Antigenic epidemiology of foot-and-mouth disease: understanding the limitations of *in-vitro* vaccine matching for Malaysian strains

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Abstract

Foot-and mouth disease (FMD) is highly transmissible between all cloven-hoofed animals and is considered a livestock disease of great importance due to its impact upon productivity and the trade restrictions. The aetiological agent is an RNA virus classified into seven serotypes: A, O, C, Asia 1, SAT 1, SAT 2 and SAT 3. Despite considerable global research efforts, serological *in-vitro* tests, such as virus neutralisation test (VNT) and liquid phase blocking ELISA (LPBE), are still the only methods for vaccine selection. Although, these assays are relatively simple to perform, they are known to be highly variable and the accuracy of the resulting relationship coefficient (r1-values) is often questioned. The overall aim of the thesis was to identify the source of variability that impacts the relationship coefficient (r1-values) and understand the limitations of the *in-vitro* vaccine-matching tests. Therefore, three main factors, thought to contribute to this variability, were investigated: (i) cellular, (ii) serological, and (iii) virus. The experimental design focused on the A/ASIA/Sea-97 lineage; a contemporary virus frequently detected in Southeast Asia that exhibits high antigenic variability in *in-vitro* vaccine matching tests. The impact of cell replication cycle on virus and neutralisation titres, reflect on the variability of vaccine-matching tests. Thus, it is recommended that fully confluent cells are used to reduce the variability. Further, the source of the variability in the vaccine-matching tests is also attributed to the low of day-to-day repeatability of the serological tests and the inherent differences between individual post vaccinal sera. Consequently, variability in *in-vitro* vaccine matching assays can be reduced by pooling sera from different animals and performing the tests for field and vaccine viruses simultaneously. Although, measuring different spectrum of antibodies, results obtained using VNT are more inconsistent than those of LPBE. The importance of FMDV antigen integrity on variability of *in-vitro* vaccine matching tests was shown to be multifactorial; even in related strains, viral capsid dissociates at different temperatures and the level of dissociated capsid particles varies in different viruses. Interestingly, the A-May-97 vaccine virus was found to be more unstable than the field strains. Field evaluation of post vaccination immune response in Peninsular Malaysia indicated that the vaccine used is effective and able to confer protection against the contemporary field virus, despite poor vaccine-matching results.

In conclusion, the current serological tests can be useful indicators for vaccine selection; there are simple and affordable but the r1 values need further attention as well as improvement, and, ideally, should be linked to protection.

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Declaration

I declare that this thesis and the research contained within it is my own work unless otherwise stated, and no part of it has been submitted as part of any other degree or qualification.

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Abbreviation

B-cells	B-lymphocytes
BCP	B-cell receptor
BEI	Binary Bthyleneimine
BVS	Bovine Vaccinal Sera
CFT	Compliment fixation test
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dpi	Days post infection
dpv	Days post vaccination
DVPs	Degraded virus particles
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
EPP	Expactancy of protection
ERAV	Equine rhinitis A virus
EuFMD	Commission which involved with the European neighbourhood and the West Eurasia Roadmap
FAO	Food and Agriculture Organization of the United Nations
Fc	Antibody receptor involved in antigen recognition
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
IFNα	Interferon alpha
IgG	Immunoglobulin
LPBE	Liquid Phase Blocking ELISA
mAbs	Monoclonal antibodies
moDCs	monocyte-derived dendritic cells

MTM	Malaysia-Thailand-Myanmar
NSP	Non- structural proteins
OIE	*Office Internationale des Epizooties*
ORF	Open reading frame
PaSTRy	Particle stability thermal release assay
PCP-FMD	FMD progressive control program
PD	Protective dose
PHEFA	*Plan Hemisférico de Erradicación de la Fiebre Aftosa* for South America
PPG	Protection against Podal Generalisation
PVM	Post vaccination monitoring
RCU	Regional Coordination Unit
RNA	Ribonucleic acid
rRT-PCR	Real-time reverse transcription-polymerase chain reaction
SAT	South African Territories
SEA	South East Asia
SEACFMD	South East Asia China FMD Campaign
SEAFMD	South East Asia FMD Campaign
SENASA	Argentine Animal Health Service
SPBE	Solid phase blocking ELISA
SPs	Structural proteins
TCID	Tissue culture infective dose
UTR	Untranslated region
VLP	Virus like particle
VNT	Virus Neutralisation Test
WAHIS	World Animal Health Information System
WRL FMD	World Reference Laboratory for FMD

Chapter 1:

General Introduction

1.1 Introduction

Antigenic variation of foot-and-mouth disease (FMD) virus impacts the performance of FMDV vaccines used to control outbreaks. Serological methods used to define antigenic relationships are relatively inexpensive and quick to perform and provide an indicator of the antibody cross-reactivity between vaccine and field strains. Although these tests are widely used, their reliability of the test have been questioned due to issues with variability of the results. This thesis investigates the inherent variability of the assay and possible impacts upon vaccine selection and FMD control in Malaysia.

1.2 History and background: disease, virus and control

In the year 1514 Girolamo Fracastoro made the first description of a disease that resembled FMD in cattle in Italy. Fracastoro also noted that this disease was able to be transmitted to other cattle in the herd (Blancou, 2002). A disease with similar clinical presentation was observed in the United Kingdom in 1839 (Henderson, 1978); whereas, the first occurrence of a disease with similar description in Peninsular Malaysia (P. Malaysia) was not observed until 1860 (Wallace, 1936). At that time, the impact of FMD was significant, indicated by the offer of a prize in 1893 amounting to 3000 Reichmarks by the Prussian Ministry. for the person who 'identified and, if possible isolated the contagious matter causing the disease and demonstrated its effectiveness by means of decisive experiments on animals' (Brown, 2003). Five years later, in 1987 after the establishment of an experimental institution in Germany for thorough investigation of the disease that for FMD was identified as filterable agent or a virus by Loeffler and Frosch providing the first knowledge of the aetiology of FMD. The highly infectious nature of FMD placed constraints on the early research work which lead to the relocation of the FMD research facilities from Greifswald Germany to an island in the Baltic Sea (Insel Reims) in 1909. United States of America also built their FMD facility in 1954 on the Plum Island for the same reason.

The aetiological agent of FMD discovered by Loeffler and Frosch in 1897 was the first virus recognised to cause animal or human disease. About 25 years after this first discovery, the antigenic diversity of FMD virus was defined by two French scientists named Valles and Carre. Initially, they described serotypes O (Oise) and A (Allemande), findings that were broadly confirmed and expanded (serotypes A, B, C) by two German scientists, Waldmann and Trautwein, in 1926. As a consequence of the work by these two groups, three serotypes of FMDV, now known as O, A and C were defined. Later in 1940's three

additional FMDV serotypes from Southern Africa were described by Galloway, Brooksby and Henderson and named after the place "Southern African Territories" as SAT1, SAT2 and SAT 3 (Brooksby and Rogers, 1957). The seventh serotype, Asia 1, was discovered at the FMD World Reference Laboratory, Pirbright in a sample from Pakistan in 1954 (Brooksby and Rogers, 1957).

FMD is a transboundary animal disease recognised as the main sanitary barrier for the international trade of livestock and livestock products. Moreover, FMD is included on the OIE notifiable disease list. Unlike many other animal diseases where national free status can be achieved through self-declaration (and mutual agreement between countries in trade), FMD freedom needs to be declared by the *Office Internationale des Epizooties* (O.I.E, 2018). Countries with FMD free status prohibit importation of susceptible animals and their related products from non-FMD free countries. This restriction also relates to FMD free countries by vaccination, unless appropriate measures are taken to remove any parts of the carcases that may contain residual FMD virus. Thus, the economic consequences of FMD is high. Fear towards this highly infectious transboundary animal disease has motivated Food and Agriculture Organisation of the United Nations (FAO) and OIE to facilitate eradication programs within FMD endemic countries. The ultimate objective is to eliminate risk of FMD spread by eradication of the disease. Examples of these programs are:

- The EuFMD Commission, which is involved in understanding risks to the European neighbourhood (such the work of the West Eurasia Roadmap).
- In southeast Asia, the South East Asia FMD (SEAFMD) Campaign that started in 1997 and its expansion to the 2020 Roadmap for Foot and Mouth Disease Control in South-East Asia and China (SEACFMD)
- The Plan Hemisférico de Erradicación de la Fiebre Aftosa (PHEFA) for South America

1.3 Foot-and-mouth disease virus

FMD virus (FMDV) belongs to the Aphthovirus genus within *Picornaviridae* family and, like other members of this family, FMDV is non-enveloped and small in size (approximately 30nm in diameter). The family is currently divided into at least 40 proposed genera that cause both human and animal disease (Knowles, 2018). The classification of picornaviruses is currently defined by molecular techniques in combination with an assessment of virus sensitivity to pH. Among the generas that belong to *Picornaviridae* family the Enterovirus genus has the largest number of member while, 23 of the defined

genera have only single member of virus (MacLachlan, 2017). A member of the Enterovirus genus, poliovirus that causes poliomyelitis in human, has been studied most extensively due to its severe negative impact upon human quality of life. The efforts in poliovirus vaccinology research have resulted in the development of an effective vaccine that has significantly combated the disease and eradicated two out of the three poliovirus types. Among the animal viruses classified under the umbrella of the *Picornaviridae* family is Equine rhinitis A virus (ERAV). ERAV shares physiochemical properties such as base composition and acid lability as well as buoyant density with FMDV (Newman et al., 1973; Newman et al., 1977). In addition, the nucleotide sequence of these two viruses (FMDV and ERAV) share a high degree of identity (Li et al., 1996; Studdert and Gleeson, 1978; Wutz et al., 1996). These two factors have placed ERAV and FMDV in the same Aphthovirus genus alongside two other bovine viruses (Bovine rhinitis A virus and Bovine rhinitis B virus) of the *Picornaviridae* family. FMDV like other RNA viruses, are prone to errors during replication which is thought to underpin the constant development of mutant strains resulting in antigenic variability within the different serotypes (Domingo et al., 2004).

1.3.1 The genome organisation of foot-and-mouth disease virus

All viruses within *Picornaviridae* family have capsids with icosahedral symmetry. The capsid is constructed from 60 copies each of four capsid proteins to contain the positive-sense single-stranded Ribonucleic acid (RNA) genome. As member of the *Picornaviridae* family, FMDV has similar overall capsid structural organisation as other picornaviruses but has several unique features that can be observed by X-ray diffraction techniques at a resolution of 2.9 Å (Acharya et al., 1990). At present, FMDV can be visualized in crystallographic structure representing serotype A, O and C (Fry et al., 2005). The dimensions of the virus are approximately 30 nm in diameter. The virus particle is non-enveloped, spherical in shape with icosahedral symmetry made of major structural capsid VP1, VP2 and VP3 and a smaller peptide of VP4 in mature virus. Unlike other members of the *Picornaviridae* family such as enteroviruses and Mengovirus that have large depression or protrusions on the capsid surface, FMDV surface structures appeared smoother with no deep canyons in the capsid. The lack of the canyon-like structure revealed as important for FMDV to attach itself to the host cells and plays a part in its immunogenic function (Fry et al., 2005; Olson et al., 1993; Rossmann, 1989). FMDV has a single-stranded positive-sense RNA genome of about 8500nt in length that includes two untranslated regions (UTR) that flank a single open reading frame (ORF) at both ends (5'UTR and 3'UTR). Similar to the other picornaviruses, the RNA of FMDV is uncapped and a small viral protein (VPg) is covalently attached to the 5´end of the genome. The ORF is divided

into four regions according to the presence of their cleavage sites namely Lpro, the P1, P2 and P3 (Figure 1.1). P1 encodes the proteins that comprise the viral capsid shell of the virus. The Lpro and the non-structural proteins are involved in RNA synthesis and create conditions within the host cell favourable for FMD virus replication (Mason et al., 2003). Both P2 and P3 are precursors that make up the additional non-structural protein of the virus that promote viral replication and assembly (Belsham, 2005).

