requires a freeze-thaw cycle to collect MJ for analysis. In terms of collection and cost, MJ is more accessible than serum. Tools such as serum separation tubes, needles, and pharmaceuticals to subdue animals are not necessary for the collection of meat since it can be obtained directly at abattoirs or markets. This eliminates the need for extensive travel to farms for collection of typical samples like blood and oropharyngeal fluid. Information on circulating serotypes or isolates can be easily obtained with MJ compared to other sample types. In some instances, farms are located remotely in areas with poor infrastructure. These areas are difficult to access and can increase the time and cost of surveillance. Remote locations may also pose a risk to safety in countries with criminal activity. These benefits make MJ a more cost effective option for managing FMD.

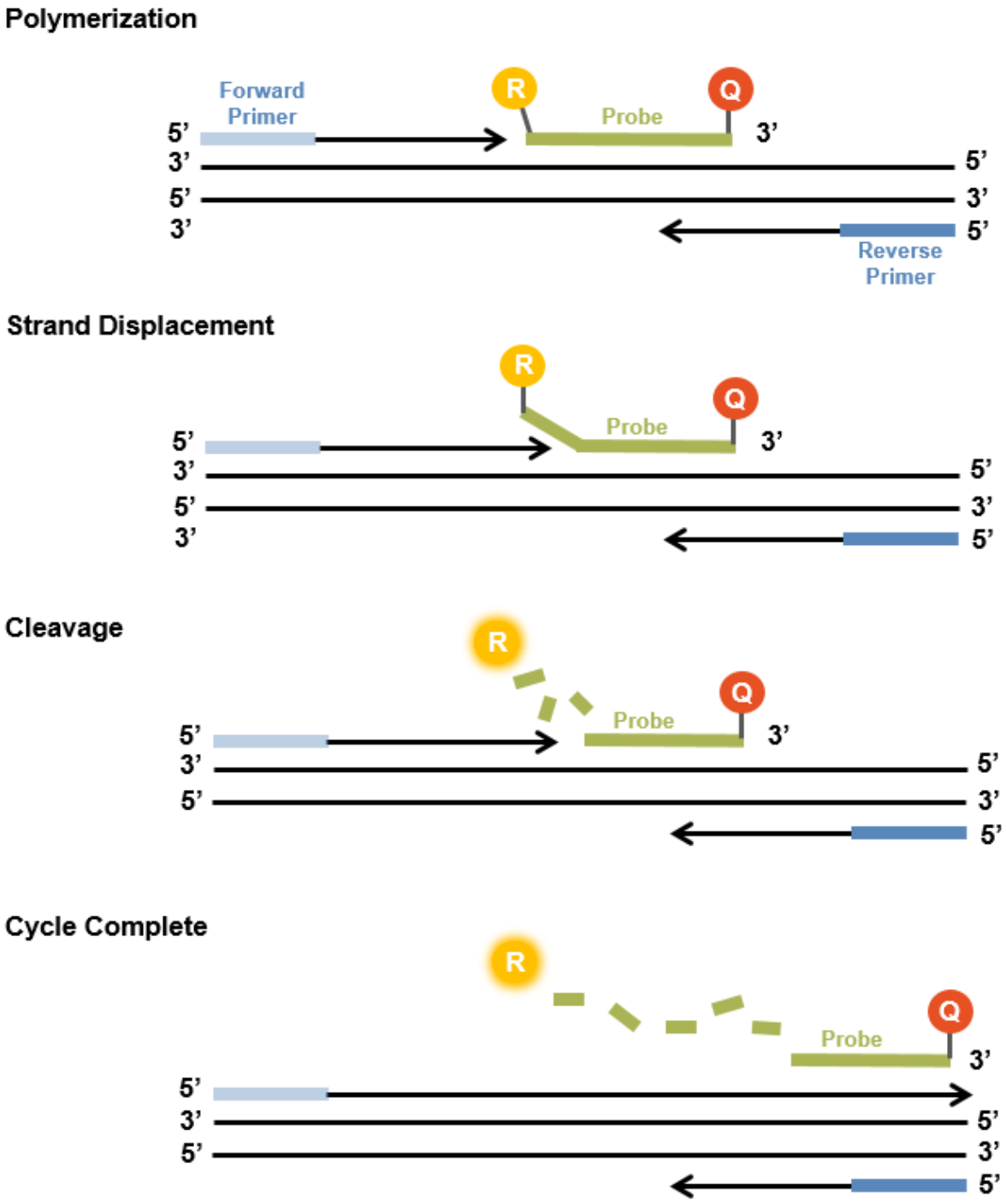
However, MJ is not without its flaws. The volume of MJ obtained has been found to vary from sample to sample (Mousing et al., 1997; Wacheck et al., 2012). Some meat samples may not produce MJ which increases uncertainty for diagnostic purposes (Mousing et al., 1997). In terms of diagnostic testing, a greater amount of MJ was necessary for serological testing in comparison to usual samples (Yonemitsu et al., 2019). The use of MJ as a sample for detection of CSFV by rRT-PCR was found less sensitive and less specific than serum (Lohse et al., 2011). MJ is known to contain several contaminants that can interfere with certain diagnostic tests (Yonemitsu et al., 2019). In particular, the contamination found in MJ likely impacts assays that do not require processing of the sample.

1.1.11. Diagnostic Test Methods

1.1.11.1. Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR)

rRT-PCR is one of the most efficient and effective test methods in use to test directly for FMDV nucleic acid. rRT-PCR can output results in real-time through the measurement of fluorescence. rRT-PCR require an initial step where the target region of the template RNA is first converted into complimentary DNA by reverse transcriptase. The target complimentary DNA (cDNA) is then amplified through repetitive cycling of extension, denaturing, and annealing steps.

Figure 3.1 Steps showing the amplification steps of a rRT-PCR. Sample RNA is first converted into cDNA (not shown) and followed by target amplification. cDNA synthesis begins at the forward primer and continues in the 5’ to 3’ direction. Probes are conjugated to a fluorophore (R) and a quencher (Q). These probes are displaced and cleaved by DNA polymerase exonuclease activity during the extension of cDNA. This step releases the fluorophore (R) and subsequent fluorescence that is measured by the thermocycler.



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During each cycle, extension of the new cDNA by DNA polymerase causes displacement and degradation of the labelled probes by 5' to 3' exonuclease activity (Figure 3.1). This releases the fluorescent molecule from the probe and separates it from the quencher (Figure 3.1). This separation allows for fluorescence which can be measured by the thermocycler and indicates presence or absence of target RNA. A threshold is established for the assay such that any sample which produces more fluorescence than the specified cutoff is considered positive. This is termed the cycle threshold (Ct) and refers to the number of cycles that a sample has undergone before meaningful levels of fluorescence have been detected. Lower Ct values indicate greater quantities of target RNA while higher Ct values indicate lower quantities. Samples that produce no Ct values are considered negative while samples that have a Ct value below the established threshold are considered positive.

The FMDV rRT-PCR test uses a specific forward primer, reverse primer, and fluorescent labeled probe to amplify the conserved 5'UTR region or the 3D gene (Callahan et al., 2002; Howson et al., 2017; Moniwa et al., 2007; Rios et al., 2018; Vandenbussche et al., 2017). These regions are chosen because of high sequence conservation between serotypes, subtypes, or isolates since they are key components of FMDV that are necessary for replication (Carrillo et al., 2005; S. M. Jamal et al., 2015). The 5'UTR facilitates translation while the 3D polymerase is responsible for genome replication (Carrillo et al., 2005; S. M. B. Jamal, G.J., 2013; Mahy, 2005). Mutations that arise in these regions cause the virus to become inviable as these components are necessary for FMDV infection (Carrillo et al., 2005). Therefore, targeting of these conserved regions within the FMDV allows these particular rRT-PCR to be pan-serotype (S. M. Jamal et al., 2015). The 3D gene is the preferred target for the FMDV rRT-PCR as it is more sensitive at detecting FMD positive samples than when the 5' UTR target is used (Veronika Dill et al., 2017; Gorna et al., 2016; Hindson Benjamin et al., 2008).

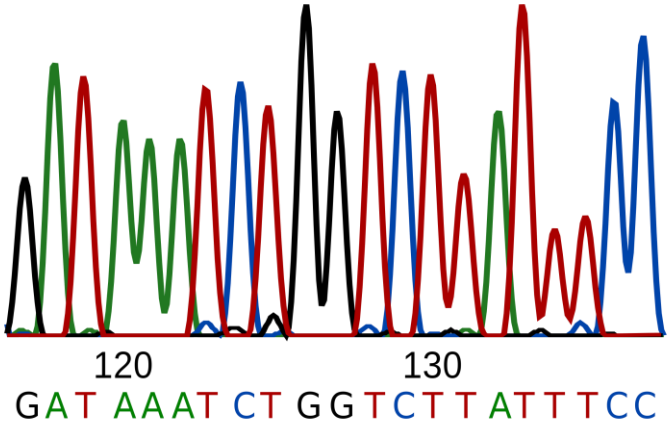
1.1.11.2. Sanger Sequencing

The viral nucleic acid can also be sequenced directly to determine presence or absence of FMDV; however this method is laborious and time consuming. Sequencing can be

employed in cases where the pan-serotype rRT-PCR has given a positive result and identification of the specific serotype is required.

Sanger sequencing is carried out by amplification of the desired region within the genome. For FMDV, this is the VP1 gene which is known to vary between serotypes and subtypes (Ludi et al., 2014). Amplification of this region by conventional RT-PCR is necessary to obtain higher quantities of template for the end-point PCR amplification that is needed for sanger sequencing (N. J. Knowles, Wadsworth, J., Bachanek-Bankowska, K., King, D. P., 2016). The amplification also converts the RNA sequence of the FMDV genome into cDNA. Amplified template is used to produce various sizes of the VP1 PCR products for sequencing. The sequencing PCR incorporates unique nucleotide analogs that are fluorescently labelled and modified. These analogs are di-deoxynucleotide triphosphates (ddNTPs) that terminate de novo assembly of cDNA when they are incorporated into the growing strand (Heather et al., 2016). Each of the four major ddNTPs can be detected through fluorescence. These ddNTPs are labelled with a unique fluorophore that emit varying wavelengths that is detected by the sequencing device. The device distinguishes and associates these reads with a specific base (Figure 3.2). To obtain sufficient coverage over a region of interest, several truncated cDNA products must be produced to provide adequate coverage to obtain base identification at each position.

Figure 3.2 Example of a short stretch of trace file output showing unique fluorescence readings at each position that corresponds to a base. Black peaks indicate guanine (G), green peaks represent adenine (A), red peaks indicate thymine (T), and blue peaks indicate cytosine (C) at each respective position.



1.1.11.3. Antigen ELISA

The viral capsid itself can be detected using antigen detection Enzyme-Linked Immunosorbent Assay (Ag ELISA). This ELISA is designed as a double-antibody sandwich where antibodies specific to FMDV are coated on the plate surface followed by the sample containing the virus, and then another FMDV specific antibody (Figure 3.4) (OIE, 2021). These sandwiches would then be bound by horseradish peroxidase conjugated monoclonal antibodies (OIE, 2021). Colour development is via substrates such as 3,3′,5,5′-Tetramethylbenzidine (TMB) or o-phenylenediamine dihydrochloride (OPD) that is measured by the corresponding wavelengths 450 nm and 490 nm respectively. Virus antigen is quantified based on relative absorbance between the sample and diluent control, (Figure 3.4). Samples that contain more virus antigen produce higher optical density (OD) values.

1.1.11.4. Virus Isolation

The gold standard test for FMDV detection is by VI, where virus can be detected through CytoPathic Effect (CPE) of cultured cells (Kabelo et al., 2020). This test is considered the gold standard because of the amplification of virus by continuous cell lines, and is confirmed visually under a light microscope (Kabelo et al., 2020). Samples collected from suspected animals are applied to cultured cells such as LFBKαVβ6 and monitored for 2 – 3 days. Any development of CPE indicates that virus is present in the sample.

Subsequent confirmatory testing by rRT-PCR and Ag ELISA must be done on the cell culture supernatant before the animal is declared infected. Samples that do not produce CPE are frozen, thawed, and blind passaged again on freshly cultured cells (OIE, 2021).

Although this test is the gold standard, turnaround times are significantly longer than by other assays like the rRT-PCR. In addition, the time period where live virus can be isolated from samples is limited. Viremia also dissipates approximately 5 to 7 days after infection (Arzt et al., 2017). This time period can easily be missed, therefore other tests must be done in combination with VI to obtain accurate diagnosis.

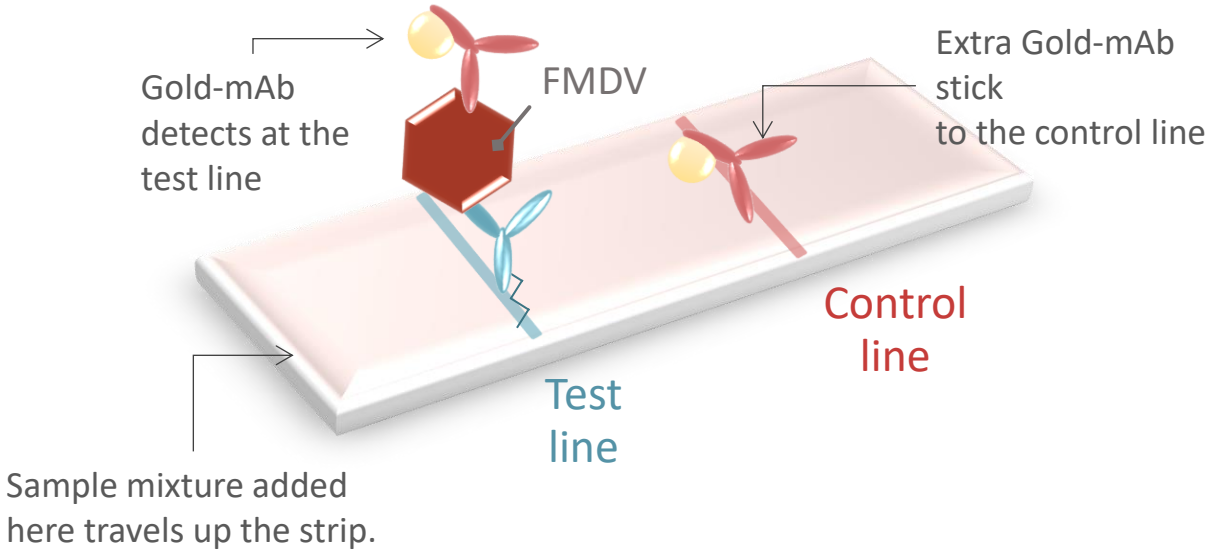
1.1.11.5. Lateral Flow Immunoassays

Lateral Flow Immunoassays (LFIs) are another method to rapidly test for the presence of FMDV in animals. These tests use FMDV specific mouse monoclonal antibodies that bind the virus in a sample.