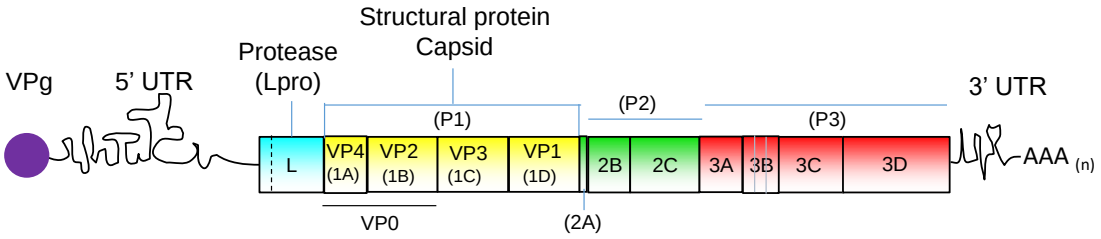

Figure 1.1: A schematic representation of FMDV genome organisation. The genome is covalently linked to the protein VPg at the 5' end and is polyadenylated at the 3' end. The FMD virus RNA coding regions are shown in the form of a single large open reading frame. The poly-proteins flanked both end by untranslated regions (5' and 3' UTR). The capsid coding (structural protein of VP 1-4) contained in the P1 region while the rest are the non-structural proteins. Figure adapted from (Belsham, 2005).

1.3.2 Diversity of FMDV

At present there are seven FMDV serotypes that have been identified by serological differences; A, O, Asia 1, C, South African Territories (SAT) 1, SAT 2 and SAT 3. These seven serotypes were determined using serological method. These serotypes can also be discriminated on the bases of 30 - 50% nucleotide sequences difference within the VP1-coding region between the different serotypes (Knowles and Samuel, 2003; Marquardt and Adam, 1990). Similar to other picornaviruses, FMDV undergoes constant mutation and evolution that leads to considerable sequence variability within a serotype. FMD viruses can also undergo genomic recombination within and between serotypes. These mechanisms enable the virus to rapidly adapt to changes in their environment (Duarte et al., 1994). Diversity in FMDV as the outcome of high mutation rate has direct impact on FMD control in endemic countries particularly when vaccination is used.

Each FMDV serotype can be further classified on the basis of phylogenetic comparison of the VP1 coding sequences. These analyses, combined with geographical regional information of the disease occurrence has led to the sub-classification of FMDV sequences into topotypes, lineages and sub lineages (Knowles and Samuel, 2003). Serotype A is considered the most antigenically and genetically diverse (Pereira, 1976). There are three

major topotypes of the FMDV serotype A: EURO-SA, ASIA and AFRICA (Knowles and Samuel, 2003).

Generally it is understood that antibodies produced against one serotype either by vaccination or infection have no demonstrable cross-protection against other FMDV serotypes and in some cases antibodies do not cross-protect even within the same serotype (Kitching et al., 1988; Knowles and Samuel, 2003). However, multiple challenge with three different FMDV serotypes demonstrated to induce protection against further challenge with FMDV (Cottral and Gailiunas, 1972). Furthermore, sequential multiple vaccination with different heterologous FMD viruses of serotypes A, O and Asia-1 elicited humoral antibody responses that could recognise FMDV serotype SAT-1 and C detectable by ELISPOT (Grant et al., 2017). In addition, administration of bivalent vaccine containing serotype O and A has been demonstrated to elicit neutralization titre against FMDV serotype SAT 1 in naïve animals (Kalmar et al., 1972).

1.3.3 FMDV capsid assembly and dissociation

Similar to the other Picornaviruses, FMDV has structural capsid proteins VP1, VP2, VP3, and VP4 also termed as 1D, 1B, 1C and 1A, respectively. During maturation, the 3C NSP protease processes the P1–2A precursor to produce VP1, VP3 and VP0 (that is subsequently cleaved into VP4 and VP2). The VP1, VP2 and VP3 form a trapezoid and are positioned externally on the viral capsid. VP4 is concealed internally and is predicted to be in contact with the viral RNA inside the capsid structure (Mateu, 2017). The VP4 protein is the smallest capsid protein and is myristoylated at the N-terminus (Acharya et al., 1990). The major capsid (VP1, VP2 and VP3) of FMDV are smaller as compared to capsid proteins of other picornaviruses.

The mature FMDV capsid is made up of 60 copies each of the four structural proteins. These four proteins assemble in wedge-shaped to form a protein sub-unit known as protomer (Figure 1.2d) which later forms a pentamer (Figure 1.2c) when five of the protomer joint together. The whole capsid forms when twelve pentamers join up together (Figure1.2c) (Fry et al., 2005; Rueckert and Wimmer, 1984). The intact capsids are held together by non-covalent hydrogen bonds and weak hydrophobic interactions between the inter-pentameric subunits (Acharya et al., 1990; Ellard et al., 1999).

Intact FMDV capsid has a sediment size of 146 based on its sediment rate in sucrose gradient centrifugation (sediment at coefficient 146S) in sucrose gradient and are hence also known as 146S particles (Figure 1.5a). Empty capsids, capsid shells without encapsidated viral RNA, can also form and are known as 75S particles (sediment at a

sucrose gradient coefficient of 75). These 75S particles are often referred to as "natural empties" as they are naturally produced in cells during FMDV infection. The virus neutralising antibody responses are elicited by antigenic sites on the viral capsid, known as epitopes. The majority of these epitopes are associated with the intact viral capsids rather than disassociated capsid components. Although the 75S particles resemble the 146S in structure and antigenicity, they are inherently less stable (Basavappa, Syed et al. 1994). Furthermore, a study has demonstrated that 75S particles of FMDV serotype A Cruzeiro are able to produce useful level of immunity but are less effective compared to the 146S (Doel and Chong, 1982). The intact capsid (146S particles) is vulnerable to low pH (below 6.8) (Brown and Cartwright, 1961) as well as to high temperatures (Bachrach et al., 1957). These conditions cause the 146S particle to split into pentamers (Figure 1.2b) resulting in generation of another type of FMDV particle known as 12S to form (Figure 1.2c) (Cartwright et al., 1980).

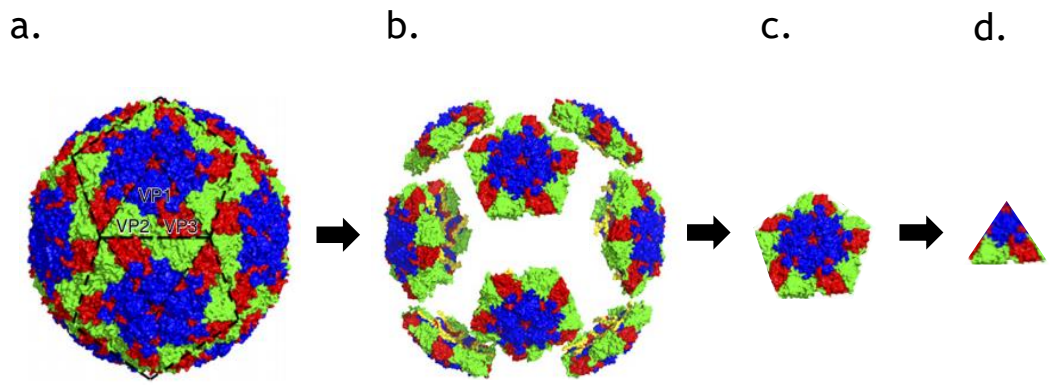

Figure 1.2: A schematic representation of FMDV capsid dissociation. The intact capsid (a) contains viral RNA is known as 146S. Dissociated capsids (b) contain 12 pentamers. The pentamers (c) are assembled from 60 copies of protomers (d). A capsid protomer comprise of VP1 (blue), VP2 (green) and VP3 (red) and VP4 which is concealed inside. Figure adapted from (Kotecha et al., 2015).

Unlike the 146S, antibodies raised against the 12S particles have been demonstrated to have low neutralising activity (Cartwright et al., 1982; Rao et al., 1994). FMDV protein particles of various sizes exist in FMDV preparations (such as FMDV virus stocks and FMDV vaccine preparations) (Doel and Chong, 1982). Therefore, the proportion of 146S, 75S and 12S will affect the efficiency of a vaccine. Stability of the FMDV capsid differs markedly between serotypes. The FMDV serotype A and Asia-1 are relatively more stable upon heat and pH change as compared to FMDV serotype O and SATs (Doel and Baccarini, 1981; Kotecha et al., 2016).

1.3.4 Survival of FMDV in the environment

FMD virus is sensitive to heat and desiccation but is stable at a humidity above 55 to 60% (Bachrach, 1968). Survival of FMDV in fomites is influenced by the nature of the materials as a high concentration of organic material helps the survival of the virus (Donaldson and Ferris, 1975). Wool that is kept at 4ºC allows FMDV to survive for an average period of two months, but survival of the virus decreases considerably by increasing the temperature to 18ºC (McColl et al., 1995). At a temperature greater than 20ºC, it was estimated that FMDV was able to survive for about three months in soil surface while at below 0ºC FMD virus can survive about six months beneath the soil surface, as reviewed by Bartley (Bartley et al., 2002). FMD virus can survive in bovine faeces and slurry (Haas et al., 1995; Parker, 1971). Study by Dowson indicated that transmission of FMD among dairy cattle during the FMD epidemic in the UK in 1967-1968 has been associated with bulk milk tanker during milk collection. FMD transmission via milk can occur through drinking contaminated milk, inhalation of droplet or aerosol as reviewed by Donaldson (Donaldson, 1975). In addition, FMDV can survive in milk and cream collected from FMD infected cows even after pasteurisation process at 72°C for 0.25 minutes (Blackwell and Hyde, 1976).

1.4 Clinical signs of FMD

FMD virus causes acute vesicular disease affecting livestock and wild cloven-hoofed animals with an average mortality of about 1%. However, the disease is highly infectious, and morbidity can be close to 100% in affected herds (Mahy, 2005). The disease has rapid onset causing acute fever with transmission primarily from this early febrile stage to susceptible animals (Alexandersen et al., 2003c).

For livestock in the *Bovidae* family, fever can exceed 40ºC and is associated with depression and a decrease in milk production (Donaldson, 2004; Kitching, 2002). Fever is followed by the development of vesicular lesions in the epithelial tissues of the mouth (dental pad, tongue, lips, and gums), feet (coronary bands, interdigital and bulbs of the heals) and teats (Kitching, 2002). Occasionally vesicular lesion form in the nostrils or vulva (Kitching, 2002). In severe cases of FMD, large areas of the dorsal surface mucosa of the tongue may slough leaving red and raw painful lesions that leads to excessive salivation and a refusal to feed. At the same time, lesions in the feet can cause acute lameness and a reluctance to move. Rapid loss of body weight is often observed mainly associated with

the painful lesions in the mouth and the feet. Mortality usually involves neonates associated with myocarditis (also known as tiger heart). Indigenous breeds of cattle or buffalo in endemic areas can often exhibit milder lesions than the high-productivity breeds that are derived from temperate countries (Geering et al., 1995; Kitching, 2002). In order to estimate the time of initial infection, the progress of vesicular lesions (commonly in the epithelial tissue of the mouth) can be aged in cattle.

Early clinical signs of FMD in pigs include fever, depression and lameness (Donaldson, 2004). Unlike cattle, the marked vesicular lesions are usually found on feet that cause acute lameness and recumbence particularly when pigs are housed on a hard floor surface. Vesicular lesions that occur around the coronets can cause the keratinised layer of the hoof to detach from the corium. Complete detachment of the hoof can sometimes occur in severe cases. Vesicular lesions can also be seen on the snout, but these can be difficult to see if a lesion forms at the base of their tongue. Lesions on the teat as well as abortion is often a consequence of FMD infection in pregnant sow. Sudden death in suckling pigs may also occur (Donaldson, 2004). The aging of FMD lesion in pigs often estimated by the lesion of the keratinised layer detachment of hoof (Donaldson, 2004).

Clinical signs and lesions are usually not obvious in small ruminants such as sheep and goats. For these species, foot lesions are on commonly confuse with foot-rot (Donaldson, 2004) and mouth lesions can be hard to distinguish (Grubman and Baxt, 2004; Kitching, 2002).

1.5 Transmission of FMD

FMD disease transmission pathways can be divided broadly into two distinct mechanisms; (i) direct and (ii) indirect transmission via fomites. The direct transmission occurs through direct contact between infected and susceptible animals. This is the most common route of transmission in pigs, goats and sheep (Aggarwal et al., 2002). Although FMDV is considered acid labile, the virus can survive outside the host and become air borne. The main source for the air borne FMDV transmission include excretions and secretions of infected livestock, such as breath, saliva, semen, milk, faeces, urine, and vaginal secretions (Bedson and Maitland, 1927; Brown, 2004; Paton et al., 2018). A limited number of FMDV infective particles are required for FMD infection in susceptible animals particularly cattle (Sellers, 1971). Therefore, airborne transmission through inhalation is considered a common route in cattle transmission, unlike pigs which are relatively resistant to infection via the airborne route and need higher virus dose to get infected

(Donaldson, 1987). Although air borne/aerosol transmission is not an efficient route of infection in pigs, in an acute infection, pigs excrete more FMD virus particles per day than cattle and other ruminants (Donaldson et al., 1970; Kitching et al., 2005; Sellers and Parker, 1969) As a consequence, while infection in pigs is more difficult to initiate, FMD in pigs has a greater potential to seed the disease on other susceptible animals. Spread of FMDV from goats and sheep to other susceptible animals can commonly occur via clinical or sub-clinically infected animals (Barnett and Cox, 1999). Furthermore, the virus may be excreted intermittently during recovery and thereafter (Donaldson, 1987). The typical spread of FMDV within a herd or flock takes 2 to 6 days for all species (Alexandersen et al., 2003a).

FMD can also be transmitted indirectly by mechanical dissemination through contaminated clothing and other fomite routes such as contaminated vehicles, agricultural tools and many more (described in the section below). In addition to these "mechanical" transmission routes, FMDV can persist in the human nasal passages for up to 28 hours after exposure (Sellers et al., 1970) which may pose a risk of FMDV transmission (Amass 2003). However, a more recent study indicated low risk of FMDV survival in human nasal cavity at 16 to 22 hours after exposure to FMDV infected animals (Wright et al., 2010). In addition, ingestion of infected animal products by susceptible animals either through water or feed can spread the disease (Alexandersen et al., 2003b; Schijven et al., 2005). Even though FMDV has also been detected in the semen of boars, the risk of spread of the disease through sexual transmission is considered low (Guerin and Pozzi, 2005).

1.6 Incubation Period of FMD

The incubation period is important in the epidemiology of FMD due to the fact that FMDV can be excreted during this period particularly in pigs housed in groups (Stenfeldt et al., 2016b). The incubation period of FMD is highly variable depending on agent factors particularly the dose and route of transmission, the host factors including the species and pre-existing immunity as well as environmental factors such as husbandry management (Alexandersen et al., 2003b; Hughes et al., 2002). For example, the incubation period within a farm is 2 to 14 days (Alexandersen et al., 2003c; Hugh-Jones and Tinline, 1976). However, the incubation period in experimental pig challenge with high dose can be as short as 24 hours (Alexandersen and Donaldson, 2002; Alexandersen et al., 2003c). Another study in non-vaccinated piglets and dairy cattle reported excretion of FMDV one to two days after challenge, whereas non-vaccinated lambs took three to three and a half

days (Orsel et al., 2009). However, a study based on paired calves (a one to one transmission system) showed that animals are less likely to be infectious before the appearance of clinical sign (Charleston et al., 2011).

1.7 Subclinical infection versus persistent / carrier stage in FMD

In addition to clinical disease, infection by FMDV can also be unapparent. Animals that transmit FMD virus but do not show clinical signs of FMD are said to have a subclinical FMDV infection. The other type of unapparent FMDV infection is known as a persistent or so-called carrier stage animal that was first defined by van Bekkum in 1959 (Bekkum 1959). Persistent stage of FMD is common in ruminants, particularly in FMD endemic area but does not exist in pigs (Salt, 2004; Stenfeldt et al., 2016a). FMD Persistent state is defined as live FMDV being able to be recovered from the oropharyngeal fluid by the probang sampling method for more than 28 or more days after acute infection (Alexandersen et al., 2002; McVicar and Sutmoller, 1969; Woodbury, 1995). Using an experimental infection model, it was shown that similar numbers of vaccinated and naïve cattle develop persistent infection and that it is possible to define whether animals will become persistently infected before 28 days post infection (dpi) (at 21 dpi and 10 dpi for naïve and vaccinated cattle, respectively) (Stenfeldt et al., 2016a). Persistently infected animals add to the complexity of FMD control and eradication processes, since the currently used serological tests are only useful at herd level, do not differentiate sub-clinical from persistently infected animals. The complication occurred during post vaccination monitoring (PVM) for the FMD control where vaccine is used particularly to identify an uninfected herd. For FMD eradication process inability to discriminate subclinical from persistent animal may lead to unnecessary culling. Furthermore, the existence of persistent stage in vaccinated animals impede livestock and livestock product trading rights of FMD free countries with vaccination.

The duration of FMD persistence stage is variable in different species of ruminants. It can last for 3.5 years in cattle, nine months in sheep, four months in goat (Alexandersen et al., 2002) and five years in African buffalo (Syncerus caffer) (Bastos et al., 2000). African buffalos that are in persistent stage of FMD, have been reported to cause clinical FMDV infection in cattle in the field (Dawe et al., 1994a) and experimentally (Dawe et al., 1994b).

1.8 Host immune response against FMD

The host immune response against FMD involves both the cellular and humoral immune components. Unlike many other viruses that are typical monocytotropic, the agent (FMDV) does not use host immune cells such as monocytes (macrophages) and dendritic cells to propagate itself in the host (McCullough et al., 2009).

Recruitment of immune cells during inflammation associated with the development of vesicular lesions together with cellular damage of the host provide a "danger signal" which can be recognised by the host innate immune response mechanism. Initiation of the local inflammatory reaction is self-amplifying and leads to the recruitment of blood monocytic, granulocytic cells and blood dendritic cells (DCs). The local immune response against FMD enhances the recruitment of lymphocytes resulting in a transient leucopenia in infected animal (Bautista et al., 2003). In response to FMDV infection, DCs in skin were shown to produce type 1 interferon (Bautista et al., 2005). The involvement of both B-lymphocytes and T-lymphocyte in immune response against FMDV are dependent on these DCs (McCullough et al., 2017). DCs present the FMD antigen directly to B-lymphocytes through B-cell receptors (BCR) in the blood that stimulate differentiation of the B cells to plasma cells. Plasma cells produce FMDV-specific antibodies that can form complexes with the virus to activate the complement pathway to prevent FMDV infection (McCullough et al., 1992a). The interferon (IFNα) responses, stimulated by FMDV-antibody complexes, have been demonstrated to be produced by DCs (Guzylack-Piriou et al., 2006).

On the other hand, monocyte-derived dendritic cells (moDCs) process FMDV proteins into peptides before they can be presented to T-lymphocytes (Banchereau et al., 2000). These cellular interactions of DCs with live virus or vaccine are vital functions for functional adaptive immune response. However, the antibody-FMDV complexes hinder the host immune response by attachment to the Fc receptor to enter and kill the cells (moDCs). It was reported that the antibody-FMDV complexes killed bovine moDCs six hours after entry, resulting in inability of moDCs to present the B-lymphocytes in the lymph nodes (Robinson et al., 2011).

1.8.1 Epitopes and antibody escape mutant of FMDV

FMDV needs to be recognised as foreign body by the host's receptor, in order to initiate the host immune responses. Structural analysis of FMDV, through the three-dimensional modelling of viral capsid proteins, has provided information that has helped uncover the

important antigenic properties of FMDV. The antigenic sites recognised to date are located at the surface of capsid proteins VP1, VP2 and VP3 (Kitson et al., 1990).

Antibodies interact mainly with the loop located at the carboxy-termini of VP1, VP2 and VP3 proteins. The VP1 contains the main antigenic site, the G-H loop, which can elicit virus neutralising and non-neutralising antibodies (Bolwell et al., 1989; Ouldridge et al., 1984; Thomas et al., 1988). The G-H loop is a flexible structure that protrudes out from the FMDV capsid making it exposed and highly accessible by the host immune responses. There are about 20 amino acids on the G-H loop that make several overlapping epitopes. These epitopes can be classified as continuous (linear epitopes) and discontinuous or conformational epitopes. Additionally, a conserved, trypsin-sensitive amino acid triplet Arg-Gly-Asp (RGD) that located at the surface of VP1, close to the G-H loop, is identified as the primary site for promoting cell attachment via integrin (Burman et al., 2006; Cavanagh et al., 1977; Fox et al., 1989; Jackson et al., 2004; Laporte et al., 1973).

Antibody-escape mutants are often used to determine FMDV functional epitopes. Most of these escape mutant studies were conducted experimentally using monoclonal antibodies to generate resistant mutants. Many neutralisation antigenic sites were then determined by sequencing of the monoclonal antibody resistant mutants (Martinez-Salas and Belsham, 2017; Reeve et al., 2010).