Lateral Flow Immunoassays (LFIs) are useful for rapid diagnosis of FMDV onsite. Tests previously developed at the National Centre for Foreign Animal Diseases (NCFAD) were found to produce results within 30 minutes of sample application (M. Yang et al., 2013; M. Yang et al., 2019). With rapid diagnosis, immediate action can be taken to quarantine herds while samples are sent to an approved lab for confirmatory testing.

LFIs involve mouse monoclonal antibodies (mAbs) to FMDV that are conjugated with capture components and visualization components. LFIs developed by Yang et al. (2013) utilize a biotin conjugated mAb for capture, and gold nanoparticle conjugated mAb for visualization (Figure 3.3).

Figure 3.3 Schematic showing the working components of the FMDV LFI developed at the NCFAD. One monoclonal antibody (mAb) to FMDV is conjugated to biotin (shown in blue) while another is conjugated in gold (shown in red) for visualization on the strip. Both mAbs are combined with sample containing FMDV before being applied to the sample pad. Sandwich complexes form and are captured at the test line (shown in blue) and indicate a positive sample. Excess gold conjugated anti-FMDV mAbs travel down the strip and are captured at the control line to prove the LFI is functional.



Note: Image produced by Kaye Quizon and reused with permission.

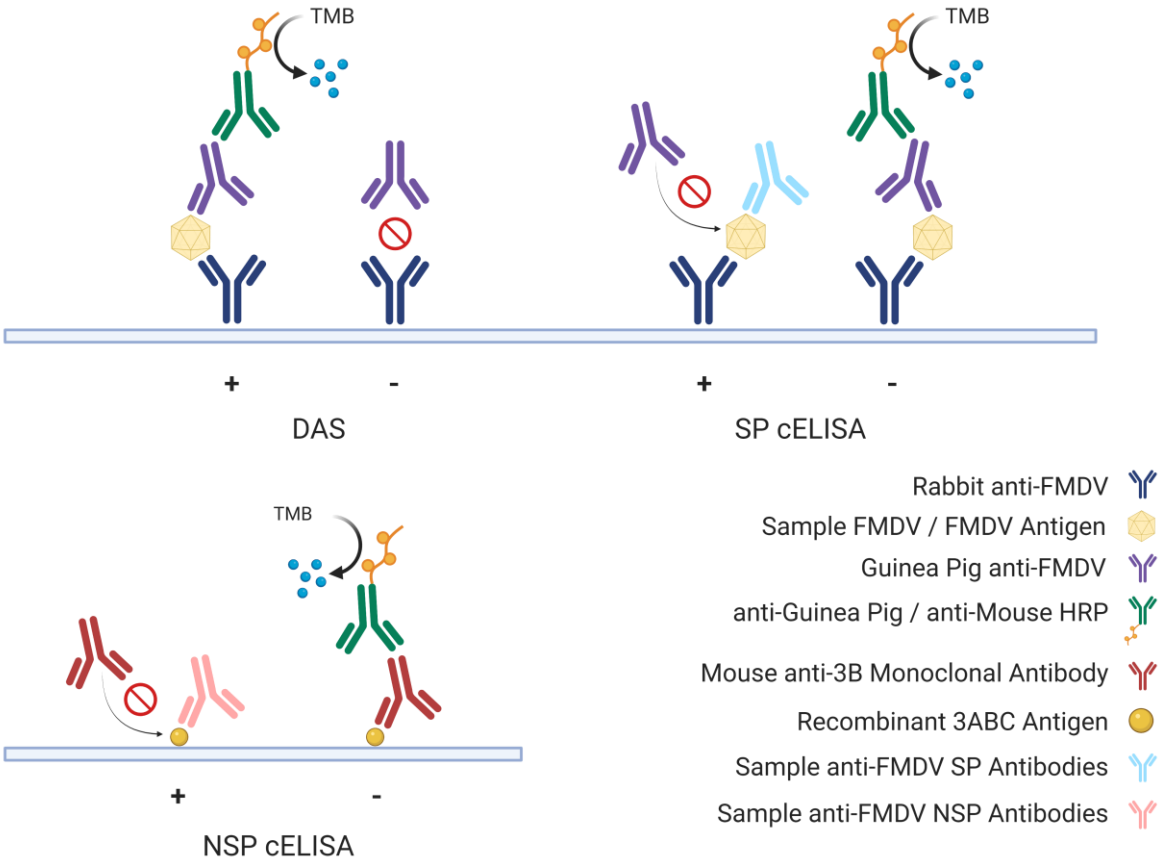
Both conjugated anti-FMDV mAbs are mixed with sample containing FMDV and form sandwich complexes as seen in Figure 3.3. This mixture is added onto the sample pad of the LFI and given 30 minutes to travel across the strip. Any antibody-FMDV sandwiches will be captured at the test line by streptavidin. Streptavidin associates with the biotin conjugated mAb to fix the sandwich complex in place. The control line is a region of the strip that is coated with anti-mouse antibodies. Thus any excess gold conjugated anti-FMDV mAbs are captured at the control line. Both lines are visualized by the gold nanoparticle component of the conjugated mAb. Samples that contain FMDV will be positive based on the development of the bands. Coloured bands at the test and control lines indicate a positive result. If only one band at the control line is present, this indicates that the sample is negative for FMDV antigen.

1.1.11.6. Non-Structural Protein (NSP) ELISA

FMDV can also be detected indirectly by measuring antibody levels that are specific to different components of the virus. Animals that become infected by the virus eventually develop a humoral immune response by producing antibodies that bind to various parts of

the virus or its proteins. These antibodies may bind to components of the capsid/structural proteins (SPs), or to non-structural proteins (NSPs). NSP ELISAs can be used for high throughput screening of many animals. These ELISAs target conserved FMDV NSPs such as 3ABC. The NCFAD 3ABC cELISA measures antibody levels in serum to the 3ABC protein of FMDV (Figure 3.4) (Ming Yang et al., 2015). Antibodies can be detected using Histidine-tagged recombinant 3ABC antigen coated onto a microtitre plate' (Ming Yang et al., 2015). Serum samples are then added together with a competing mouse anti-3B mAb. Antibodies present in serum compete with the mouse anti-3B mAb for binding to the 3ABC antigen on the plate. A peroxidase conjugated anti-mouse antibody to the known competing antibody is added next. Substrate is added as a last step to measure the level of binding by the mouse mAb compared to the anti-3ABC antibodies in serum. No colour development indicates a high level of anti-3ABC antibodies while strong colour development indicates low levels of anti-3ABC antibodies within the sample. High levels of anti-3ABC indicate that the serum sample collected from an animal is positive. This means the animal is infected or was previously infected by FMDV.

Figure 3.4 Schematic showing the various ELISAs used for the detection of FMDV or antibodies to components of the virus. The Double Antibody Sandwich (DAS) ELISA detects FMDV in samples, the Structural Protein (SP) ELISA detects antibodies to the capsid proteins of FMDV, and the Non-Structural Protein (NSP) ELISA detects antibodies to the non-structural proteins of FMDV.



Created with BioRender.com

This assay is also useful in cases where differentiation of infected from vaccinated animals is required. Vaccines are typically inactivated virus which has been purified to remove NSPs and does not replicate when introduced into animals. The lack of viral replication means that any NSPs that may develop in a normal infection are absent with vaccination. Therefore antibody responses develop only to the structural components of the virus. Hence, animals that have been vaccinated do not produce an antibody response to 3ABC. Vaccinated animals tested by the NCFAD 3ABC would produce high OD values which corresponds with a negative result.

1.1.11.7. Solid Phase Competitive ELISA

Solid Phase Competitive ELISA (SPCE) are serological tests that are employed for screening suspected animals (OIE, 2013, 2021). This test detects for antibodies within

serum samples in a competitive format. The NCFAD SPCE uses serotype specific capture antibodies found in rabbit antiserum that is coated on plates (D. K. J. Mackay et al., 2001). After coating of the plate with rabbit antiserum, the corresponding FMDV antigen is added and incubated (D. K. J. Mackay et al., 2001). FMDV antigen forms a solid support complex with rabbit anti-FMDV antibodies. Samples from suspected animals are then added onto the plate together with guinea pig antiserum (D. K. J. Mackay et al., 2001). This simultaneous addition of guinea pig and sample serum leads to competition between the antibodies in the sample from the suspected animal and the antibodies from guinea pig antiserum (Figure 3.4). Animals that have been infected by or vaccinated against FMDV are expected to produce an antibody response. Thus test sera from infected or vaccinated animals should contain antibodies that bind to the FMDV antigen and inhibit binding of the anti-FMDV guinea pig antibodies. Conjugated anti-guinea pig antibodies followed by chromogenic substrate are added to determine the amount of guinea pig antibody binding. Inhibition of the guinea pig antibodies by sample antibodies will vary. The amount of inhibition is inversely proportional to the amount of anti-FMDV antibodies within the test sample. The minimum inhibition (cutoff) that classifies a sample as positive is 50% across all serotypes of FMDV. Therefore any sample that produces a level on inhibition greater than 50% is positive for antibodies to the SPs of FMDV. As with the NSP ELISA, colour development indicates a negative sample while no colour development indicates a positive sample. This test can also be used to determine the serotype of FMD that infected the animal since structural proteins vary between serotypes (D. K. J. Mackay et al., 2001).

1.1.11.8. Virus Neutralization Test

The Virus Neutralization Test (VNT) is an internationally and OIE accepted gold standard test for neutralizing antibody detection that proves that an animal was infected by or vaccinated against FMDV (S. M. B. Jamal, G.J., 2013). This assay is designed such that antibodies in a sample will bind to live virus and prevent infection of cultured cells (Payne, 2017). The NCFAD VNT uses live virus combined with serum from an infected animal that is then applied to IB-RS-2 cells and monitored over 2 days. Back titrations of

the virus used in this assay confirms the amount of virus used while serum with a known antibody titre is used as a positive control. Serum from uninfected animals is used as a negative control. These are necessary to monitor test performance.

Serum from infected animals contains antibodies approximately 7 days after an infection (Cox, 2000). These antibodies bind to FMDV, may prevent adhesion of the virus to the FMDV-binding receptors of the IB-RS-2 cells, and stop development of CPE. If no CPE is observed, it proves that an animal was infected by or vaccinated against FMDV. This assay confirms the presence of neutralizing antibodies in serum. However this test cannot detect non-neutralizing antibodies since these antibodies, if present, will not block binding of the virus to the IB-RS-2 cells (Payne, 2017).

This test while reliable, is time consuming and requires a high containment laboratory with highly trained staff (Basagoudanavar et al., 2013). Therefore this test is used only for confirmatory purposes when other rapid assays produce suspicious results (OIE, 2019).

2. Hypothesis and objectives

2.1. **Hypothesis**

Due to the fact that skeletal muscle is perfused by blood vessels, MJ can serve as a suitable alternative to conventional samples for the detection of FMDV. Therefore,

- The FMDV genome and antigen can be detected in MJ of infected animals by currently available diagnostic tests such as rRT-PCR, antigen ELISA, and LFIs during the acute phase of FMD infections.
- Antibodies to FMDV can be detected in MJ from infected animals using currently available diagnostic tests such as serotype specific structural protein and NSP ELISAs during the convalescent phase of the disease.

Therefore, MJ can be a valuable sample matrix for FMDV detection in unique situations where traditional samples are not available.

2.2. Objectives

The goal was to analyse swine MJ harvested from the *biceps femoris* to determine its suitability as a sample matrix for the detection of FMDV and the antibodies produced to FMDV. Established assays were used to accomplish this objective. Detection of FMDV genome was tested for in MJ by rRT-PCR. FMDV antigen detection in MJ was assessed using pen-side LFI and antigen ELISA. Antibodies to the SP and NSP of FMDV in MJ was detected for by SPCE and NSP ELISAs, respectively.

3. Manuscripts

3.1. Detection of Foot-and-Mouth Disease Virus in Swine Meat Juice by RRT-PCR and Lateral Flow Immunoassay (LFI)[2]

3.1.1. Abstract

Foot-and-mouth disease virus (FMDV) is a highly contagious agent that impacts livestock industries worldwide, leading to significant financial loss. Its impact can be avoided or minimized if the virus is detected early. FMDV detection relies on vesicular fluid, epithelial tags, swabs, serum, and other sample types from live animals. These samples might not always be available, necessitating the use of alternative sample types. Meat juice (MJ), collected after freeze-thaw cycles of skeletal muscle, is a potential sample type for FMDV detection, especially when meat is illegally imported. We have performed experiments to evaluate the suitability of MJ for FMDV detection. MJ was collected from pigs that were experimentally infected with FMDV. Ribonucleic acid (RNA) was extracted from MJ, sera, oral swabs, and lymph nodes from the same animals and tested for FMDV by real-time reverse transcription polymerase chain reaction (rRT-PCR). MJ was also tested for FMDV antigen by Lateral Flow Immunoassay (LFI). FMDV RNA was detected in MJ by rRT-PCR starting at one day post infection (DPI)

[2] Modified from original publication
DOI: 10.3390/pathogens9060424

and as late as 21 DPI. In contrast, FMDV RNA was detected in sera at 1–7 DPI. Antigen was also detected in MJ at 1–9 DPI by LFI. Live virus was not isolated directly from MJ, but was recovered from the viral genome by transfection into susceptible cells. The data show that MJ is a good sample type for FMDV detection.