Most of the escape-mutant data has been obtained for serotype O viruses with limited data also available for serotype A. However, it is thought that similarities between locations of antigenic sites exist among all FMDV serotypes. The five known neutralisation antigenic sites for serotype O are located within VP1–3 and are described as Sites 1 to 5. Site 1 is associated with the (G– H) loop of VP1 and the C terminus of VP1 at amino acid positions 144, 148, 154 and 208; these residues are linear and trypsin-sensitive. On the other hand, Sites 2 - 5 are conformation-dependent and trypsin-resistant. Site 2 involves VP2 at amino acids positions 70–73, 75, 77 and 131, whereas the Site 3 involves the B-C loop of VP1 at amino acids positions 43 and 44. Site 4 involves amino acids positions at 56 and 58 of VP3. Finally, Site 5 involves VP1 at amino acids position 149, which is probably formed by interaction of the VP1 G–H loop region with other surface-located amino acids (Baxt et al., 1989; Crowther et al., 1993; Kitson et al., 1990; Mateu et al., 1990; Xie et al., 1987). More recent studies, using reverse genetics techniques, have identified an additional new neutralising epitope for FMDV serotype O, at the threefold axis of VP2 (Asfor et al., 2014). Structural analysis and antigenic escape mutant studies have determined significant antigenic variability between and within FMDV serotypes. However, application of this information to improve the design of FMD vaccines in order to provide wider antigenic coverage, is yet to be achieved.

1.9 Diagnosis for FMD

Rapid spread of the disease after introduction of FMD virus to susceptible animals highlight the need for a rapid and accurate diagnostic method. Besides, initiatives to understand the epidemiology of FMD and to control the disease rely on accurate reporting. FMD is often initially diagnosed clinically in the field. Suitable samples are collected for confirmatory diagnosis according to the recommendations of the OIE Manual of Diagnostic Test and Vaccine for Terrestrial Animals (OIE, 2017). In some instances, confirmatory tests can be performed at the field using portable devises, providing rapid results. However, in order to confirm FMD, series of laboratory diagnostic tests need be performed in designated laboratory premises (Figure 1.3).

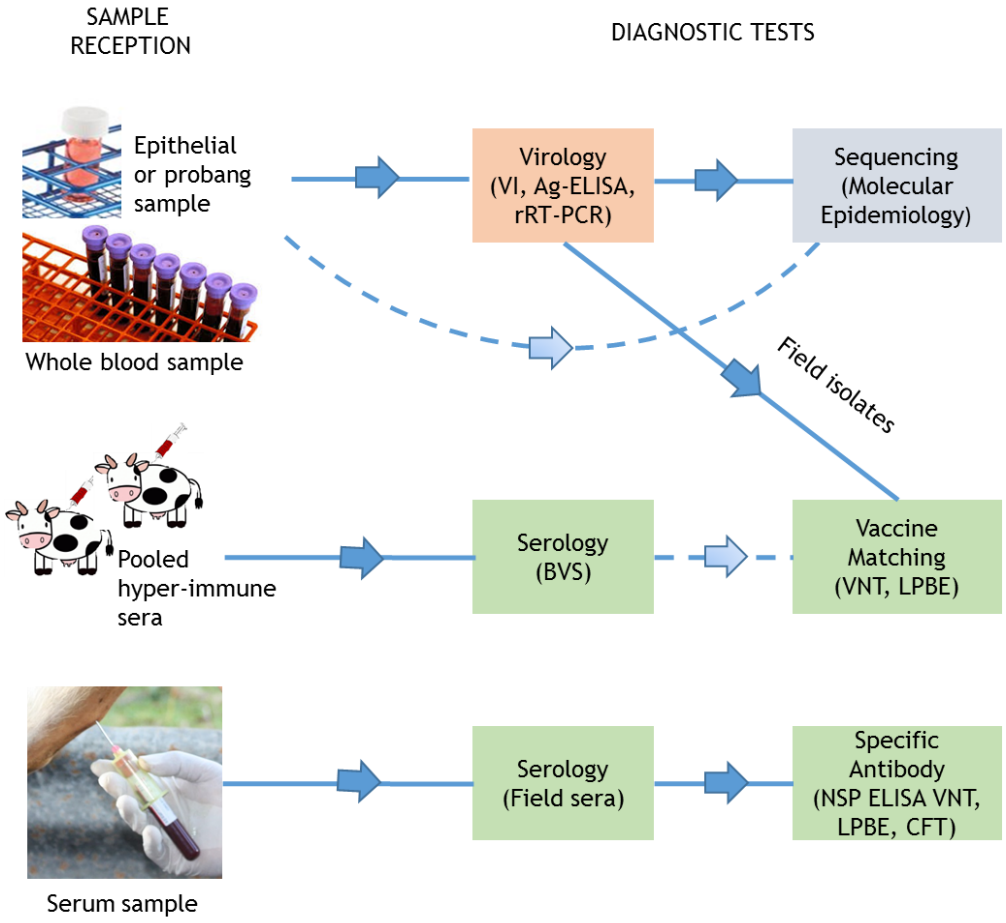

Figure 1.3: The workflow of FMD laboratory diagnostics. Blue lines with arrow indicate standard diagnostic procedures. Blue dashed lines with arrow indicate optional diagnostic procedures.

These tests are commonly performed simultaneously even though virus isolation (VI) is considered as the gold standard. The tests include antigen detection methods (Antigen enzyme-linked immunosorbent assay (ELISA)) and real-time reverse transcription-

polymerase chain reaction (rRT-PCR), a method to detect FMDV nucleic acid. Sequencing, particularly of VP1 coding region, can be performed for detail virus characterisation, often used in molecular epidemiology of FMDV. Occasionally, sequencing may also be carried out on a clinical specimen, without the need for prior virus propagation. Serological methods can be used to confirm the presence (or absence) of FMDV specific antibodies, either against structural proteins (SPs) or against non-structural proteins (NSPs). Currently, specific antibody tests against FMDV NSPs is only available as ELISA method. On the other hand, more serological methods have been developed to detect and measure specific antibody against FMDV SPs that include virus neutralisation test (VNT), liquid phase blocking ELISA (LPBE), solid phase blocking ELISA (SPBE) and complement fixation test (CFT), with VNT being recognised as the gold standard. These serological methods are perform routinely in FMD laboratories for various purposes, mainly (i) for certification of FMDV absence for animal import-export activities, (ii) determination of antigenic matching between the field isolates and vaccine virus, (iii) demonstration of previous FMD virus infection or post vaccination monitoring (PVM) particularly in endemic countries that use vaccination as part of FMD control measures, and (iv) determination of FMD serological prevalence for routine surveillance, particularly in endemic countries.

1.10 Global distribution of FMD

FMD distribution is broadly correlated with the geographical location of the countries with least developed economy. Resolution No 22 for the OIE General Assembly Session described four categories for official FMD status for member countries is currently divided into four categories. These are (i) countries recognised as FMD free where vaccination is not practised, (ii) countries recognised as FMD free where vaccination is practised, (iii) countries having FMD free zones where vaccination is not practised and (iv) countries having FMD free zones where vaccination is practised. These four categories are defined within Chapter 8.8 of the *Terrestrial Animal Health Code* and are subject to annual revision. Out of the 182 member countries of the OIE, there are only 68 countries in category (i) and only two countries that belong to category (ii). A total of 11 countries have free zones where vaccination is not practised and eight countries have FMD free zones where vaccination is practised. Out of the eight countries that have FMD free zones where vaccination is practised, only three countries do not have zones in category (iii) (OIE, 2018) (Figure 1.4). The rest of the member countries are without OIE official status for FMD.

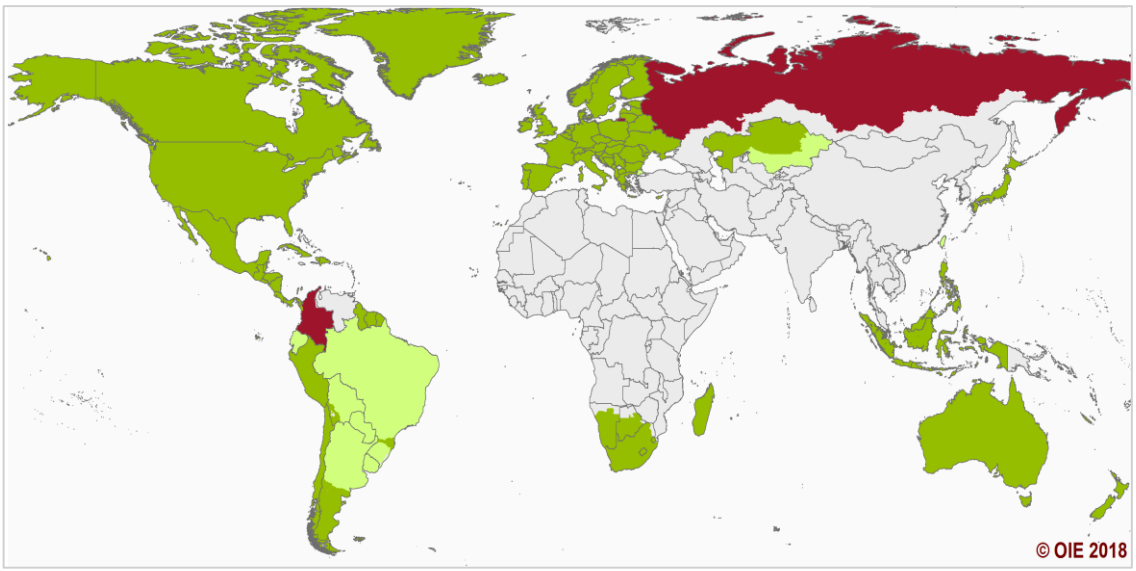

Figure 1.4: Map of OIE member countries with official FMD status for the year 2018. Marked in dark green, are the countries and zones that are recognised as FMD free where vaccination is not practised; in light green, are countries and zones that are recognised as FMD free where vaccination is practised; in red, are countries that were previously reconised as FMD free but this status is currently suspended due to recent or ongoing FMD outbreaks; in grey, are countries without OIE official status for FMD where FMD is endemic or causes sporadic outbreaks. Source: (OIE, 2018).

1.10.1 Endemic FMDV Pools

FMD virus circulation in endemic countries is divided into seven ecological pools (Figure 1.5); each containing defined FMD viruses. These pools share common FMDV genotypes that are circulating and evolving independently within respective geographical areas. The designation of the ecological pools facilitated more focused, regional FMD control strategies (as part of the OIE and FAO Global FMD Control Strategy) and selection of FMDV vaccine virus that are most appropriate for the respective regions. However, boundaries between pools can be fluid and some countries (e.g. Egypt and Libya) experience FMDV incursions from more than one pool (WRLFMD, 2018). The circulation of FMDV within each pool is dynamic and can be subject to incursion/migration of new FMDV genotypes. Prior to 2004, FMDV serotype A and O were the most prevalent (Kitching, 1998). Serotype C has not been reported since 2004 when outbreaks occurred in Kenya and Brazil (Rweyemamu et al., 2008). Viruses classified within the Asia-1 serotype are mostly detected within Asia (including the Middle East) but reported incidences are relatively low. Serotype SAT 1, 2 and 3 are mostly confined within Africa with incidental outbreaks occurring in the Middle East.

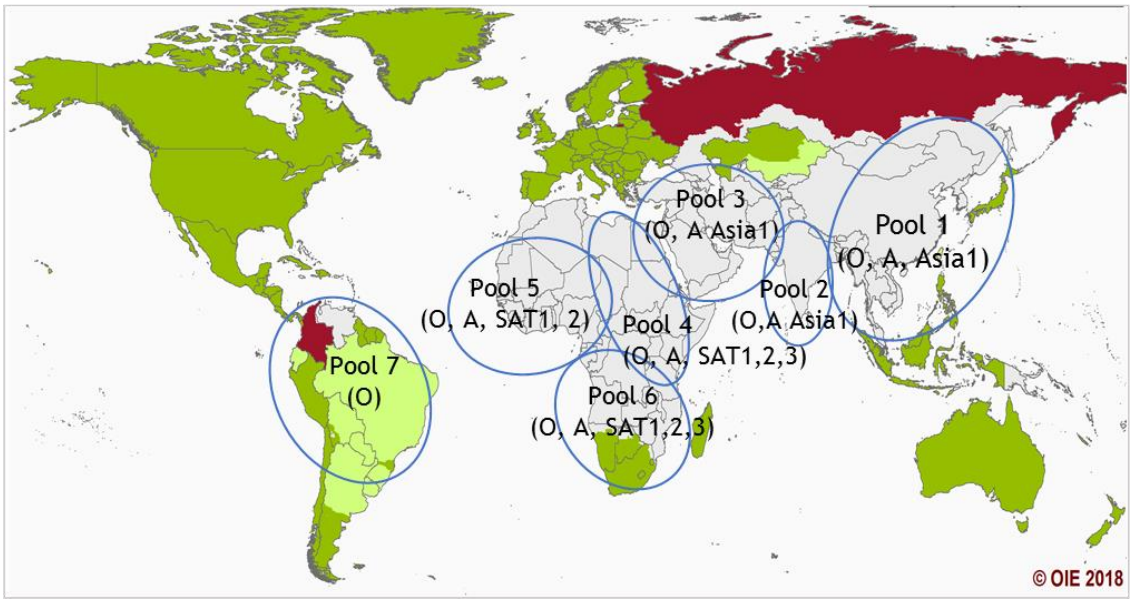

Figure 1.5: The global distribution FMDV within seven of pools. Countries and regions where FMD is endemic are indicated in grey. Map adapted from OIE website (FMD official status). The (SAT) Southern African Territories.

Long-distance, large-scale trance-pool movement of viruses occurs relatively rarely but can have severe consequences, also impacting upon FMDV vaccine selection. The recent spread of FMD viruses from Pool 2 (South Asia) is a good example of the dynamic patterns of global FMDV distribution (Bachanek-Bankowska et al., 2018a; Bachanek-Bankowska et al., 2018b). A recent study described a pandemic spread, including to mainland SEA, of two sub-lineages within the O/ME-SA/Ind-2001 lineage that normally circulates within the Indian subcontinent (Bachanek-Bankowska et al., 2018b). Another example of an FMDV moving out of Pool 2 is the spread of the A/ASIA/G-VII lineage into the Middle East region (Turkey, Iran, Saudi Arabia, Armenia and Northern Israel) (Figure 1.6) (Bachanek-Bankowska et al., 2018a; WRLFMD, 2018). Within Pool 1, migration of the A/ASIA/Sea-97 lineage from mainland SEA has caused outbreaks in the Republic of Korea in 2017 and 2018 (Figure 1.7).

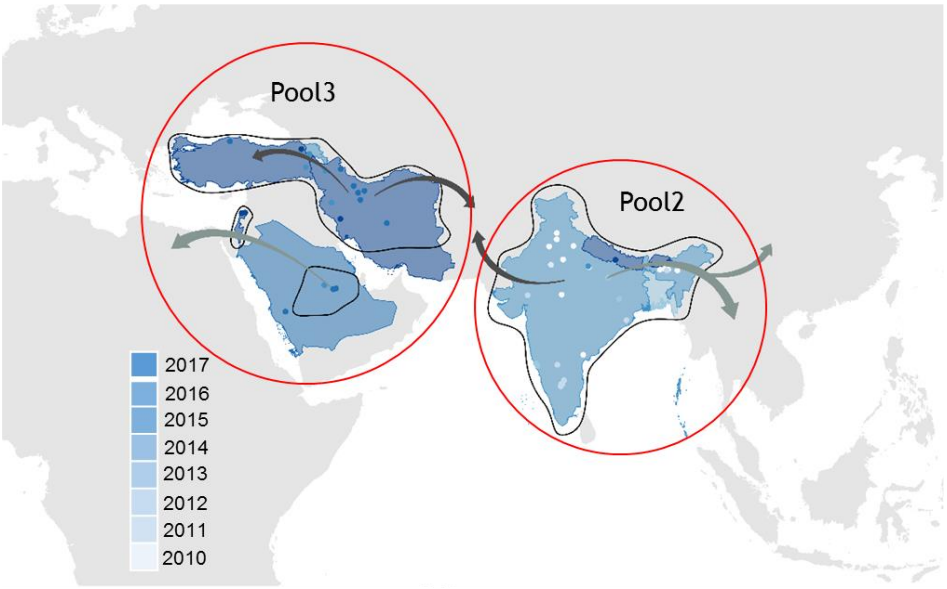

Figure 1.6: Recent movements of the A/ASIA/G-VII lineage. The A/ASIA/G-VII lineage normally exists in the Indian sub-continent (Pool 2), but it was introduced to the Middle East (Pool3) in 2013. Since the introduction, the lineage not only persists in the countries affected but also spreads into new territories, with outbreaks in Northern Israel recorded in 2017. Confirmed FMD reports due to the A/ASIA/G-VII lineage between 2010 and 2017 are indicated in shades of blue. Black arrows represent confirmed movements of the virus, while grey arrows indicate predictions of a possible spread. The map is adapted from the OIE/FAO FMD Laboratory Network annual report (WRLFMD, 2018).

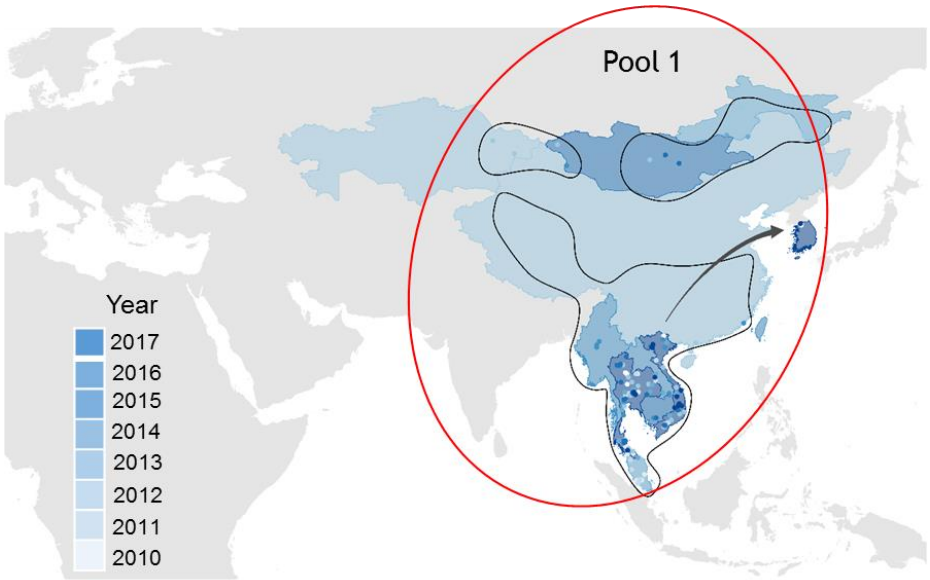

Figure 1.7: Recent movements of the A/ASIA/Sea-97 lineage. The A/ASIA/Sea-97 lineage circulates within mainland SEA (Pool 1), but its normal distribution within the Pool has recently changed and outbreaks were recorded in South Korea in 2017 and 2018. Confirmed FMD reports due to the A/ASIA/Sea-97 lineage between 2010 and 2017 are indicated in shades of blue. Black arrows represent confirmed movements of the virus, while grey arrows indicate predictions of a possible spread. The map is adapted from the OIE/FAO FMD Laboratory Network annual report (WRLFMD, 2018).

1.10.2 Regional distribution: FMDV in mainland Southeast Asia

Countries within mainland SEA (Pool 1) are largely affected by common FMD viruses. These viruses belong predominantly to serotypes O (five different lineages), less commonly to serotype A (two different lineages) and, infrequently, to serotype Asia 1 (Table 1.1).

Table 1.1 Summary of recent occurrences of FMDV individual lineages in Southeast Asia (Pool 1). The data was collated based on the World Reference Laboratory for FMD (WRLFMD) and the OIE/FAO FMD Laboratory Network reports, accessed from the WRLFMD webpage (WRLFMD, 2018).

	Cambodia	Laos	Malaysia	Myanmar	Thailand	Vietnam
O/ME-SA/In-2001		2015	2018	2016	2016	2017
O/SEA/Mya-98	2014	2016	2016	2016	2016	2017
O/CATHAY					2012	2017
O/ME-SA/PanAsia	2015	2012			2015	2017
O/ME-SA/PanAsia2			2006			
A/ASIA/May-97	2015	2015	2014	2015	2016	2017
A/ASIA/unnamed				2010		
Asia 1				2017		2006

However, there are subtle differences in the distribution of the FMDV lineages within the region. For example, the latest confirmed detection of serotype Asia 1 was in 1998 in Thailand and in P. Malaysia in 1999, while this serotype was detected recently, in 2017, in Myanmar. It is important to note, that the Asia 1 virus detected in Myanmar was genetically linked to the Asia 1 virus currently circulating in South Asian countries (Pool 2) such as India and Bangladesh. These recent outbreaks in Myanmar might occurred after the withdrawal of serotype Asia 1 (Asia 1 Shamir) component from the FMD vaccine used in the country (Bo et al., submitted) and might linked to the reduced protection in animals. These events reinforce the importance of using vaccines that contain serotype Asia 1 virus particularly in parts of mainland SEA with trade connections to South Asia (Pool 2). Additionally, in 2005, the O/CATHAY lineage was last detected in P. Malaysia and Thailand. The same lineage was also reintroduced to Thailand in 2012 but it was not detected in Malaysia on that occasion. However, the O/CATHAY lineage was isolated in

Vietnam in 2012, 2016 and 2017 (Table 1.1). The difference in the occurrence of O/CATHAY in Vietnam as compared to P. Malaysia is believed to be due to the strict import regulation (ban of import) of live pigs from Vietnam into Malaysia introduced after the incidence in 2005.