3.1.2. Introduction

Foot-and-Mouth Disease (FMD) is a condition caused by foot-and-mouth disease virus (FMDV) that affects cloven-hoofed animals such as cattle, pigs, and sheep (Alexandersen, Kitching, et al., 2003). FMDV is a ribonucleic acid (RNA) virus that is highly contagious among susceptible animals and produces symptoms which include fever, reduced appetite, or vesicular lesions (Alexandersen, Kitching, et al., 2003). Mortality rates of animals infected by FMDV are generally low among adults, but very high in neonates (Alexandersen, Kitching, et al., 2003). Animals infected with FMDV have greatly reduced milk production, decreased draught power, and lose significant weight (Alexandersen, Kitching, et al., 2003; Grubman et al., 2004). FMDV remains endemic in several countries around the world and impacts their respective agricultural industries (FAO/EuFMD, 2019). Countries that have obtained FMDV-free designation by the World Organization for Animal Health (OIE) always face the risk of potential outbreaks. These FMDV-free countries utilize preventative measures to minimize the risk of an outbreak. Animals suspected of having a vesicular disease are tested to ensure the absence of FMDV as the clinical signs are indistinguishable from its differentials (Grubman et al., 2004). Procedures recommended by the OIE to identify FMDV are antigen enzyme-linked immunosorbent assays (Ag ELISAs), virus isolation (VI), real-time reverse transcription polymerase chain reactions (rRT-PCRs), or pen-side tests such as lateral flow immunoassays (LFI). These tests require samples such as vesicular fluid, epithelial tags, blood, milk, and swabs of the nares, oral cavity, or lesions, but these are not always accessible. Meat juice (MJ) is a potential alternative that can be used in situations where blood cannot be collected due to animal death or when the entire carcass is absent. This is common in the case of illegally imported meat into countries free of FMDV. Illegally imported meat contaminated with FMDV can potentially infect

domestic farm animals and induce an outbreak. The 2001 UK FMDV outbreak was attributed to a single farm that fed untreated waste to pigs (Gibbens et al., 2001). This epidemic resulted in culling of millions of animals to contain the virus and prevent further spreading (Tildesley et al., 2009).

MJ is a liquid transudate that can be collected from tissues after a freeze-thaw cycle. The cycling allows formation of ice crystals to disrupt cell membrane integrity and subsequent release of the intracellular contents, serum, and lymphatic fluid into the external environment (Gutierrez et al., 2008). Success has been demonstrated previously with MJ for the detection of various viruses by rRT-PCRs and ELISAs. Both assays have proven successful for detecting porcine reproductive and respiratory syndrome virus (PRRSV) in MJ from infected animals (Strutzberg-Minder, 2007). The RT-PCR detects the viral genome, while the ELISA detects antibodies produced by the animal in response to the virus. Virus detection with PCR was also successful for MJ samples containing African swine fever virus (McKillen et al., 2010). Other pathogens have also been detected by measuring the antibody response against hepatitis E virus (Wacheck et al., 2012), Japanese encephalitis virus (Yonemitsu et al., 2019), Aujeszky's disease virus (Panyasing et al., 2018), porcine circovirus type 2 (Fabisiak et al., 2013), classical swine fever virus (Lohse et al., 2011), *Toxoplasma gondii* (Berger-Schoch et al., 2011), and *Salmonella enterica* (Mousing et al., 1997). MJ has also been used for the measurement of porcine C-reactive proteins as a method of monitoring health status (Gutierrez et al., 2008). However, the use of MJ for the detection of FMDV has not yet been characterized. We report on the feasibility of MJ as a sample matrix for the detection of FMDV by rRT-PCR, lateral flow immunoassay (LFI), and virus recovery through transfection of cultured cells using extracted viral RNA from MJ. The rRT-PCR provides evidence of FMDV RNA in MJ, while LFI confirms the presence of viral antigen. The presence of FMDV RNA was confirmed through VP1 sequencing and recovery of live FMDV by transfection of cultured cells with extracted RNA from MJ.

3.1.3. Materials and Methods

3.1.3.1. Viruses

FMDV Serotype A (A22 IRQ 24/64) and Southern African Territories 2 (SAT2 ZIM 5/81) were used in separate studies. The viruses were obtained from the World Reference Lab for FMDV, Pirbright Institute. Each virus was amplified in the baby hamster kidney 21 (BHK-21) cells, the cell culture supernatant harvested after cytopathic effect (CPE) was observed, and the virus titre determined as previously described (Reed et al., 1938).

3.1.3.2. Experimental Animals and Inoculations

The Animal Care Committee at the Canadian Science Centre for Human and Animal Health approved this study under the Animal Use Document (AUD) number C-18-004. Thirty-six pigs at 5-6 weeks old were used for each FMDV serotype (A and SAT2). The pigs were purchased locally from Manitoba farmers and transported to the laboratory following the Canadian Council on Animal Care guidelines. These pigs were separated into six groups of six pigs and each group was housed together in a cubicle. The animals were given one week to acclimate to the environment prior to inoculation with the virus. Feed was provided twice daily and water was provided *ad libitum*.

3.1.3.3. Animal Inoculations

FMDV inoculum was prepared by diluting the virus to 5 $x10^3$ tissue culture infectious dose 50 (TCID50)/mL in cell culture media. Three of the six pigs in each cubicle were each anesthetised with isoflurane before inoculation with 0.1 mL of inoculum in each heel bulb of the left hind limb for a total of 0.2 mL of inoculum per pig.

3.1.3.4. Monitoring and Sample Collection

Animals were monitored daily for clinical signs and their rectal temperature recorded. The lameness scoring system by Main et al. (Main et al., 2000) and modified by Kilbride et al. (KilBride et al., 2008) was used where 0 is normal and 5 is no movement. Blood, orals swabs, and oral fluid collection was done once before inoculation and once-daily

post-inoculation from day 1–7, 12 or 14, 21, and 28 or when an animal reached the humane endpoint.

Ten millilitres of blood were collected from the anterior vena cava with a 20-gauge needle into BD Vacutainer Blood Collection SST Serum Separation Tubes (B367988, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Tubes were centrifuged at 3000 × g for 20 min, 4°C for separation of serum. Oral swabs were taken with Dacron tipped swabs and stored in 1 mL of BD Universal Viral Transport Media (Becton, Dickinson, and Company). Four pigs were euthanized each day from days 1–5, 7, 12 or 14, 21, and 28. Animals with clinical signs and/or animals that reached humane endpoints were euthanized first. Shortly after euthanasia, approximately 2 cm^2 of muscle tissue from *biceps femoris* were collected and stored in re-sealable plastic bags. In addition, approximately 2 g of tonsil, submandibular lymph nodes, prescapular lymph nodes, and popliteal lymph nodes were stored in tubes containing 3 mL of Universal Transport Media (UTM). All samples were kept frozen at −70°C.

3.1.3.5. Meat Juice and Tissue Processing

Muscle tissue in a re-sealable plastic bag was removed from −70°C and allowed to thaw at room temperature. Liquid exudates from the thawed muscle (MJ) released into the plastic bag were harvested with a sterile pipette and transferred into cryovials. Then, 10% tissue suspensions of the lymph nodes and tonsils were prepared using Precellys Lysing Kits (BER-P000918LYSK0A0, ESBE Scientific, Markham, ON, USA) as previously described (Hole et al., 2019; Kittelberger et al., 2017). Briefly, excess fatty tissue was removed from samples and the appropriate amount of lymph nodes or tonsils cut and placed with 0.01M PBS in Precellys Lysing Kits. Lysing Kit tubes were shaken at 5,000 RPM in 10 second intervals 6 times using a Precellys 24 Homogenizer (P000669-PR240-A, Bertin Corp, Rockville, MD, USA), then followed with centrifugation at 3,000 x g for 20 minutes at 4°C. Tissue suspension supernatant is collected post centrifugation into clean sterile tubes. MJ and tissue suspensions were stored at −70°C until they were used for testing.

3.1.3.6. Real-Time Reverse Transcription PCR

RNA extractions were performed on MJ, serum, oral swabs, and tissue suspensions using the Applied Biosystems MagMAX-96 Viral RNA Isolation Kit (AMB1836-5, Life Technologies, Burlington, ON, USA) together with a MagMAX Express-96 Deep Well Magnetic Particle Processor (Life Technologies) as described previously (Hole et al., 2019).

The commercial Tetracore VetAlert™ FMDV RNA Test Kit (Tetracore Inc., Rockville, MD, USA) real-time reverse transcription polymerase chain reaction (rRT-PCR) was used to test the extracted RNA from various samples for the presence of FMDV genome. The assay was performed according to the manufacturer's instructions using the Applied Biosystems 7500 Real-Time PCR System (4351106, Life Technologies). Briefly, mastermix was combined with polymerase enzyme and aliquoted into plates. Extracted RNA and controls are then added and the plate is run on the ABI 7500 using the following protocol: Stage 1 (48°C for 15 minutes), Stage 2 (95°C for 2 minutes), Stage 3 (45 cycles of 95°C for 10 seconds followed by 60°C for 40 seconds). rRT-PCR output was graphed linearly and cutoff was established using 4% of the total fluorescence produced from the positive control. Cycle threshold (Ct) values less than 40 were considered positive.

3.1.3.7. Antigen Detection by Lateral Flow Immunoassay (LFI)

Meat juice samples from naïve and FMDV–infected pigs were tested for antigen by LFI for FMDV serotype A (M. Yang et al., 2013) and FMDV serotype SAT 2 (M. Yang et al., 2019) following the published protocols for each serotype. Briefly, conjugated monoclonal antibodies are mixed with MJ and then added to the sample pads of the strips. 30 minutes is given for the sample to disperse across the entire strip before photos are taken. Each test utilizes two conjugated serotype-specific monoclonal antibodies. One antibody is conjugated to biotin, which is captured at the test line by streptavidin, while the other antibody is conjugated to gold for visualization of bound antigen (Figure 3.3).

Limit of detection for the test is 103.77 TCID50 for serotype SAT2 and 105.50 TCID50 for serotype A (M. Yang et al., 2013; M. Yang et al., 2019).

3.1.3.8. Virus Recovery from Nucleic Acid by Transfection

Transfection reagents (Lipofectamine 3000, L3000001, Invitrogen, Carlsbad, CA, USA) were prepared according to the manufacturer's protocol. Briefly, Lipofectamine 3000 reagent (Invitrogen) was mixed with Opti-MEM (31985-062, Gibco, Life Technologies) as instructed in the kit protocol. RNA extracted from MJ, as described above, was diluted in Opti-MEM and then mixed with P3000 reagent. The RNA and P3000 mixture was then mixed with the Lipofectamine 3000 reagent, which was previously diluted in Opti-MEM, and incubated at room temperature for 15 min. During the incubation period, 90% confluent LFBKαVβ6 cells on a 24 well plate was prepared for transfection by aspirating cell culture supernatant, followed by a wash of the cell monolayer with pre-warmed sterile PBS (311-425-CL, Wisent Inc.). PBS was aspirated and discarded, followed by another wash with supplemented DMEM. The RNA plus P3000 mixture was applied to the cells and then incubated at 37°C for 15 min. Following incubation, each well was topped up with supplemented DMEM containing 2% FBS and then incubated at 37°C for approximately 48–72 hours. The plates were monitored daily for CPE. RNA from FMDV A22 IRQ 24/64 and SAT2 ZIM 5/81 (amplified in cell culture) were used as positive controls while PBS was used as a negative control. A second passage was performed by collecting supernatant from each well after freeze-thawing cycles and centrifugation at 3,000 x g for 20 minutes and 4°C to pellet cells. Then, the supernatant was transferred onto a new plate of LFBK cells. The plate was incubated for 48-72 hours and monitored for CPE progression.

3.1.3.9. Sequencing of FMDV RNA from Meat Juice Samples

RNA was extracted from MJ samples as described previously (Hole et al., 2019). The VP1 region of FMDV was amplified using the qScript One-Step RT-qPCR Kit (95143-200, Quantabio, Beverly, MA, USA) and VP1 specific primers for each serotype of

FMDV (Table 1.1). The PCR reactions were run on an ABI GeneAmp PCR System 9700 (4339386, ThermoFisher Scientific) using the following cycling conditions: Stage 1 (48°C for 20 min), Stage 2 (94°C for 3 min), and Stage 3 (40 cycles of 94°C for 20 s, 55°C for 30 s, 68°C for 1.5 min). PCR products were run on a 1% agarose gel with 0.05M TBE buffer (tris, boric acid, EDTA) at 80V for 90 minutes. Gel was imaged using the Azure C400 (Azure Biosystems, Dublin, CA, USA). PCR reaction mixes with only a single observable band were purified using the QIAGEN PCR Purification Kit (28104, Qiagen Canada, Toronto, ON). PCR reaction mixes that contained multiple bands were excised from gels manually and purified with the QIAGEN Gel Extraction Kit (28704, Qiagen Canada). Isolated DNA was quantified using the Nanodrop One system (ND-ONE-W, ThermoFisher Scientific) and used for de novo sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (4337454, ThermoFisher Scientific) and relevant primers (Table 1.1). Sequencing reaction mixes are then cleaned up using the QIAGEN DyeEx 2.0 Spin Kit (63206, Qiagen Canada), dried using the Eppendorf Vacufuge Plus (022820109, Eppendorf Canada, Mississauga, ON) for 30 minutes, 60°C, with spinning. Dried DNA is then resuspended in Hi-Di Formamide (4401457, Applied Biosystems, ThermoFisher Scientific), and then run on the ABI 3500xL Genetic Analyzer (4406016, Applied Biosystems, ThermoFisher Scientific).

Forward and Reverse sequence outputs from the ABI 3500xL were aligned using the MUSCLE alignment in Geneious (Version 2020.1.2). Consensus sequences for each sample were then aligned with the control virus consensus sequence to determine pairwise identity.

3.1.4. Results

3.1.4.1. Clinical Signs in Pigs

Three of the 6 pigs in each of 6 groups were each anesthetised with isoflurane before inoculation with 10^3 tissue culture infectious dose 50 (TCID50) of FMDV A22 IRQ 24/64 (first experiment) or FMDV SAT2 ZIM 5/81 (second experiment) in the bulb of

the left hind limb per pig. The other pigs in each group acquired infection by close contact with the directly inoculated pigs.

For both FMDV A22 IRQ 24/64 and FMDV SAT2 ZIM 5/81, clinical signs, including a slight increase in rectal temperatures, vesicles on the feet, and lameness, were seen in pigs starting at day post infection (DPI) 2–3 (Tables 2.1-2.3, 3.1-3.3**)**. Disease progression in the pigs was as expected, with the directly inoculated pigs showing viremia and clinical signs 24–72 hours prior to the direct contacts (Figure 4.1, Tables 2.1-2.3 and 3.1-3.3). Pigs with the most severe clinical signs were selected for euthanasia and tissue collection at scheduled time points.