1.11 Economic impact of FMD

The immediate negative impact of FMD is more dramatic when the disease occurs in countries that are free of the disease or where FMD has been previously eradicated. Outbreaks of FMD that occurred in Japan in 2010 had significant economic impacts with the direct cost of eradication estimated to be US$563 million (Hayama et al., 2017; Muroga et al., 2012). FMD costs to the UK national economy have been estimated at US$9.2 billion during the UK 2001 FMD epidemic (FAO, 2002). The estimated costs of an FMD outbreak, if it is to occur in Australia is US$12.5 billion (Buetre et al., 2013). Together, the global costs of FMD incursions into FMD free countries is estimated at US$1.5 billion per year (Knight-Jones and Rushton, 2013). These costs are mainly due to the implementation of control and eradication measures particularly through the culling of infected and susceptible livestock, in addition to the ban of livestock trade activities that arises due to the loss of FMD-free status. In contrast, the negative impacts of FMD on livestock and rural economy are more subtle in endemic countries. Here, the detrimental losses of FMD outbreaks are often under estimated and less-well defined. Most farmers in these countries are smallholders (Swanepoel et al., 2002). In mainland SEA, the agriculture system is closely linked and always related to animal husbandry of smallholders with mixed farming practices. In this region, livestock are traditionally reared in household backyards in a local system that is usually integrated with crops, and deeply rooted to the in their lifestyle of the people. It represents a complex relationship between the farmer's family, their animals and their crops. In this setting, FMD causes loses through neonatal mortality of livestock, lowered fertility of animals and loss of income due to the prohibition of selling animals and animal products. Also, smallholder dairy farmers face production losses through reduced milk yields and prohibition of selling milk. Other impacts of FMD include smallholders that keep cattle or buffalo to plough rice fields where the impacts can result from a loss of draught power. In mainland SEA the estimated losses due to FMD in smallholders varies among countries. For example, in Lao PDR FMD estimated costs per animal are between US$56 and US$66 (Nampanya et al., 2016) whereas, in Cambodia these costs are higher and estimated to be between US$216 to US$371 (Forman et al., 2009). Furthermore, these losses can differ within a country.

For instance, in the highland region of Vietnam, an area with low livestock density, FMD related losses were estimated at US$84, in contrast to US$930 for the lowland areas, where livestock density is high (Forman et al., 2009). Unfortunately, no studies have been undertaken to estimate the losses due to FMD in P. Malaysia.

1.12 FMD control

Despite global attention and considerable effort in scientific research into the causative agent of FMD, the disease remains endemic in many regions of the world. These are predominantly less developed and poorer countries in Asia (including the Middle East), Africa, Middle East and South America (Gleeson, 2002; Kitching, 1998; OIE, 2018). Within the regions where FMD is endemic, there are also countries (such as Japan 2010) that are free from the disease where sporadic outbreaks have been recorded where it has been possible to control and regain FMD free status (without vaccination). On the other hand, there are countries that have struggled to eradicate FMD after these sporadic FMD episodes such as in the Republic of Korea where regular outbreaks have occurred since 2010, and recovery of FMD-free status has not been possible to achieve. In order to assist in the process of FMD control and eradication, the Progressive Control Pathway for FMD (PCP-FMD) has been introduced by the FAO and OIE (Sumption et al., 2012) (Figure 1.8) and improved in new edition (FAO/OIE, 2018). The purpose of the framework is to assist endemic countries to develop a strategy to attain free status in a step-wise manner. Thus, the PCP-FMD framework identifies an endemic country's risk and helps to develop a work plan to move progressively through development of FMD free zones in order to ultimately achieve the status of FMD free country without vaccination. The PCP-FMD framework includes vaccination as well as other FMD control measures. However, in order to implement this PCP-FMD framework, FMD endemic countries require affordable and reliable tools to carry out post-vaccination monitoring activity alongside affordable and reliable but more manageable vaccine. Therefore, improvements of the existing serological diagnostic and surveillance tools are needed to increase specificity and reliability of the *in-vitro* methods.

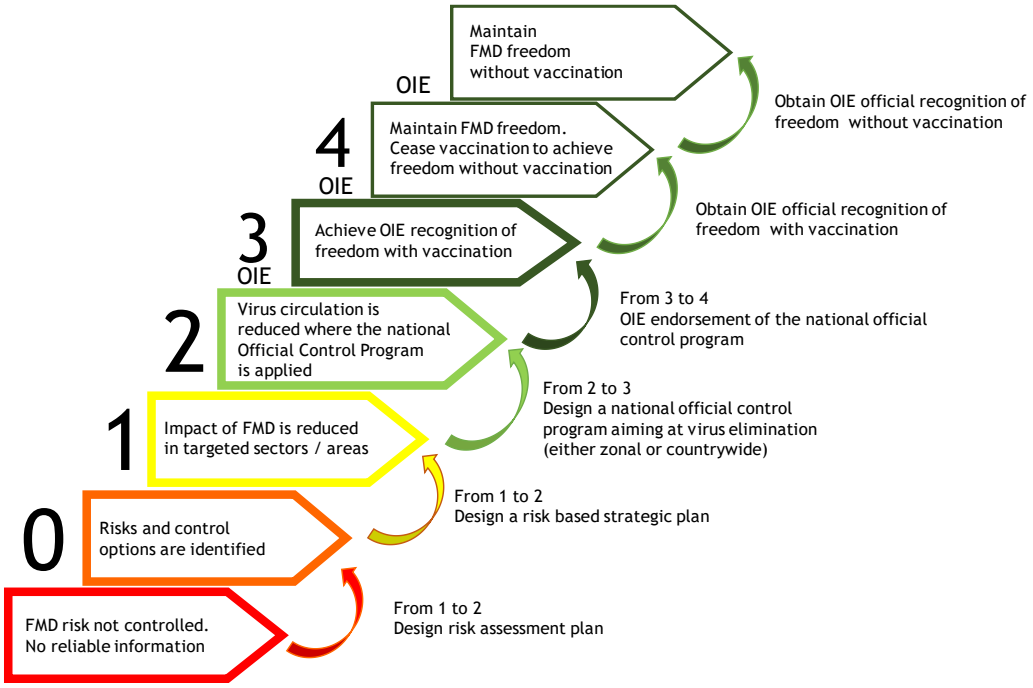

Figure 1.8: Principle, Stage Description and Standard of Progressive Control Program for FMD (PCP-FMD) framework. Adapted from Progressive control pathway for Foot-and-Mouth Disease guideline 2nd Edition 2018.

1.12.1 Review of FMD control initiatives

Developed countries deployed stringent policies in order to control FMD by culling of infected animals and animal movement restrictions. These methods were first applied in France in 1739, and later adopted by Great Britain in 1869. These stringent measures against FMD are still in place in developed countries.

In contrast to these successful polices to control FMD in Europe, FMD control in the most developing countries (including P. Malaysia) has progressed more slowly. For example, in P. Malaysia, the first two disease resembled FMD records were in cattle in 1860 and in 1909. Then, 26 years later, in 1936 an outbreak of a disease resembling FMD was recorded in 551 cattle in the states of Perak and Selangor (Wallace, 1936) and was followed by reports of similar nature, involving 238 cattle in Perak in 1938 (Wallace, 1939). The latest incidence was linked to the movement of animals from neighbouring countries. These events resulted in the development of policies to control livestock movement in four northern states of P. Malaysia (Perlis, Perak, Kedah and Kelantan).

P. Malaysia changed its policy to slaughter coupled with strict sanitary procedures after an outbreak by FMDV subtype A_{22} in Perlis in 1973. These measures lead to effective control of FMD outbreaks, with no FMD cases recorded for next five years. However, in October 1978 another FMD case was recorded in the state of Kelantan which borders

Thailand. The disease was then spread through trading routes, to the district of Muar in Johor and to the district of Ipoh in Perak. Another FMD case was then reported in the district of Tumpat in Kelantan, which spread to the states of Perlis and Kedah in late 1978. These FMD cases were identified as being caused by FMDV subtype O_1. The extension of this spread lead to the adoption of a stamping out policy by Department of Veterinary Services (DVS) which was applied in the affected area. In total, 18,117 animals were slaughtered of which 7,511 (41.5%) were cattle (Chong, 1979; Thuraisingham, 1977). However, this stamping out policy was unpopular and strongly objected by the people. Therefore, in January 1979 the policy was changed to prophylactic vaccination (Chong, 1979). However, the vaccination policy was found not to be effective since more FMD cases were identified (due to serotype Asia 1) which were linked to importation of cattle to P. Malaysia for slaughter (Babjee, 1994).

Realising the complexity of FMD control across countries that share common borders (Ozawa, 1994), SEAFMD Campaign was proposed and established in 1994, with the first meeting being held in 1995. Subsequently, a Regional Coordination Unit (RCU) was also established in Bangkok in 1997 to supervise the FMD control program in the SEA region (Abila and Foreman, 2006; Edwards, 2004). In 2001 an area was identified by OIE that could as the potentially gain FMD free status in the SEA region by the OIE. This area, include the northern P. Malaysia and isthmus of Kra involving three countries: Malaysia, Thailand and Myanmar. The five-year program, named Malaysia-Thailand-Myanmar (MTM) Campaign for FMD Freedom, was officially established (Edwards, 2004; Suseno and Wongsathapornchai, 2004; Wongsathapornchai et al., 2008). In Malaysia, the MTM FMD free zone involved the entire states of Perlis and Kedah and selected districts located in additional four states in the northern part of the country. In Thailand two regions, 8 and 9, were selected in addition to a buffer zone involving Prachuapkirikhan province that located in region 7. In Myanmar, Kawthung district of Tanintharyi division was involved in addition to the buffer zone in Myeik district (Turton, 2004) (Figure 1.9).

In 2010, the SEAFMD Campaign was expanded to involve China and renamed as SEACFMD Campaign. Guided by the SEACFMD Roadmap, the implementation of the eradication campaign is divided into five phases that will run up to 2020 (OIE, 2016). Now, with only about two years left before the end of the campaign, the number of FMD cases in mainland SEA countries are still increasing adversely affecting the livelihoods of the local populations. Furthermore, incursions of new, previously unreported in mainland SEA lineages of FMDV, such like the O/ME-SA/Ind-2001 lineage, are reported (WRLFMD, 2018). Despite the efforts, out of the six FMD endemic countries in mainland SEA and China (People's Rep of), only Thailand and China currently have an official national control program for FMD that is endorsed by resolution no. 23 of the general assembly of OIE.

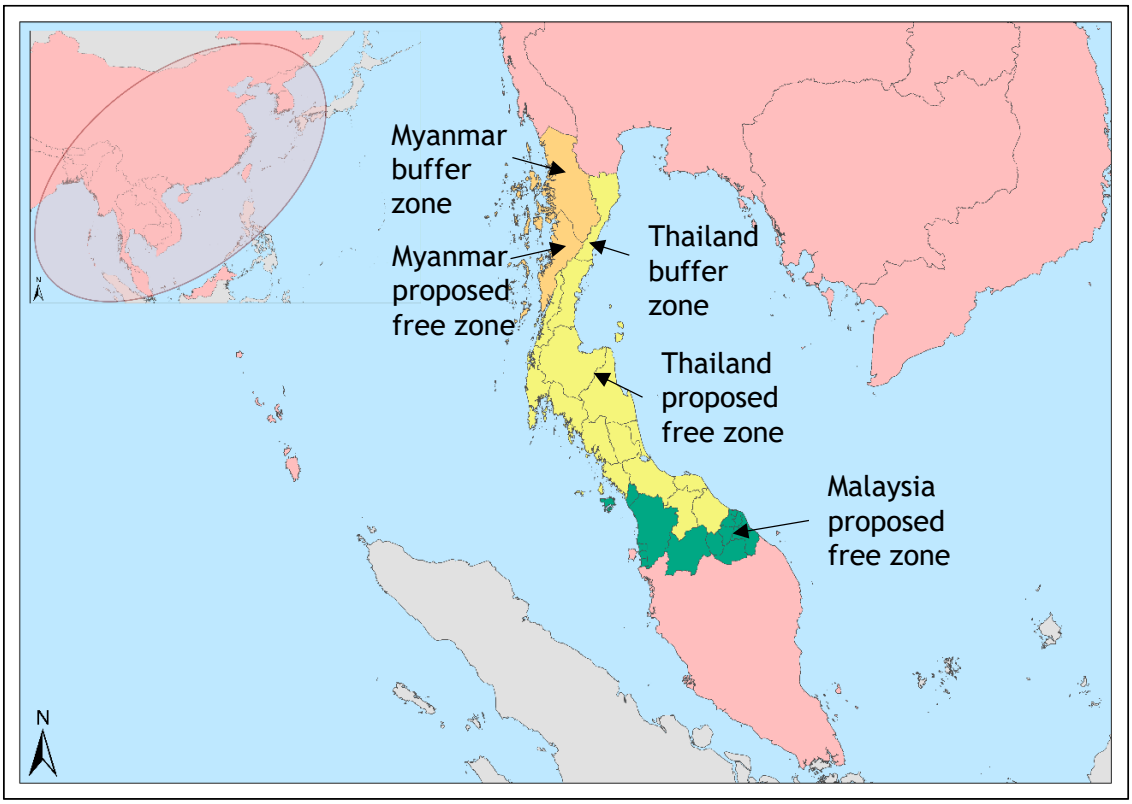

Figure 1.9: The geographical area of the Malaysia-Thailand-Myanmar (MTM) Campaign for FMD Freedom. Map is courtesy of Dr Di Nadro.

1.12.2 Challenges of FMD control

The Food and Agricultural Organization of the United Nations (FAO) and OIE has recognised FMD as a threat to world food security, and has therefore made FMD control a global priority (Sumption et al., 2012). Although there are various different control measures that can be adopted there is no standard FMD control and eradication program that is suitable for all FMD endemic countries. The epidemiology of FMD involves a complex and dynamic interaction of the aetiological agent, susceptible hosts, and environment (including livestock trade). Unlike other animal diseases, the environmental factor in the epidemiological triad of FMD is wider and more complex. It is not only limited to the direct interaction of the agent or host with the environment but it includes economic status, trade, cultural and political situation of the society that manage the host (non-direct environmental factors). In fact, the success of FMD control and eradication program of a country is almost solely dependent on these non-direct environmental factors. The current global distribution of FMD mirrors the geographical location of countries with low economic status. In these countries, FMD cases are often

under-reported and animal health services are generally under-resourced. Therefore, it is almost impossible for these countries to adopt the stringent FMD control measures.

In mainland SEA, control measures adopted include mainly vaccination and restrictions in animal movements (Gleeson, 2002). The development of the PCP-FMD control framework through vaccination and free zoning by the FAO and OIE can be used to facilitate FMD control program. This approach has worked in some countries in SEA such as Indonesia and the Philippines. However, these countries comprise of islands with the sea/ocean acting as natural barriers. Countries that share common land borders face more challenges. It is established that the most effective way for the spread of FMD is through direct contact of the susceptible animals with either clinically or sub clinically infected animals, or with animals incubating the disease (Alexandersen and Donaldson, 2002; Brown, 2004; Donaldson, 1987; Orsel et al., 2009).

Movement of animals, either via legal or illegal trade, has been demonstrated to be an effective mechanism of FMD spread (Mansley et al., 2003; Rweyemamu et al., 2008). For example, the 1990s spread of FMDV serotype Asia 1 was shown to follow the movement of livestock from India, to Myanmar via Bangladesh and then, following the route of livestock movement, down to P. Malaysia (Rweyemamu et al., 2008). Another example is the introduction of the O/ME-SA/PanAsia-2 lineage to P. Malaysia, a virus linked with previous outbreaks in India and Bangladesh (Knowles et al., 2005). Cultural practices pose great challenges to the deployment of restrictions on animal movements to control FMD in Malaysia (Sasaki, 1994), particularly when imports of livestock and meat products from FMD free countries more expensive than from adjacent countries.

Another hurdle faced by FMD endemic countries is that livestock are mainly reared by smallholder farmers. For example, in P. Malaysia, even though pigs and poultry are generally reared on farms with good husbandry and zoo sanitary practices, ruminants are reared differently. For beef cattle 23% are reared under the oil palm plantations and beef cattle reared traditional system contributes 57% of the ruminant population. This indicates the majority of ruminants in the country are reared by smallholders in their backyards. The livestock husbandry practices are not very different in other countries in mainland SEA. In these settings, animals are free to roam and co-mingle with other animals within the village and, to some extent, with other villages. Under these conditions, the probability of effective FMD spread is very high, while implementation of effective control measures, such as vaccination, can be difficult. For instance, to perform vaccination in these traditional and integration systems, which do not have basic facilities to restrain the animals, the vaccinator's team often has to build temporary corrals before the actual vaccination can be performed (Figure 1.10 and 1.11).

The existence of persistent/carrier state of FMDV infection in naïve and vaccinated ruminants adds to the complication of FMD control. The problem occurs especially during post vaccination monitoring (PVM) and manifests itself in the lack of the ability of the currently available serological tests to discriminate a subclinical infection from a persistent infection at an individual animal level. In addition, FMDV serotype cannot be distinguished based on clinical manifestation of the disease. This results in the need for regular PVM to determine whether the vaccine virus currently in use is appropriate against the serotype that is causing contemporary outbreaks. Furthermore, a spectrum of viruses with their own antigenic and epidemiological characteristic can also exist within a serotype making the FMD control measures and preparedness even more complicated requiring regular in-depth laboratory characterisation of the currently circulating FMD viruses.

Figure 1.10: Vaccination of cattle reared in integration system in P. Malaysia. Pictures are courtesy of Pn Salamiah Binti Sarif.

Figure 1.11: Vaccination of buffalo reared in traditional system in P. Malaysia. Pictures are courtesy of Pn Salamiah Binti Sarif.

1.12.3 FMD vaccines

In countries where FMD is endemic, vaccination is commonly used as a primary tool to suppress clinical signs of the disease. Vaccines can be applied to induce herd immunity, reduce clinical infection and viral transmission (Orsel et al., 2007). In addition, systematic vaccination in combination with movement controls and culling of FMD infected animals, has been implemented to effectively control and eradicate FMD from endemic regions, such as Western Europe and most courtiers of South America (Clavijo et al., 2017; Leforban and Gerbier, 2002; Saraiva, 2004). For countries with FMD free status, the use of vaccine as a "vaccinate-to-kill policy" has reduced the spread of the disease, and subsequently led to the reinstatement of the FMD free status (such as in The Netherlands [2001] and Japan [2010]). However, FMD control by vaccination has challenges and to date there are no countries that have achieved an FMD freedom status by using vaccination alone.

There are many factors associated with the success of FMD control by vaccination such as vaccine quality, potency and cross-reactivity between a vaccine virus and field isolate (Barnett and Carabin, 2002). The fact that diverse FMDV field isolates needs to be

matched to the homologous vaccine virus, highlights the importance of a sound and reliable vaccine matching test (Paton et al., 2005). In addition to antigenic match, the type of adjuvant used in the vaccine formulation also contributes to the performance of a vaccine (Jamal et al., 2008). Besides these points, there are two more main factor that influence the successfulness of FMD vaccination. One is host related factor such as the impact of maternally derived immunity and interference with other vaccine if it were to be given at the same time. The second point relates to human factors, such as the maintenance of a cold chain from the vaccine plant to the field and correct vaccine administration (the dose, route, frequency and time) (Heininger et al., 2012; Lyons et al., 2016).

1.12.4 Vaccine potency for FMD

In order to quantitatively measure the specific ability of the vaccine to confer protection (vaccine immunogenicity), a vaccine potency test is usually performed. There are currently two different methods that are described to measure vaccine potency. Following the standard set by the European Pharmacopeia that FMD vaccine potency is expressed in half of protective dose (PD) more frequently called PD_{50} (Goris et al.). In South-America FMD vaccine potency testing is guided by Argentine Animal Health Service (SENASA). The SENASA expressed FMD vaccine potency by Protection against Podal Generalisation (PPG) (Goris and De Clercq, 2008). Both of these methods involve vaccination and experimental challenge of animals (usually cattle). According to the OIE terrestrial manual chapter 2.1.8 section C requirement for vaccine, FMD vaccines can be classified into two classes: (i) standard and (ii) higher potency vaccines (OIE, 2017). The standard potency vaccine for FMD should have minimum of $3PD_{50}$ or 75% PPG (protection against generalised foot infection) whereas, the higher potency vaccine should have > $6PD_{50}$.

In order to achieve 75% PPG (which is broadly an equivalent of >$3PD_{50}$), a minimum of 16 animals need to be vaccinated with a full dose of the test vaccine. The animals are challenged at four weeks or more with 10^4 bovine infectious doses (BID_{50}) after which 12 animals have to be protected, without clinical lesions, on the feet for 7 days. The PD_{50} test is generally performed by dividing a group of animals into three; (i) a group of animals that receives full vaccine dose, (ii) a group of animals that receives quarter vaccine dose and (iii) a group of animals that receives sixteenth vaccine dose. This method allows usage of fewer animals in total. However, comparison of the two vaccine potency methods, using large number (>60) of animals tested, showed that PPG was more reliable as compared to PD_{50} (Filho et al., 1993; Goris et al., 2008). Additionally, the PD_{50} method

requires a higher number of animals per vaccine dose; a group up to 25 animals is needed in order to distinguish between 3, 6 or 10 PD_{50} in a single trial (Goris et al.). Therefore, alternative *in-vitro* method has also been suggested. The alternative for PD_{50} using the indirect potency test, for example, the use of neutralisation or total antibody titre and protection against challenge (Robiolo et al., 1995).

1.12.5 The current FMD vaccines

Conventional FMD vaccines used worldwide are derived from *in-vitro* passaged FMDV isolates that are chemically inactivated and purified. These "killed" vaccines use binary ethyleneimine (BEI) to inactivate the FMDV and are often processed to deplete non-structural proteins (NSPs). These are produced as purified monovalent or polyvalent FMDV products that are formulated as oil or aqueous based vaccines with adjuvants. Vaccines can be stored as concentrated antigens that are suitable for long term preservation (~5 years) when kept in liquid nitrogen, but can have a shorter shelf life when kept at 4°C. Furthermore, once prepared into a formulated product the shelf life of the vaccine is usually about 12 twelve months. The potency of the vaccine varies depending on the supplier and purpose of vaccination and ranges from those standard vaccines that provide half protective dose of least three ($3PD_{50}$) to high ($6PD_{50}$) suitable for emergency vaccination. Vaccines that contain at least $3PD_{50}$ are reported to confer protection against homologous virus challenge in cattle within seven days (Golde et al., 2005). Further research has shown that very high potency vaccines of at least $30PD_{50}$ confer good protection against heterologous virus (Brehm et al., 2008) irrespective of a poor antigenic match.