3.1.4.2. FMDV Detection in Meat Juice and Other Samples by Real-time Reverse Transcription Polymerase Chain Reaction

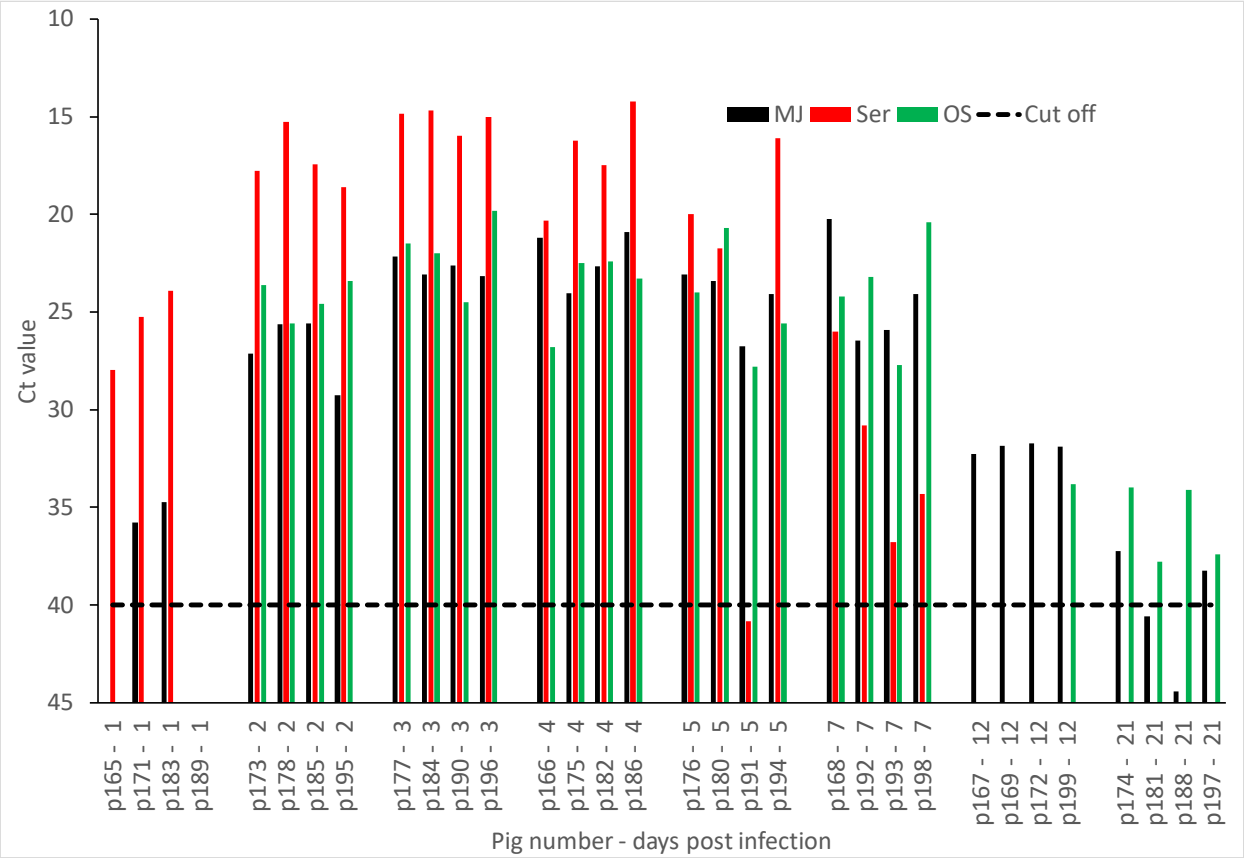
Skeletal muscle (*biceps femoris*) was collected from animals experimentally infected with FMDV and MJ harvested after freeze-thaw cycles of skeletal muscle. RNA extractions were performed on MJ, serum, oral swabs, and tissue suspensions. Real-time reverse transcription polymerase chain reaction (rRT-PCR) was used to test the extracted RNA from these samples for the presence of FMDV genome.

3.1.4.3. FMDV A22 IRQ 24/64 Experiment

In the FMDV A22 IRQ 24/64 experiment, FMDV genome was detected in MJ as early as DPI 1 to as late as DPI 21 (Figure 4.1). Viremia based on FMDV RNA detection in sera started at DPI 1 (Figure 4.1) and was cleared within 4–5 days after first detection. FMDV RNA was also detected in oral swabs starting at DPI 2 (Figure 4.1) and was still detectable at 21 DPI in oral swabs. FMDV RNA was detected in MJ and oral swabs longer than in serum. The VP1 sequence of FMDV from MJ was >99% when aligned to the A22 IRQ 24/64 inoculum.

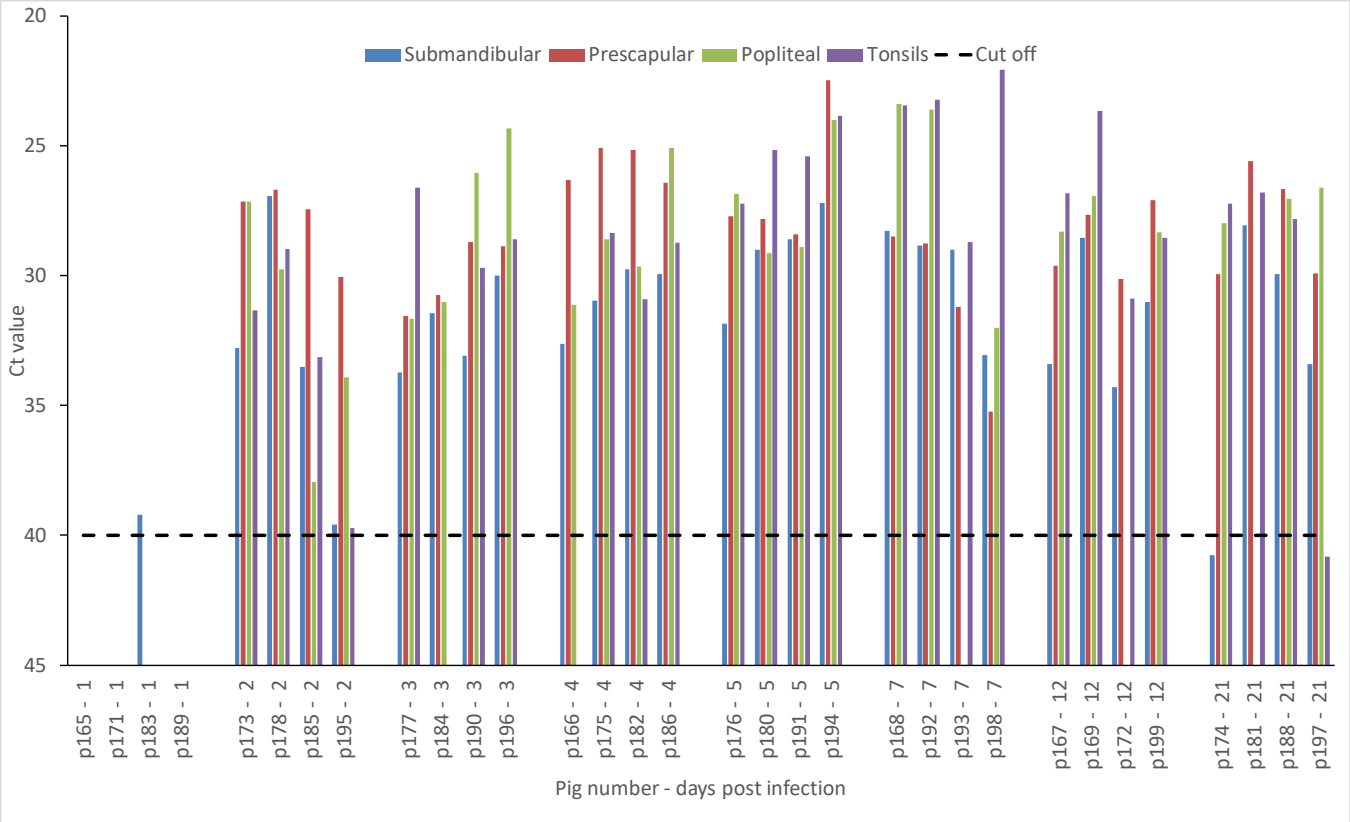
Figure 4.1 Detection of Foot-and-mouth disease virus (FMDV) in meat juice (MJ), serum (Ser), and oral swabs (OS) by rRT-PCR. Skeletal muscle (*biceps femoris*) was

collected from animals experimentally infected with FMDV A22 IRQ 24/64 and MJ harvested after freeze-thaw cycles of skeletal muscle. Ribonucleic acid (RNA) was extracted from MJ, Ser, and OS collected at corresponding time points and tested by each rRT-PCR. The cut-off Ct value for the rRT-PCR is 40. Samples with Ct values less than 40 are considered positive for FMDV.



FMDV RNA was detected in the submandibular lymph node from one animal at DPI 1. By DPI 2, all tested lymph nodes (submandibular, prescapular, and popliteal) and tonsils were positive for FMDV RNA and stayed positive up to 21 DPI (Figure 4.2).

Figure 4.2 Crossing threshold (Ct) values for rRT-PCR for various tissue suspensions (submandibular lymph nodes, prescapular lymph nodes, popliteal lymph nodes, and tonsils) from FMDV A22 IRQ 24/64 infected animals. 10% tissue suspensions were prepared in PBS and clarified by centrifugation. RNA was extracted from tissue suspensions and tested by rRT-PCR. The cut-off Ct value for the rRT-PCR is 40. Samples with Ct values less than 40 are considered positive for FMDV.

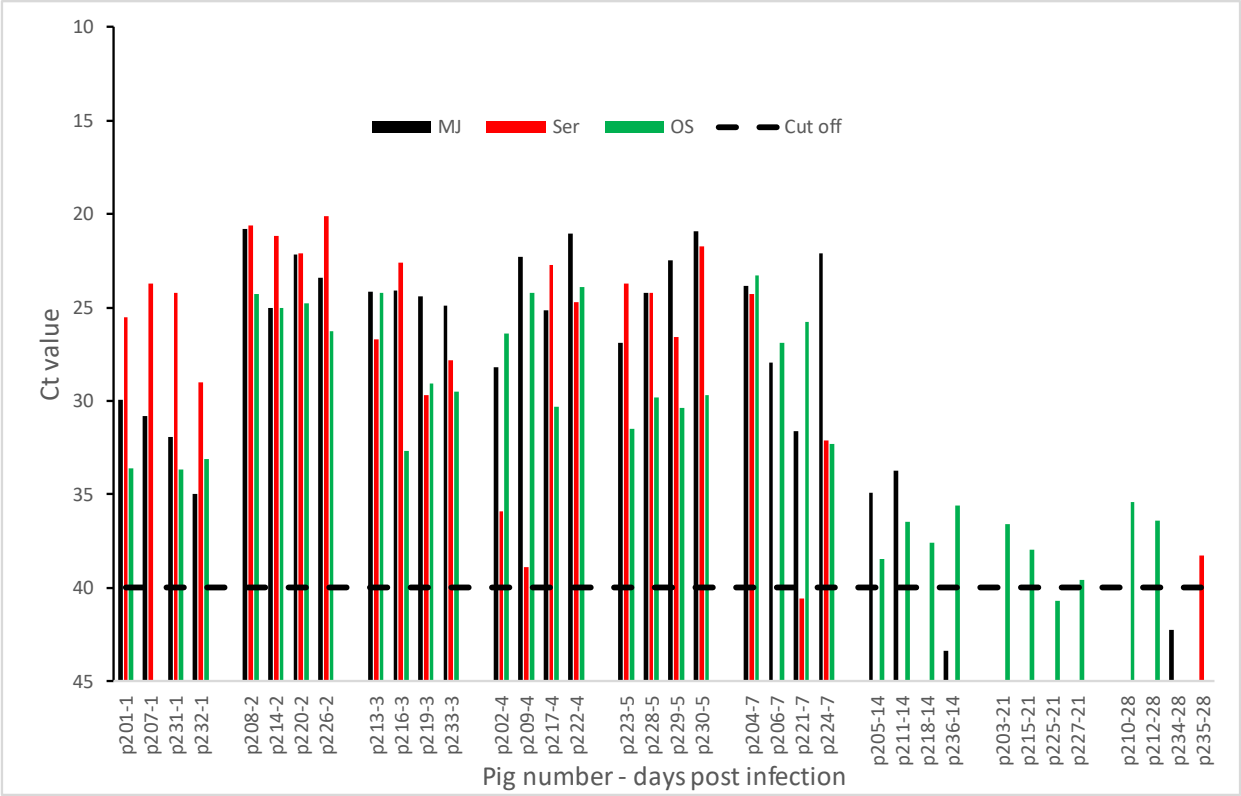


3.1.4.4. FMDV SAT2 ZIM 5/81 Experiment

In the FMDV SAT2 ZIM 5/81 experiment, FMDV genome was detected in MJ as early as DPI 1 to as late as DPI 14 (Figure 4.3). A fragment of the VP1 sequence of FMDV from MJ was successfully determined and shown to be up to >99% identical to the SAT2 ZIM 5/81 inoculum.

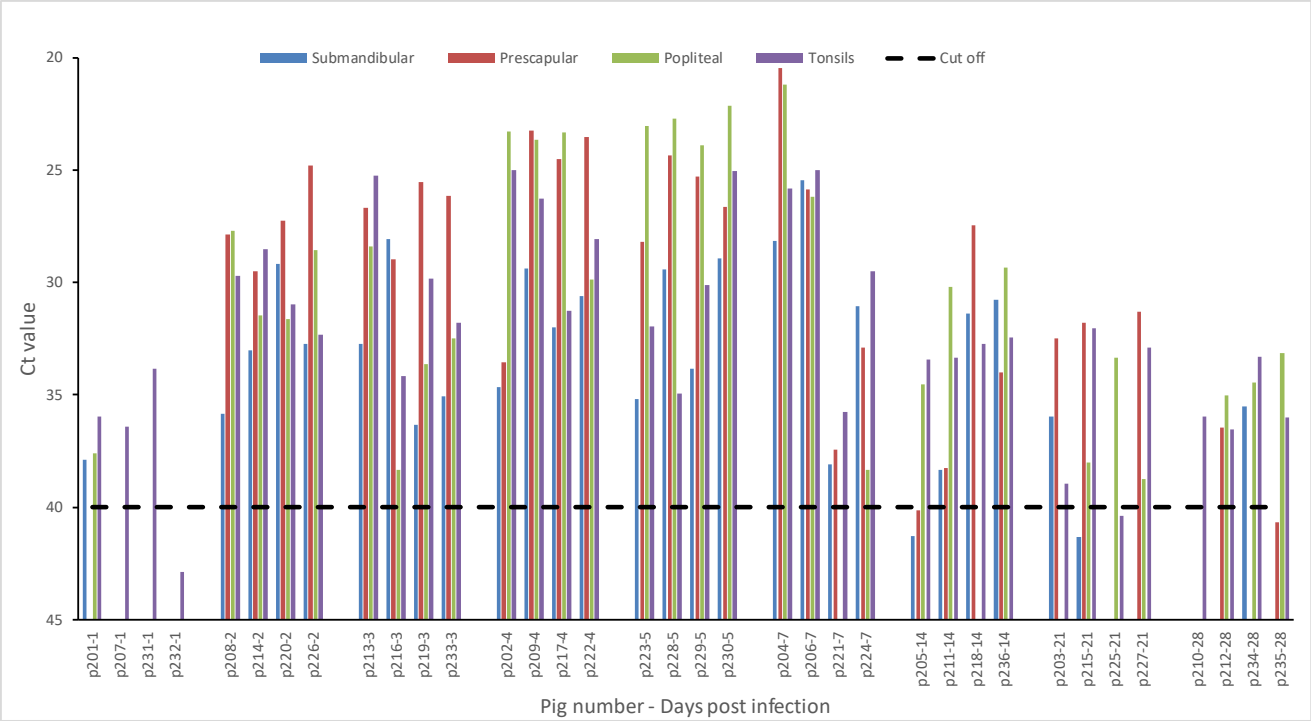
Viremia started at DPI 1 (Figure 4.3) and was cleared within 4–5 days after detection. FMDV RNA was detected in oral swabs at DPI 1–14.

Figure 4.3 Detection of FMDV in meat juice (MJ), serum (Ser), and oral swabs (OS) by rRT-PCR. Skeletal muscle (biceps femoris) was collected from animals experimentally infected with FMDV SAT2 ZIM 5/81 and meat Juice (MJ) harvested after freeze-thaw cycles of skeletal muscle. RNA was extracted from MJ, Ser, and OS collected at corresponding time points and tested by each rRT-PCR. Cut off Ct value for the rRT-PCR is 40. Samples with Ct values less than 40 are considered positive for FMDV.



FMDV RNA was detected in the lymph nodes and tonsils starting at DPI 1. By DPI 2, all tested lymph nodes and tonsils were positive for FMDV RNA and stayed positive up to 28 DPI (Figure 4).

Figure 4.4 Crossing threshold (Ct) values for rRT-PCR for various tissue suspensions (submandibular lymph nodes, prescapular lymph nodes, popliteal lymph nodes, and tonsils) from FMDV SAT2 ZIM 5/81 infected animals. 10% tissue suspensions were prepared in PBS and clarified by centrifugation. RNA was extracted from tissue suspensions and tested by rRT-PCR. Cut off Ct value for the rRT-PCR is 40. Samples with Ct values less than 40 are considered positive for FMDV.

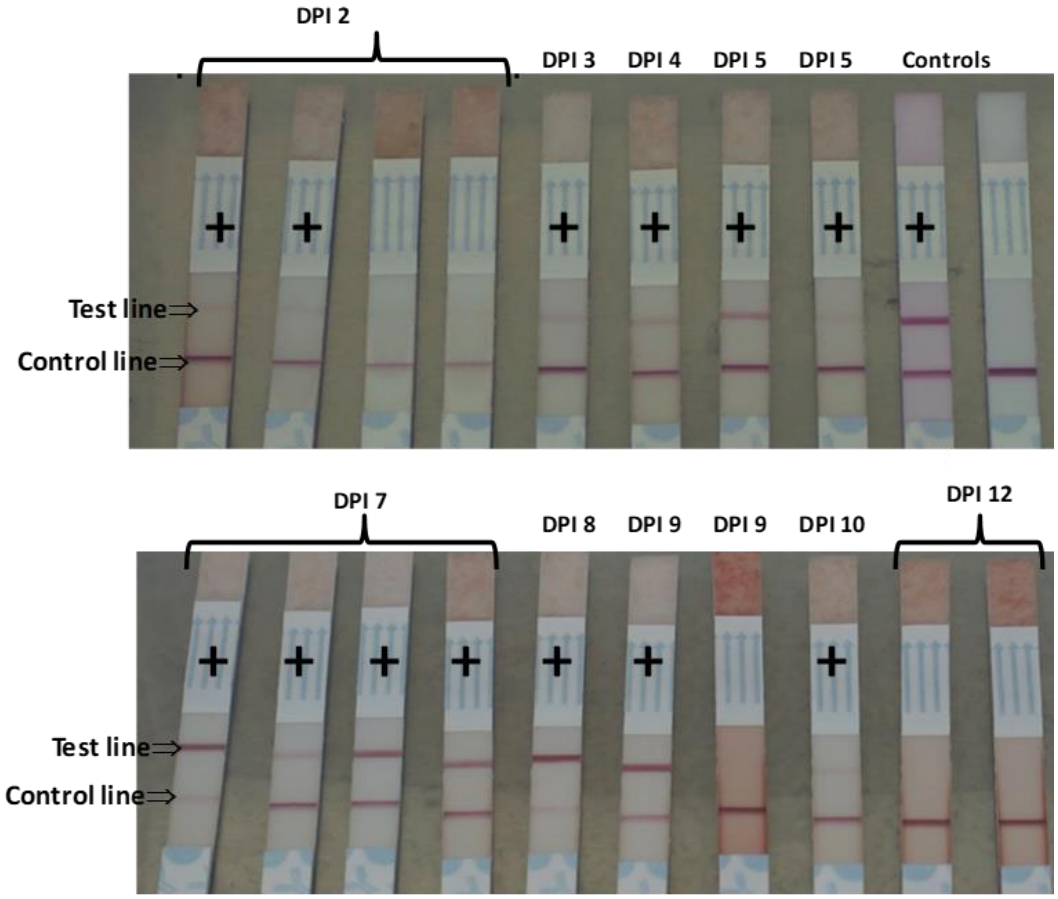


3.1.4.5. Antigen Detection by Lateral Flow Immunoassay

MJ samples from naïve and FMDV-infected pigs were tested for antigen detection by serotype-specific lateral flow immunoassay (LFI). Twenty-four MJ samples from naïve pigs were negative on both FMDV A22 IRQ 24/64 and SAT2 ZIM 5/81 LFIs (data not shown).

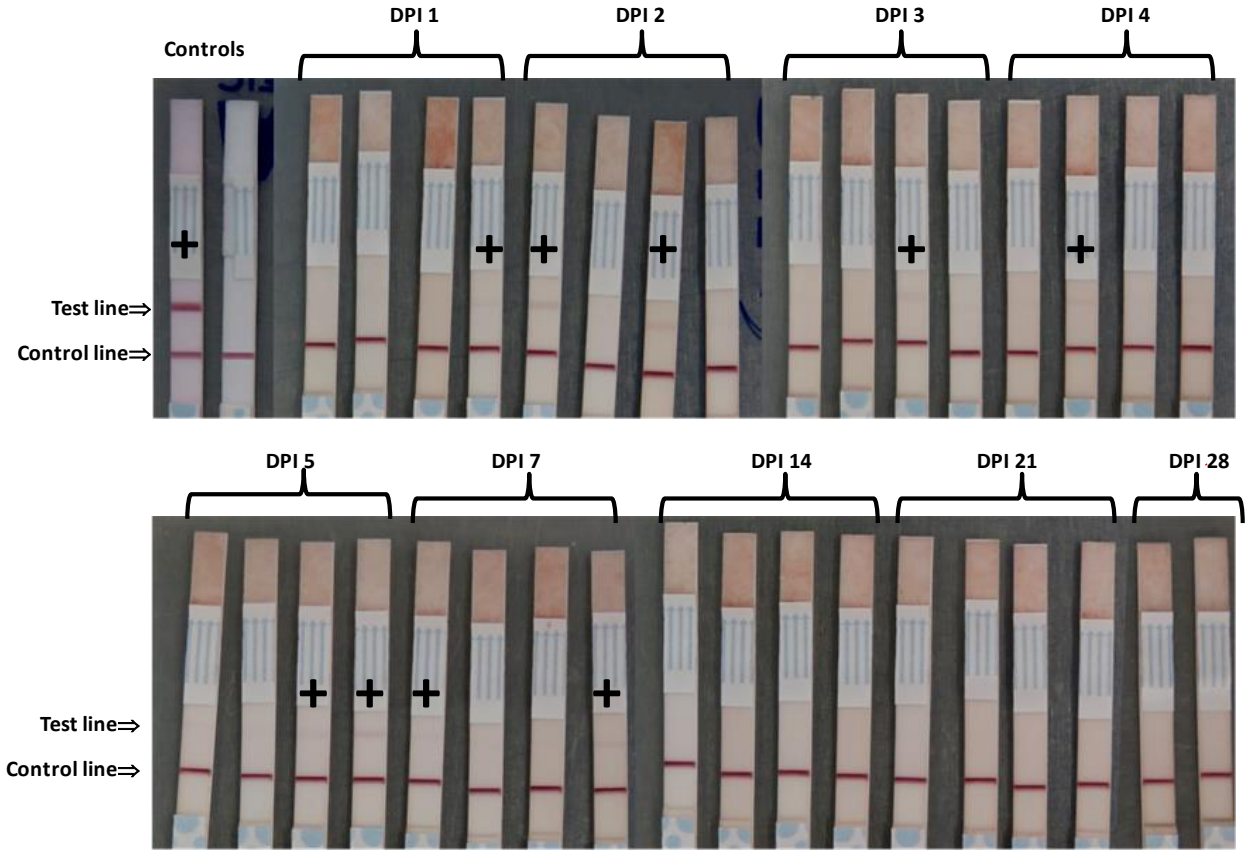
With the FMDV A22 IRQ 24/64 experiment, FMDV antigen was detected in MJ at 3 DPI with faint test bands. These bands increased in intensity at DPI 5–9 (Figure 4.5).

Figure 4.5 Detection of FMDV serotype A antigen in meat juice by lateral flow immunoassay. Meat juice was harvested from pigs inoculated with FMDV A22 IRQ 24/64 and tested for FMDV serotype A antigen by lateral flow immunoassay. + = positive for FMDV antigen, DPI = days post-infection.



For the FMDV SAT2 ZIM 5/81 animal experiment, FMDV antigen was detected at 2–7 DPI, but the test bands were all faint compared to the positive control (Figure 4.6).

Figure 4.6 Detection of FMDV SAT 2 antigen in meat juice by Lateral Flow Immunoassay. Meat juice was harvested from pigs inoculated with FMDV SAT2 ZIM 5/81 and tested for FMDV serotype SAT 2 antigen by lateral flow immunoassay. + = positive for FMDV antigen, DPI = days post-infection.



3.1.4.6. Virus Recovery from Nucleic Acid by Transfection

Virus isolation from MJ was attempted without success despite the presence of FMDV RNA as detected by rRT-PCR. In an effort to recover infectious material from rRT-PCR-positive MJ, susceptible cells were transfected with RNA from MJ.

For the FMDV A22 IRQ 24/64 experiment, CPE was observed after 2 passages in LFBKαVβ6 cells for RNA from MJ samples collected at DPI 3, 7, and 12 (Table 4.1). CPE was observed after the first passage for the positive control (Table 4.1).

For the FMDV SAT2 ZIM 5/81 experiment, CPE was observed on the first passage for RNA from MJ samples collected at DPI 2 and 5 (Table 4.1). CPE was also observed after the first passage for the positive control (Table 4.1). The recovered virus was confirmed positive for FMDV by rRT-PCR and LFI.

3.1.5. Discussion

The primary objective of this study was to assess MJ as a sample type for FMDV detection in situations where commonly used samples are not available. To obtain these MJ samples, pigs were experimentally inoculated with FMDV A22 IRQ 24/64 and SAT2 ZIM 5/81. Our data shows that FMDV can be detected in MJ primarily by rRT-PCR. In addition, FMDV antigen can be detected in MJ at specific time points using LFI. FMDV could not be isolated from MJ, but infectious material could be recovered by the transfection of LFBKαVβ6 cells with nucleic acids from MJ. Failure to isolate FMDV from MJ could be attributed to virus inactivation by lactic acid. Indeed, MJ had a pH of 6. MJ is inherently acidic due to lactic acid build-up caused by rigor mortis. FMDV is inactivated in muscles from acid buildup (Cottral, 1969). The acid causes degradation in the protein structure of the FMDV capsid. Without proper adhesion molecules, viral attachment is not possible for delivery of the FMDV genome into susceptible cells. Freezing of samples temporarily suspends acid formation until thawing (Callis, 1996). However, once tissue samples are thawed, acid formation proceeds and inactivates any virus present (Callis, 1996). In these acidic conditions, RNA has been shown to remain stable while DNA becomes degraded through depurination (Lindahl et al., 1972).

Based on the rRT-PCR results, MJ and serum samples from the same pigs were positive for FMDV RNA from DPI 2–7. Therefore, it can be assumed that the presence of a virus in the blood implies that it will most likely be present in muscles since blood flows into the latter. This would probably be true for the early time points post-infection. However, even in the absence of viremia, MJ samples from DPI 12–21 were positive for FMDV genome. It is possible that the clearance of the virus from muscles happens at a slower rate than from blood, possibly because the virus is located within the myocytes or adipocytes and is only released when cells are disrupted by freeze-thawing. Attempts to locate FMDV within the muscles by immunohistochemistry and in situ hybridization were unsuccessful. The second possibility is that the virus could have originated from the regional lymph nodes and was released into muscles through the lymphatic system. FMDV is known to persist in popliteal lymph nodes for more than 28 days post-infection (Stenfeldt, Pacheco, et al., 2016). We also showed that lymph nodes from infected pigs

were positive for FMDV RNA at the end of the experiments at DPI 21 and 28 for FMDV A and SAT2, respectively.

Detection of FMDV antigen in MJ by LFIs shows great potential for use of LFI as a point of need test in circumstances of illegal importation of meat. However, antigen detection by LFI was short-lived compared to rRT-PCR, implying that LFI might fail to detect low levels of viral antigen in MJ. rRT-PCR is inherently a more sensitive assay (M. Yang et al., 2019) and thus explains the detection of FMDV RNA over a wider duration of the experiment. The fact that LFI can detect FMDV in MJ is a good reason to collect both MJ and sera when possible, considering that FMDV antigen detection in serum samples by LFIs has not been successful (M. Yang et al., 2013).

In some circumstances, virus isolation from clinical samples might be necessary for downstream analysis. However, virus isolation from MJ samples was attempted without success. Transfection is an alternative rescue system where the viral genomic RNA can be transfected into susceptible cell lines to allow replication of positive-sense RNA and production of progeny virus. Previous research confirmed the ability to recover FMDV by transfection (V. Dill et al., 2019; J. Horsington et al., 2020). Through transfection with Lipofectamine 3000, FMDV was recovered using RNA extracted from vesicular material stored at room temperature over three weeks (V. Dill et al., 2019). This model was used to recover FMDV from MJ and proved that infectious FMDV can be obtained from MJ when FMDV RNA is present in the tissues.

The presence of FMDV RNA in MJ was further confirmed by sequencing of the VP1 gene of FMDV. Consensus sequences from four different MJ samples showed >98% similarity to the FMDV inoculum that was used to infect the pigs.

In conclusion, MJ is a useful sample for FMDV detection alongside traditional sample types and in exceptional circumstances when the usual samples are not available. The virus might be rapidly inactivated in MJ, but infectious virus is recoverable by transfection of FMDV RNA into susceptible cells. The prolonged detection of FMDV RNA in MJ demonstrates the additional benefit of using this sample type, in addition to any available conventional samples, for rRT-PCR to test for FMDV.

The success of this study demonstrates that the detection of FMDV in MJ is possible in the acute phase of the disease. The next question was whether MJ can still be useful for FMD diagnosis after the virus has been cleared. Therefore, the next steps are to determine if antibodies to FMDV can be detected in MJ during the convalescent phase. Assessment of MJ is done with the SPCE and the NSP ELISA. These ELISAs will provide information on the antibody response in FMDV infected swine and whether antibodies to SP and NSP of FMDV can be detected during the convalescent phase of disease. MJ can be used directly on these assays and compared with serum as a reference sample type for serology.

3.2. Detection of Antibodies to Structural Proteins of Foot-and-Mouth Disease Virus in Swine Meat Juice[3]

3.2.1. Abstract

Exponential increase in the consumption of meat and meat products has led to increased animal rearing and trade to keep up with growing demand. Rise in global trade and increased crowding of animals introduces greater risks for highly contagious diseases such as foot-and-mouth disease (FMD). To ensure an uninterrupted supply chain, suspected cases are tested to confirm that animals are free of FMDV. Established test methods rely on sample collection from live animals, however circumstances exist where it is not possible to collect the desired samples. Meat juice (MJ) has been explored as an alternative for the detection of FMDV and has previously proven successful for FMDV detection by real-time reverse transcription polymerase chain reaction and Lateral Flow Immunoassay. MJ has not yet been assessed for detection of antibodies to FMDV. Hence, this study evaluated MJ for detection of antibodies to structural proteins (SP) by existing serotype-specific solid phase competitive ELISAs (SPCE). Antibodies to FMDV SP were detected in MJ from experimentally infected pigs beginning 6 or 7 days post infection (DPI) and continued until 21-28 DPI. Sera were tested in tandem and followed similar

[3] Modified from a short communication accepted for publishing in the Canadian Journal for Veterinary Research

patterns of antibody detection. The results show that MJ can be used for detection of anti-FMDV SP antibodies.

3.2.2. Introduction

Global consumption of meat and meat products increases every year as much of the world becomes more developed (Delgado, 2003). Meat has become a prominent part of the average modern-day diet as poverty levels decrease (Delgado, 2003). Animal trade between countries has become commonplace to satisfy the increased consumption of meat (Zhang, 2018). To supply the rising demand, increased animal production and improved growth efficiency methods have been employed (Ali et al., 2015). However surges in animal rearing pose a greater risk for infectious disease outbreaks. Among cloven-hoofed animals, Foot-and-Mouth Disease (FMD) is an infectious disease that can cause reduced appetite, fever, vesicular lesions, and death in infected animals (Gibbens et al., 2001). The disease is caused by Foot-and-Mouth Disease Virus (FMDV) (Gibbens et al., 2001). FMD outbreaks can decimate agricultural industries and severely affect supply chains (FAO/EuFMD, 2019).

World Organization for Animal Health (OIE) recommended methods of testing suspected animals for FMDV include real-time reverse transcription polymerase chain reactions (rRT-PCRs), antigen enzyme-linked immunosorbent assays (Ag ELISAs), and virus isolation (VI). These tests typically use blood, vesicular fluid, milk, epithelial tags, or swabs when they are available. In certain situations, desirable sample types may not be accessible. The importation of animal products is one situation that presents a risk as FMDV is known to remain viable for more than 120 days in tissues such as lymph nodes and bone marrow if they are kept at 4°C (Cottral, 1969). The fast-paced nature of the meat industry can result in the detection of FMD outbreaks after animals have already been slaughtered. In these unique circumstances, meat juice (MJ) as a sample type can minimize disruption at abattoirs while maintaining traceability. MJ has been proven to be an effective alternative for the detection of FMDV by rRT-PCR (Yeo, 2020). The effectiveness of MJ for detection of FMDV by other test methods such as ELISAs has not yet been explored. We report successful detection of antibodies to FMDV structural

proteins (SP) in MJ using an adapted diagnostic serotype specific solid-phase competitive ELISA (SPCE).

3.2.3. Materials and Methods

3.2.3.1. Experiment Sample Collection

Swine meat juice and sera were obtained from pigs experimentally inoculated with FMDV serotype A (A22 IRQ 24/64) and Southern African Territories 2 (SAT2 ZIM 5/81) in two separate studies. The viruses were obtained from the World Reference Lab for FMD, The Pirbright Institute, and propagated at the NCFAD as previously described (Yeo, 2020). The experimental procedures, sample collection, processing, and storage have been previously described (Yeo, 2020). In addition, 48 MJ samples from naïve pigs were tested to establish cut off values for each SPCE.

3.2.3.2. Testing of Experiment Samples by Competitive ELISA

Sera and meat juice samples were tested using a SPCE developed at the NCFAD as previously described (Kittelberger et al., 2017), with minor modifications. Briefly, 96-well Nunc Maxisorp microplates (439454, ThermoFisher Scientific, Waltham, MA, USA) were coated with 100 µL per well of an optimal dilution of rabbit anti-FMDV serum specific for serotype A or SAT2 in coating buffer (0.06 M carbonate buffer, pH 9.6) overnight at 4°C. The plates were then washed five times using 0.01 M phosphate buffered saline (pH 7.2) with 0.05% tween (PBST) followed by the addition of 100 µL per well of antigen for each FMDV serotype optimally diluted in blocking buffer (5% normal rabbit serum, 10% normal bovine serum in PBS-T) and incubated at 37°C on an orbital incubator shaker. After 1 hour, the plates were again washed 5 times with PBST. Duplicate wells of 50 µL per well of each serum sample (diluted 1 in 5 in blocking buffer) or meat juice sample (diluted 1 in 2.5 in blocking buffer) or blocking buffer alone (diluent controls) were added to specific wells, immediately followed by the addition of 50 µL per well of guinea pig anti-FMDV serum diluted in blocking buffer to all wells. Plates were incubated for 1 hour at 37°C on an orbital incubator shaker, washed 5 times

with PBST, and followed with 100 µL per well of polyclonal donkey anti-guinea pig IgG (H&L) conjugated to horseradish peroxidase (706-035-148, Jackson ImmunoResearch, West Grove, PA, USA) diluted in blocking buffer. Plates were again incubated for 1 hour at 37°C on an orbital incubator shaker. After washing 5 times with PBST, 100 µL of 2-component TMB substrate (KP-50-76-03, Mandel Scientific, Guelph, ON, Canada) was dispensed into each well and incubated for 10 min at room temperature in the dark with shaking. The TMB colour change was stopped with 100 µL of TMB stop solution (KP-50-85-06, Mandel Scientific) and the OD450 of each well determined on a Molecular Devices SpectraMax Plus 384 Microplate Reader. The result for each sample was expressed as a percent inhibition (PI) where PI = 100 – (mean OD of 2 replicate test samples ÷ mean OD of the 2 replicate diluent controls) × 100.

3.2.3.3. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.4.2. Receiver Operating Characteristic (ROC) curve analysis was used to estimate sensitivities and specificities at various cut-off PIs. Multiple comparisons of MJ samples from various DPIs were also performed by 2-way ANOVA (Tukey's multiple comparison test). Correlation analysis of antibody detection in serum and MJ was performed to determine Pearson correlation coefficients.

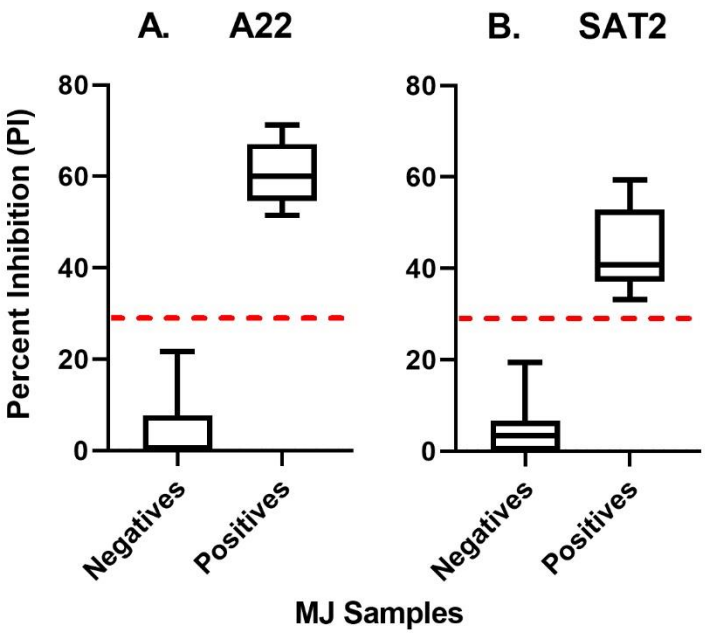
3.2.4. Results

3.2.4.1. Antibody Detection in Meat Juice and Serum

Using both ROC curve analysis, mean + 3 standard deviation and the distribution of the PI for 40 negative MJ samples (Figure 5.1A and 5.1B), the cut off PI value was 30 for SPCE A22 and SAT2. The cut off PI for serum SPCE has previously been established as 50 (Kittelberger et al., 2017).

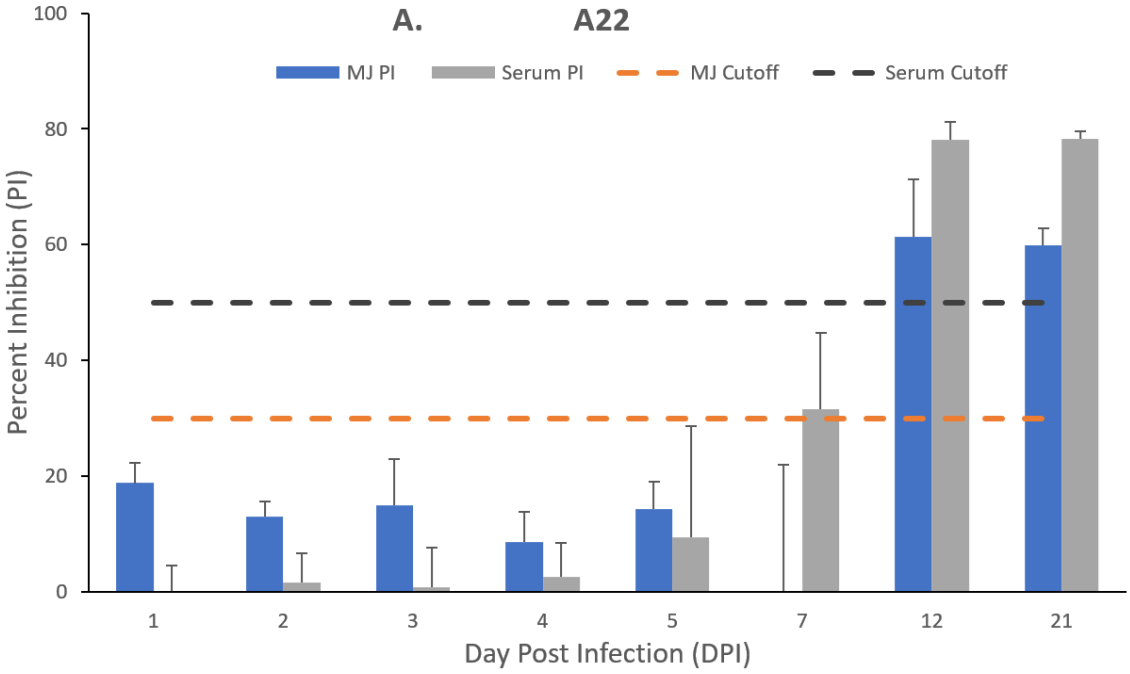
Figure 5.1 Relative levels of antibodies to the structural proteins of FMDV in swine meat juice (MJ) from the *biceps femoris*. Antibody levels are expressed as Percent

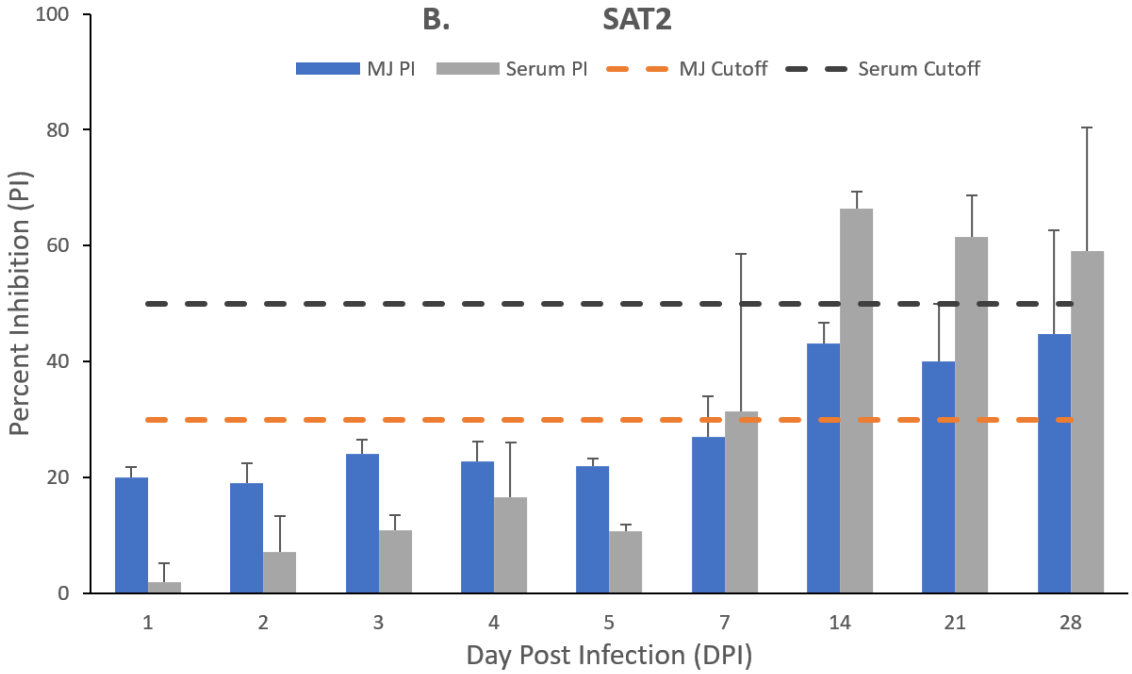
Inhibition (PI) of negative and positive MJ samples from (A) A22 IRQ 24/64 and (B) SAT2 ZIM 5/81 experimentally infected pigs. Cutoff PI was set to 30 based on ROC curve analysis of MJ samples.



Positive levels of anti-FMDV SP antibodies were detected in MJ at DPI 12 and remained relatively high at DPI 21 for the A22 IRQ 24/64 experiment (Figure 5.2A). The rise in antibody response at DPI 12 and 21 were significant compared to DPI 1 ($P<0.0001$). There was a correlation between anti-FMDV SP antibody detection in MJ and sera from corresponding animals ($r^2 = 0.77$; $P<0.0001$) (Figure 6.1A).

Figure 5.2 Percent Inhibition (PI) values showing the level of antibodies to structural proteins of FMDV in meat juice (MJ) collected from the *biceps femoris* and serum from pigs experimentally infected with (A) A22 IRQ 24/64 and (B) SAT2 ZIM 5/81. The cutoff PI value for MJ is 30. The cutoff value for serum is 50.


A.
A22
MJ PI
Serum PI
MJ Cutoff
Serum Cutoff
Percent Inhibition (PI)
100
80
60
40
20
0
1
2
3
4
5
7
12
21
Day Post Infection (DPI)


B.
SAT2
MJ PI
Serum PI
MJ Cutoff
Serum Cutoff
Percent Inhibition (PI)
100
80
60
40
20
0
1
2
3
4
5
7
14
21
28
Day Post Infection (DPI)

For the SAT2 experiment, positive levels of anti-FMDV SP antibodies were detected in one of the MJ samples at DPI 7, 100% at DPI 14 and 21, and 75% at DPI 28 (Figure 5.2B). There was a significant difference in antibody response at DPI 14 and later time points compared to DPI 1 ($P<0.05$). Similarly, there was a correlation between anti-FMDV SAT2 SP antibody detection in MJ and sera from corresponding animals ($r^2 = 0.84$; $P<0.0001$) (Figure 6.1).

3.2.4.2. Cross Reactivity

No cross reactivity was observed between FMDV A22 antibody positive MJ when tested against the FMDV SAT2 SPCE. Similarly, FMDV SAT2 antibody-positive MJ did not cross react on the FMDV A22 SPCE. Due to the lack of MJ samples, we were unable to assess the analytical specificity of the assays against other vesicular disease agents such as SVDV, SVA, and VSV.

3.2.5. Discussion

We have previously shown MJ to be a good sample matrix for detection of FMDV antigen and nucleic acids. This means MJ can be used in parallel with preferred sample types but will be most useful in situations when the preferred samples are not available. We have now shown that antibodies to FMDV SP can be detected in MJ concurrent with detection in serum. Antibodies to FMDV SP begin circulating in serum of most infected animals at 5 - 7 DPI (Alexandersen, Zhang, et al., 2003). Since MJ is a mixture of serum and other cellular exudates (Wallander et al., 2015), presence of antibodies in serum should result in antibodies in MJ as shown by our data for 11 DPI or later. The level of antibodies in MJ was also lower than in serum. The probable reason is that any serum in MJ is diluted by other liquid components contained in myocytes. This can also explain the detection of positive antibody levels in serum, but not in some MJ samples at 6 and 7 DPI. It has been shown that antibody levels in MJ are significantly lower than in serum (Kaden et al., 2009; Meemken et al., 2014).

Sera from FMDV-infected animals usually permit for diagnosis of ongoing infection through viral nucleic acid, and resolved infection through the detection of antibodies (Alexandersen, Zhang, et al., 2003). As revealed by our previous (Yeo, 2020) and current data, MJ provides similar diagnostic opportunities as serum with the additional benefit that FMDV RNA is detected in MJ for a longer period than in serum. This allows for a longer overlapping period (DPI 6/7 - 21) during which both FMDV nucleic acids and antibodies to FMDV SP can be detected in the same MJ sample. FMDV detection in serum is usually short-lived, with viremia disappearing before DPI 7 (Yeo, 2020).

Furthermore, the detection of FMDV SP antibodies in MJ implies that these antibodies can potentially be detected in MJ from seropositive FMD vaccinated pigs. However unlike sera, at this point, MJ cannot be used for differentiation of infected and vaccinated animals since detection of antibodies to FMDV non-structural proteins (NSP) in MJ was unsuccessful. Further assay optimization may be required to determine the potential use of MJ for the detection of antibodies to FMDV NSPs. Complement proteins in MJ are potentially interfering with NSP ELISAs. Serum is usually heat inactivated to prevent interference by complement proteins. However, MJ cannot be heat inactivated due to the high protein content that results in a coagulated matrix not suitable for an ELISA.

In conclusion, swine MJ is a suitable sample for the detection of antibodies to FMDV SP. Taken together with our previous detection of FMDV RNA in MJ (Yeo, 2020), this sample type is valuable for the diagnosis of FMD during the acute and convalescent phases of the disease. Therefore, in the absence of traditional samples such as in a retroactive disease investigation or for imported meat and meat products, MJ can be used for the diagnosis of FMD when necessary.

4. OVERALL DISCUSSION AND CONCLUSION

4.1. Discussion

The aims of this study were to evaluate the viability of MJ for use in existing diagnostic assays. The results show that MJ is a suitable sample for the detection of FMDV by rRT-

PCR and performs better than serum at the later stages of infection. MJ samples remained positive after DPI 7 in both the A22 IRQ 24/64 and SAT2 ZIM 5/81 experiments. This contrasts with what was found in previous work assessing MJ for detecting CSFV where MJ samples and serum samples from the same animal tested negative and positive respectively (Lohse et al., 2011).

The presence of FMDV RNA in MJ was confirmed by sanger sequencing of the FMDV VP1 region from MJ extracted RNA. Basic Local Alignment Search Tool of the consensus sequences produced high percent similarity and supports the findings of the rRT-PCR. Some samples did not produce any sequence even though DNA amplification of the VP1 region was done. One of the potential causes for the lack of sequence could be due to low DNA yields for samples that required gel excision.

Blood is the likely source of virus in MJ during the acute phase of FMD since it delivers necessary nutrients to the myocytes. During the convalescent phase, it is likely that the virus originated from nearby lymph nodes and spread through the lymphatic system into muscle tissue. Nonetheless, detection of FMDV by rRT-PCR is feasible with MJ as an alternative in cases where more commonly collected samples are not available. In situations such as at international borders, illegally imported meat can be assessed quickly to rule out viral presence. More importantly, MJ can be directly assessed from samples at abattoirs with little disruption to the industry and downstream market supply. The broad detection range of the FMD rRT-PCR with MJ also allows detection in later stages of the infection where clinical signs may have resolved or in situations of delayed outbreak response.

LFIs have proven to be a rapid and convenient test that can be performed on location (M. Yang et al., 2013; M. Yang et al., 2019). The performance of this test with MJ proved to be exceptional. The LFI was able to detect presence of FMDV in most of the MJ samples over the duration of the animal experiments where samples were positive by the rRT-PCR (Figure 4.5, 4.6).

LFIs were found to detect FMDV in MJ over a broader range than is possible with other sample types. The sensitivity of this test is limited as many samples considered positive by rRT-PCR did not give a positive result by LFI. However, MJ is still advantageous in

cases where rapid diagnosis by LFI is needed. MJ also has a step up over serum with FMDV LFIs since serum cannot be used due to the high amounts of interfering proteins that are typically found. In addition, LFIs have potential to be extremely useful in outbreak situations which would normally impact slaughterhouse operation. LFIs would allow for quick diagnosis on site with minimal interruptions. LFIs also reduce the need of highly trained staff as would normally be required for other diagnostic tests.

Virus isolation using MJ was attempted but did not produce any results. Several difficulties were encountered with the isolation assay. Observation of cell monolayers under light microscope was very difficult with MJ samples due to the high amounts of proteins present in MJ. The method of *biceps femoris* collection also introduces bacterial contamination in cell culture. The main reason for lack of success with viral isolation from MJ is the acidity of the sample. MJ naturally has a pH that is around 6 or lower due to lactic acid buildup after the animal has been euthanized (Donaldson et al., 2013). This acidic pH was confirmed with pH strips (35237, Alfa Aesar, Tewksbury, MA, USA), proving MJ collected from experimentally infected animals was acidic. At this pH, FMDV capsids are unstable and become inactivated by conformational changes in the capsid structure (Bachrach et al., 1957; Stenfeldt, Diaz-San Segundo, et al., 2016). The conformational change prevents FMDV from binding to receptors on cultured cells and results in no observable CytoPathic Effect (CPE).

In an effort to recover FMDV from MJ, transfection was pursued to bypass the difficulties encountered with direct VI. Transfection requires only extracted RNA from MJ samples. This RNA is introduced into competent cells and causes infection since the +ssRNA genome of FMDV can be translated directly. The entire RNA extraction process removes many of the MJ components that causes issues with direct VI. The transfection process utilizes reagents that disrupt cell membrane integrity of cultured cells, and delivers the extracted FMDV genome from MJ directly into the cell. This proved successful as CPE was observed upon transfection of LFBKαvβ6 cells. Recovery of virus from extracted MJ samples proves that FMDV is present in MJ and supports the findings of the rRT-PCR and LFI.

Antibodies to the capsid proteins of FMDV were found in MJ using an existing solid-phase competitive ELISA (SPCE) produced at the NCFAD (D. K. Mackay et al., 2001; Paiba et al., 2004). Modifications were necessary to optimize the assay for use with MJ, such as dilution levels. From initial assay optimization, MJ was found to require a lesser dilution in blocking buffer than serum. This is likely due to the lower antibody concentrations in MJ.

MJ harvested from experimentally infected animals showed an antibody response that mirrors that which is found in serum as seen in Figure 3.4. Antibody response proceeded as expected in terms of infection (Jacquelyn Horsington et al., 2017; O'Donnell et al., 1996). The presence of antibodies in MJ was anticipated as skeletal muscle contains blood vessels. Serum is also one of the components found in MJ (Gutierrez et al., 2008; Wallander et al., 2015) which explains the presence of antibodies, albeit at lower levels. The lower levels are expected because of other intra and extracellular contents in myocytes that make up MJ. This agrees with work on JEV antibody detection in wild boar MJ (Yonemitsu et al., 2019).

MJ was not without challenges when used in ELISAs. A potential problem with the collection of MJ is the volumes obtained. This varied greatly from sample to sample as previously described, and can make diagnosis difficult if no MJ can be obtained (Mousing et al., 1997; Wacheck et al., 2012). Another difficulty encountered with MJ for use in serological assays is the inability to heat inactivate samples for use in ELISAs. Heat inactivation (56°C for 30 min) is not possible with MJ due to the high protein content which causes coagulation of the sample at higher temperatures. The coagulation makes the sample unusable in serological assays.

Overall, the use of MJ to detect FMD should be considered supplementary for sample collection or when standard sample types are not available.

4.2. Conclusion

In conclusion, MJ from swine *biceps femoris* samples proves useful for the detection of FMDV by rRT-PCR and can be used to trace, diagnose, or survey FMD. MJ also proved advantageous over serum based on the longer period of detection of FMDV genome.

Sanger sequencing provided sequence specific proof that MJ samples contained FMDV RNA in the majority of samples. VI was not possible due to acid found in MJ samples – this was overcome using transfection as an alternate recovery system. LFI also proved useful for the detection of FMDV antigen in MJ. Antibodies to the SP of FMDV were detectable in MJ using the NCFAD SPCE with minimal cross reactivity between serotypes. From these findings, MJ can be used in tandem with typical samples to diagnose cases of FMDV or used in cases when preferred samples are not available.

5. FUTURE DIRECTIONS

MJ was found inviable by some established test methods. Difficulties encountered can be overcome with further investigation into assay optimization or sample processing to remove contaminants in MJ. Further processing may improve assay sensitivity and extend diagnostic capabilities.

Experimental animals used in this study were limited to pigs, therefore information obtained in this study pertains only to swine. MJ from other species that are susceptible to FMDV must also be tested to determine its suitability as a sample type for diagnosis. The priority should be for cattle, sheep, and goats as these are important animals in the livestock industry.

MJ must also be tested for differential viruses such as SVDV, VSV, and SVA to rule out cases of indistinguishable clinical signs.

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7. Supplementary Tables & Figures

7.1. Primers

Table 1.1 List of primers used for obtaining VP1 sequences from FMDV RNA extracted from MJ. Amplification and conversion of the VP1 sequence was done using either the A-1C562 or SAT-1D209F forward primers in combination with the NK61 reverse primer. The end-point sequencing used the same forward primers in combination with the NK72 reverse primer.

	Primer	Direction	Sequence
A22	A-1C562	FOR	TACCAAAATTACACACGGGAA
SAT2	SAT-1D209F	FOR	CCACATACTACTTTTGTGACCTGGA
VP1 amplification	NK61	REV	GACATGTCCTCCTGCATCTG
Sequencing	NK72	REV	GAAGGGCCCAGGGTTGGACTC

7.2. Experimental Animal Monitoring

7.2.1. **Table 2.1** FMDV A22 IRQ 24/64 experiment animal rectal temperatures in °C from day zero to twenty one. Spaces greyed out indicate animals that have been euthanized. White empty spaces without a value indicate that rectal temperatures were not taken for that given animal.

	DPI																					
Pig #	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	28
165	40	39.5																					
166	39.6	39.5	39.5	39.8	40.2																		
167	39.9	39.9	39.6	40.2	39.6	41	40	40.4	39.9	40	39.6	39.6	39.3										
168	39.5	39.9	39.5	40	39.3	40	40.3	40.2															
169	39.2	39.4	39.4	40.1	39.3	40	40.2	41.8	39.8	40.6	39.4	40.1	39.8										
170	39.3	39.5	39.4	40	39.7	41.2	42.2	40.4															
171	38.9	39.7																					
172	39.2	39.6	39.8	39.5	40.3	40.6	40.3	39.9	40.4	39.3	39.9	39	39.7										
173	39.2	39.9	40.7																				
174	38.8	40.1	39.7	39.9	39.7	40.5	40.1	40.7	41	39.6	39.9	39.8	40	40.3		39.5	38.7	39.2	39.6	39.3	39.6	40.2	
175	39.4	39.7	39.4	40.1	40.9																		
176	39.4	39.3	39.4	39.4	40	41.3																	
177	39.2	39.2	40.1	40.2																			
178	38.8	39.6	40.9																				
179	39.1	39.3	39.8	39.8	39.7	39.6	39.9	40.9	40.4														
180	39.4	39.3	39.9	40.2	41.1	41.2																	
181	38.7	39.2	39.5	39.5	39.7	39.7	40.2	39.4	40.5	40.5	40.5	39.7	39.7	40.3		39.8	39.2	39	39.7	39.5	39.1	39.9	
182	39.4	39.6	39.6	39.7	41.1																		

183	39	39.5																				
184	39.2	39.2	39.3	40.7																		
185	39.2	39.6	41.2																			
186	38.9	39.5	38.8	39.7	41																	
187	39.3	39.6	38.8	39.3	40.1	40.3	38.8	39.9	39.5													
188	39.3	39.3	39.4	39.3	39.3	40.5	41.4	40.3	38.5	39.9	39.2	39.2	40.1	39.8	39.7	39.8	39.5	38.5	39.8	39.6	39.8	39.7
189	39.1	39.3																				
190	39.3	39.6	38.9	40.3																		
191	39.6	39.6	40.2	40.7	40.4	40.6																
192	39.7	40	39.5	40	39.4	40.3	40.9	41.8														
193	39.4	39.5	39.2	39.5	40.6	40	41.2	41.7														
194	39.6	39.9	39.5	39.7	39.7	40.3																
195	39.1	39.2	40.1																			
196	38.9	39.6	39.7	39.8																		
197	38.7	39.9	40	40	39.4	39.3	39.3	39.3	40.4	40.4	40	40.3	40.3	40	39.7	40	39.5	39.5	39.9	39.2	39.6	39.4
198	38.6	39.9	39.8	39	38.6	39.2	39.9	39.9														
199	39.2	39.6	39.5	39.2	38.5	38.7	40.8	40.8	41	40	39.9	40.1	40.3									
200	38.5	40	39.7	39.9	39.4	39.8	39.3	39.3	40.7	40.7												

7.2.2. **Table 2.2** FMDV A22 IRQ 24/64 experiment animal lameness scoring from day zero to seven. Spaces greyed out indicate animals that have been euthanized. Zero indicates no lameness while five indicates severe lameness.

	DPI							
Pig #	0	1	2	3	4	5	6	7
165	0	0						
166	0	0	0	0	5			
167	0	0	0	0	4	3	0	2
168	0	0	0	0	0	0	1	2
169	0	0	0	0	0	0	0	1
170	0	0	0	0	0	1	2	1
171	0	0						
172	0	0	0	0	4	3	1	4
173	0	0	0					
174	0	0	0	0	0	0	2	4
175	0	0	0	0	5			
176	0	0	0	0	1	4		
177	0	0	2	0				
178	0	0	5					
179	0	0	0	0	4	0	0	2
180	0	0	0	0	4	5		
181	0	0	0	0	4	0	0	2
182	0	0	0	0	4			
183	0	0						
184	0	0	3	4				
185	0	0	5					
186	0	0	0	0	4			
187	0	0	0	0	4	0	0	2
188	0	0	0	0	4	0	0	2
189	0	0						

190	0	0	0	4				
191	0	0	0	4	2	1		
192	0	0	0	0	0	0	1	0
193	0	0	0	0	2	1	0	0
194	0	0	0	0	0	2		
195	0	0	0					
196	0	0	0	4				
197	0	0	0	0	3	1	0	0
198	0	0	0	0	3	2	0	0
199	0	0	0	0	3	1	0	0
200	0	0	0	0	3	1	0	0

7.2.3. **Table 2.3** FMDV A22 IRQ 24/64 experiment animal lesion scoring from day zero to seven. Spaces greyed out indicate animals that have been euthanized. Zero indicates no identified lesions. Each animal can have up to a maximum of thirty five lesions. A maximum of eight lesions can be identified on each foot, one on the lower lip, one on the snout, and one in the oral cavity.

	DPI							
Pig #	0	1	2	3	4	5	6	7
165	0	0						
166	0	0	1	0	9			
167	0	0	0	1	26	33	19	14
168	0	0	0	0	0	0	27	22
169	0	0	0	0	0	0	2	8
170	0	0	0	0	0	11	11	19
171	0	0						
172	0	0	0	0	21	33	4	32
173	0	0	1					
174	0	0	0	0	0	0	10	32
175	0	0	0	0	8			
176	0	0	0	0	12	15		
177	0	0	1	0				
178	0	0	5					
179	0	0	0	0	0	0	1	6
180	0	0	0	0	0	1		
181	0	0	0	0	0	0	1	3
182	0	0	0	0	3			
183	0	0						
184	0	0	3	5				
185	0	0	5					
186	0	0	0	0	9			
187	0	0	0	0	9	11	9	8

188	0	0	0	0	0	9	1	4
189	0	0						
190	0	0	0	5				
191	0	0	2	7	6	14		
192	0	0	0	0	0	1	18	11
193	0	0	0	0	10	12	33	33
194	0	0	0	0	0	9		
195	0	0	0					
196	0	0	0	1				
197	0	0	0	0	0	1	0	34
198	0	0	0	0	0	1	6	32
199	0	0	0	0	0	0	9	34
200	0	0	0	0	0	0	0	17

7.3. SAT2 Daily Monitoring

7.3.1. **Table 3.1** FMDV SAT2 ZIM 5/81 experiment animal rectal temperatures in °C from day zero to twenty eight. Spaces greyed out indicate animals that have been euthanized. White empty spaces without a value indicate that rectal temperatures were not taken for that given animal.

	DPI																											
Pig #	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
201	39	39.4																											
202	39.3	39.5	39.2	39.5	40.7																								
203	39.8	39.6	39.5	40.4	41.6	41.3	39.9	39.9	39.7	39.1	39	39.9	39.5	38.9	39.7	39.3	38.9	38.9	39.4	39.1	38.9	39.5							
204	38.8	39.4	38.4	38.9	39.5	39.2	40.6	40.5																					
205	39.1	39	39.1	38.8	39.6	39.1	40.4	40.8	40.8	39.7	39.8	39.7	38.9	38.8	39.6														
206	39.4	39.1	39.1	39	39.7	40.6	40.6	40.2																					
207	39.2	39.3																											
208	39.1	39.5	39.7																										
209	38.9	39.2	39.1	39.4	41.1																								
210	39	39.4	38.9	38.7	39.6	39.3	39.9	39.9	39.4	39.3	39.7	39.7	39.5	39.4	39	39	39.5	39.5	38.9	39.6	39.4	40	40	40.4	39.6	39.3	39.4	39.6	39.9
211	39.4	39.4	39.2	39.2	39.3	40.5	39.8	40.1	40.4	39.7	40	38.9	38.7	38.7	39														
212	39.3	39.3	39.2	39.2	39.4	39.4	39.4	40.3	40.1	40.2	40.5	39.9	38.9	39.2	39.4	40	38.9	39.1	39.5	39.4	38.9	39.7	39.4	39.6	39.9	39.7	39.3	39.4	39.7
213	39.9	39.1	39.3	40.7																									
214	39.5	39.1	40.3																										
215	39.7	38.5	39.2	40.7	40.6	41.5	39.4	39.1	39.1	40	39.4	39.6	39.3	39.5	39.4	39.1	39.3	39.4	39.1	39.3	39.4	39.6							

216	39.7	38.9	38.7																										
217	39.6	38.9	39.3	39.2	40.4																								
218	39.5	38.7	39.2	39.3	39.9	39.7	39.8	39.8	39.1	39.3	39	39.5	39.5	39	38.7														
219	39.1	39.4	40.2	40.7																									
220	38.9	39.1	40.1																										
221	39.7	39.3	39.9	40.6	40.6	39.8	39.8	41.2																					
222	38.7	38.9	39.2	39.3	39.7																								
223	39	38.7	39.2	39.2	39.6	40.6																							
224	39.7	39.7	39.2	39.6	39.4	39.6	41	49.6																					
225	38.6	39.7	39.1	40.1	41.1	41.4	40.6	38.6	39.2	39.6	38.5	39.2	39	39.2	38.8	39.6	38.9	39	38.8	39	39.9	39.6							
226	39.6	39.8	39.6																										
227	39.4	39.7	39.5	41.3	41.4	40	40.2	39.3	39.5	39.8	39.1	39.4	38.7	38.9	39	39.6	38.6	38.7	38.9	39.4	39.2	39.7							
228	39.3	39.6	39.7	39.7	40.1	40.1																							
229	39.3	39.3	39.1	39.9	39.4	39.8																							
230	39.2	38.8	39	39.4	39.5	40.1																							
231	39.6	40																											
232	39	39.5																											
233	38.9	39.9	39.7	40.4																									
234	39.3	39.7	39.4	39.8	39.2	39	39.7	39.7	39.7	39.7	39.8	39.9	39.3	39.1	38.6	38.7	39.1	38.5	39.2	39.1	39.1	39.3	39.3	39.4	39.2	38.9	39	39.4	39.8
235	39.8	39.5	39.1	40	39.7	40.3	40.3	40.3	39.3	40.3	40	39.6	39.2	38.8	38.8	39.5	39.6	38.5	39.5	40	39.7	39.9	39.8	39.6	39.4	39.4	39.6	39.9	39.8
236	38.8	39.7	39.5	39.9	40.1	40.1	40.2	40.8	39.9	40.2	40.1	39.4	39.3	39.5	39.4														

7.3.2. **Table 3.2** FMDV SAT2 ZIM 5/81 experiment animal lameness scoring from day two to seven. Spaces greyed out indicate animals that have been euthanized. Zero indicates no lameness while five indicates severe lameness.

	DPI					
Pig #	2	3	4	5	6	7
201						
202	1	3	4			
203	1	3	0	0	0	0
204	0	0	0	0	0	0
205	0	0	0	0	0	0
206	0	0	0	3	0	0
207						
208	3					
209	1	2	5			
210	0	0	0	0	0	0
211	0	0	0	3	0	0
212	0	0	0	0	0	0
213	1	3				
214	4					
215	1	2	2	3	0	0
216	0	3				
217	0	1	0			
218	0	0	0	1	0	0
219	2	3				
220	3					
221	1	2	3	3	0	0
222	0	0	4			
223	0	0	0	4		
224	0	0	0	0	0	0
225	2	1	1	2	0	0

226	3					
227	2	2	2	3	0	0
228	0	0	0	3		
229	0	0	0	4		
230	0	0	0	2		
231						
232						
233	0	3				
234	0	0	0	0	0	0
235	0	0	0	1	0	0
236	0	0	0	0	0	0

7.3.3. **Table 3.3** FMDV SAT2 ZIM 5/81 experiment animal lesion scoring from day two to twenty eight. Spaces greyed out indicate animals that have been euthanized. Zero indicates no identified lesions. Each animal can have up to a maximum of thirty five lesions. A maximum of eight lesions can be identified on each foot, one on the lower lip, one on the snout, and one in the oral cavity.

	DPI								
Pig #	2	3	4	5	6	7	14	21	28
201									
202	5	22	16						
203	1	2	6	7	24	0	0	0	
204	0	0	0	1	16	25			
205	0	0	0	0	17	28	0		
206	0	1	0	11	26	34			
207									
208	7								
209	0	10	24						
210	0	1	1	0	0	0	0	0	0
211	0	0	0	6	24	32	0		
212	0	0	0	0	2	12	0	5	0
213	6	7							
214	17								
215	12	5	20	20	21	15	0	0	
216	0								
217	0	0	3						
218	0	1	2	14	18	14	0		
219	13	20							
220	12								
221	5	23	15	26	24	24			
222	0	0	19						
223	0	0	3	16					

224	0	2	0	0	16	24			
225	3	4	17	13	18	12	0	6	
226	11								
227	12	16	23	17	20	16	0	0	
228	0	0	0	8					
229	0	0	0	4					
230	1	0	0	23					
231									
232									
233	1	2							
234	0	0	2	0	0	0	0	0	0
235	0	0	1	5	16	17	0	5	0
236	0	0	0	0	7	13	0		

7.4. VP1 Sequencing

7.4.1. **Table 4.1** MJ samples tested by transfection for the recovery of FMDV. RNA was extracted from samples and applied to confluent LFBKαVβ6 cells together with transfection reagents. Samples that showed Cytopathic Effect (CPE) under a light microscope are positive (+) while samples that did not show CPE (normal cell monolayer) are negative (-). Samples were passaged twice to ensure viral recovery in cases of delayed FMDV infection. Phosphate Buffered Saline (PBS) was used as a negative control while viral inoculum (A22 IRQ 24/64 and SAT2 ZIM 5/81) was used as a positive control.

Sample	1st Passage	2nd Passage
PBS	-	-
p177-3	+	+
p168-7	+	+
p199-12	+	+
A22 IRQ 24/64	+	+
p220-2	+	+
p222-4	-	-
p230-5	+	+
p215-21	-	-
SAT2 ZIM 5/81	+	+

7.4.2. **Figure 6.1** Correlation between the percent inhibition (PI) of serum and. Samples are tested with Foot-and-Mouth Disease Solid Phase Competitive ELISAs (SPCEs) that detect antibodies to the structural proteins of FMDV **(A)** A22 IRQ 24/64, **(B)** SAT2 ZIM 5/81