The current inactivated FMD vaccines have many limitations. Among them are that these vaccines typically elicit only short-lasting serological immunity and therefore frequent vaccination are usually needed (Doel, 2003; Doel and Chong, 1982). Furthermore, these vaccines are vulnerable to degradation at elevated temperatures. At about 30°C, FMDV antigen in the vaccine rapidly converts into irreversible immunogenically incompetent 12S (Doel and Baccarini, 1981; Doel and Chong, 1982). As consequence, the FMD virus capsid's integrity requires maintenance of a cold chain from the vaccine plant to the point that the vaccine is given to individual animals. These limitations are troublesome since most of livestock in the endemic countries are kept by smallholders that have limited facilities. Another disadvantage of these vaccines is that high-containment facilities are required for their manufacture, which are expensive to build and maintain, and handling live FMDV in these facilities can increase risks of virus escapes to cause field outbreaks.

1.12.6 Other types of FMDV vaccines

Due to the limitation of inactivated vaccines, alternative vaccines have been developed. Among them are viral vectored vaccines, recombinant virus like particle (VLP) vaccines, peptide vaccines, DNA vaccines, live attenuated vaccines and attenuated DIVA-marked FMDV for inactivated vaccine production.

In principle, viral vectors can be used to deliver FMDV structural proteins in virus-like particles (VLPs) to induce an immune response in the host. There are different vectors systems that have been researched and, to date, the recombinant replication defective human adenovirus was shown to be protective against O_1 Manisa in swine (Fernandez-Sainz et al., 2017). The recombinant VLP vaccines uses baculovirus (insect cell) culture system to produce stabilised FMDV empty capsid (75S). These recombinant VLP vaccines were shown to confer protection against serotype A in challenge study in cattle (Li et al., 2012; Porta et al., 2013).

Peptide vaccines were developed using selected linear epitopes of the virus and do not involve infectious virus. This type of FMD vaccine is currently being used in pigs in China, as reviewed by Cao and colleague (Cao et al., 2016). Protection to selected linear peptide is usually limited and escape mutant can arise in FMDV.

DNA vaccine are generally safe but the technology requires large amount of DNA and multiple inoculation. Furthermore, DNA vaccines have not been tested in natural host.

Live attenuated vaccines of FMD can provide rapid long-lasting immunity against FMDV. However, the technology has not shown to be protective in the natural host such as swine and cattle (Chinsangaram et al., 1998; Mason et al., 1997). Furthermore, the fear of the virus reversing to infectious virus is one of the main factors as to why it has not been used in the field. As a consequence, attenuated DIVA-marked FMDV for inactivated vaccine production is suggested (de Los Santos et al., 2018). The principle of this approach is replacement of the wild type inactivated whole FMDV with attenuated derivatives that can be easily propagated in a cell culture system.

Despite extensive research in FMD vaccinology, the only other type of FMD vaccine currently available on the market is the peptide vaccine that only use in China and limited in pigs.

1.12.7 Control measures, to complement vaccination

It is established that FMD control programs in countries where the disease is endemic is complicated. For example, in mainland SEA, many different suggestions have been made in order to develop and implement an effective FMD control program. It has been suggested that systematic quarantine procedures coupled with vaccination (minimum 21 days prior the quarantine procedure) and the establishment of FMD free zones is the appropriate approach to eradicate the disease in mainland SEA (Sasaki, 1994). This study argues that, due to cultural constrains and lifestyle of the people, movement restriction on live animals should not be implemented if the disease would to occur in the exporting country. However, others argue that the introduction of animal movement restriction from FMD affected country is necessary for prevention of spread of the disease (Edwards, 2004). However, in both of these scenarios, vaccination is paramount.

There are other methods that are well established and proven to be effective in FMD control, including the application of stamping out policies. However, due to economic as well as cultural reasons, it would be difficult to include stamping out policy in mainland SEA and/or in other FMD endemic countries. FMD control in endemic countries needs to extend beyond the current policy of vaccination and systematic quarantine practices. Thus, despite the socio-economic and cultural restrictions in the region, the implementation of animal movement restriction from FMD affected regions needs to be considered. In addition, an application of simulation models to aid the choice of control policies during an outbreak might be useful (Morris et al., 2002). However, in order to develop reliable models suited for supporting FMD control in mainland SEA, good epidemiological data is required.

1.13 Vaccine matching

In order to have an effective FMD vaccine, the vaccine virus has to be matched with the circulating field isolate through a vaccine matching method. This method needs to be reliable, affordable and relatively easy to perform. FMD reference laboratories undertake vaccine matching tests for the selection of vaccines.

According to the OIE terresterial manual section chapter 2.1.8 secion D, there are four main options for the vaccine matching test:

i. Two dimensional (chequerboard) VNT method.

ii. Expected precentage of protection (EPP), determination using one-dimensional VNT; the method is widely used in South America and correlation tables between serological responses and protection are developed
iii. ELISA
iv. CFT (only used for screening purpose), although this method is not widely practiced.

The antigenic relationship between the vaccine virus and field virus is determined by a mathematical formula that determines a relationship coefficient (r1-value). The r1 values generated by serological methods (most commonly either VNT or ELISA) are used as a guide to predict whether or not the vaccine virus will confer protection against the field virus (Rweyemamu and Hingley, 1984; Rweyemamu et al., 1977). The formula used to determine the relationship coefficient is expressed as follows (OIE, 2017):

$$\text{r1 value} = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithmetic titre of reference serum against vaccine virus}}$$

Determination of fitness for purpose of a vaccine using EPP method (ii) is only possible when correlation studies have been carried out for the particular vaccine viruses tested. Post-vaccination antibody titres (primary or booster) measured by VNT or ELISA are related to the probability of protection using the established correlation tables for the vaccine viruses available. For primary vaccination, protection level of vaccinated cattle is established at more than 75%, whereas for post booster vaccination, the protection level of vaccinated cattle at more than 50% (OIE, 2017).

1.13.1 *In-vitro* vaccine matching methods

Direct *in-vitro* vaccine matching methods using susceptible animal species that are vaccinated and challenged is ideal, however this is expensive, time consuming and creates animal welfare concerns. As an alternative, serological relationship between field and vaccine viruses can be adopted as indirect methods: such as liquid phase blocking ELISA (LBPE)(Crowther and Abuelzein, 1979), complement fixation test (CFT) and VNT (Booth et al., 1978). However, CFT was found to be less specific than VNT (Rweyemamu et al., 1978) and is not widely used by FMD Reference laboratories.

Sequence analysis as well as antigenic profiling using monoclonal antibodies (mAbs) may also be useful to indicate whether an FMDV isolate is a match with a vaccine virus (Paton et al., 2005). However sequencing analysis techniques are expensive and complicated; more detail information is needed for analysis using mAbs (Mahapatra et al., 2008). Furthermore, current modelling techniques such as antigenic cartography cannot yet accurately predict vaccine matching, although future discovery may allow for this (Ludi et al., 2014).

Virus neutralisation tests utilise susceptible cell culture to measure the ability of serum to neutralise a virus dose of a 100 $TCID_{50}$. Cytopathic effect (CPE) is used as the indicator for neutralisation. The 100$TCID_{50}$ is chosen for the standard protocol of the test because at this value that the linear part of the sigmoid curve is located in the chart that described the relationship between neutralization titre on the Y axis and the virus dose on the X axis (Booth et al., 1978). This is the preferred method (gold standard) for vaccine matching of FMDV field isolates since it is a better predictor of protection (Mattion et al., 2009; O.I.E, 2012; Robiolo et al., 2010). However, the test is laborious and time consuming and requires high-containment facilities to handle live FMDV.

An alternative test is the liquid phase blocking ELISA where, guinea pig polyclonal antibody raised to serotype specific FMDV is used to bind a fixed dose of virus. It is the detector antibody and that it can't bind if the FMD antigen-antibody complexes get formed. The binding form antigen-antibodies complexes, preventing the colour appearance, produce by the substrate/chromogen solution in LPBE testing. Enzyme-linked immunosorbent assay (ELISA) produces more reproducible results than VNT (Tekleghiorghis et al., 2014; Van Maanen and Terpstra, 1989). Both VNT and LPBE tests measure antigen-antibody complexes using serial dilutions. However, there are remarkable differences between these two methods. VNT measures only neutralising antibodies whereas LPBE measures binding of the antigen antibody complexes which include neutralising and non-neutralising antibodies. The fact that it is not able to differentiate neutralising from the non-neutralising antibody makes ELISA less preferred compared to VNT. Neutralising antibodies are more closely related to protection alongside interaction with the complex immune response and cell reactions (McCullough et al., 1992b; Reading and Dimmock, 2007). In addition to indirect serological test, ELISA measuring bovine serum immunoglobulin subtype ELISA particularly IgG1/IgG2 ratio and avidity ELISA have also been explored as approaches that may better predict protection (Capozzo et al., 1997; Lavoria et al., 2012).

1.13.2 Review of retrospective r1 values generated from VNT

Vaccine matching tests measures the relationship between a vaccine virus (homologous virus) and the virus isolated from the field (heterologous virus) using hyper-immunised bovine vaccinal sera that is homologous to the vaccine virus. Vaccine matching is being carried out routinely at the World Reference Laboratory for FMD (WRLFMD) to determine the most suitable vaccine for a field FMD virus. The serological test used is the virus neutralisation test (VNT). The ratio of the neutralisation titre determined against the homologous virus and the heterologous virus are used to generate the quantitative relationship coefficient (r1 value) as a predictor of potential coverage. Traditionally r1 value with minimum acceptable cut-off of 0.3 are accepted as a match, as recommended by OIE (OIE, 2017). It is assumed that the closer the r1 value is to 1 the more antigenically similar the two viruses are. However some researchers find the use of r1-values questionable (Brito et al., 2014), while others have suggested to improve r1-values calculation by using statistical models (linear mixed effects models) that utilised sequencing data (Reeve et al., 2010). During the early part of this study a preliminary analysis of retrospective r1 values for ten years (2006 - 2017) of serotype A linage A/ASIA/Sea-97 generated at WRLFMD for mainland SEA were analysed to assess the repeatability of the r1 values generated. The preliminary analysis shows that the r1 values generated from the neutralisation titres for A/ASIA/Sea-97 vaccine virus were uncorrelated above and below the protective cut-off point at 0.3 (OIE, 2017) (Figure 1.12). The proportion of the r1 values of A/ASIA/Sea-97 viruses showed majority (62%) were below the cut-off point (no-match), while 38% of field virus tested matched the vaccine virus (Figure 1.13).

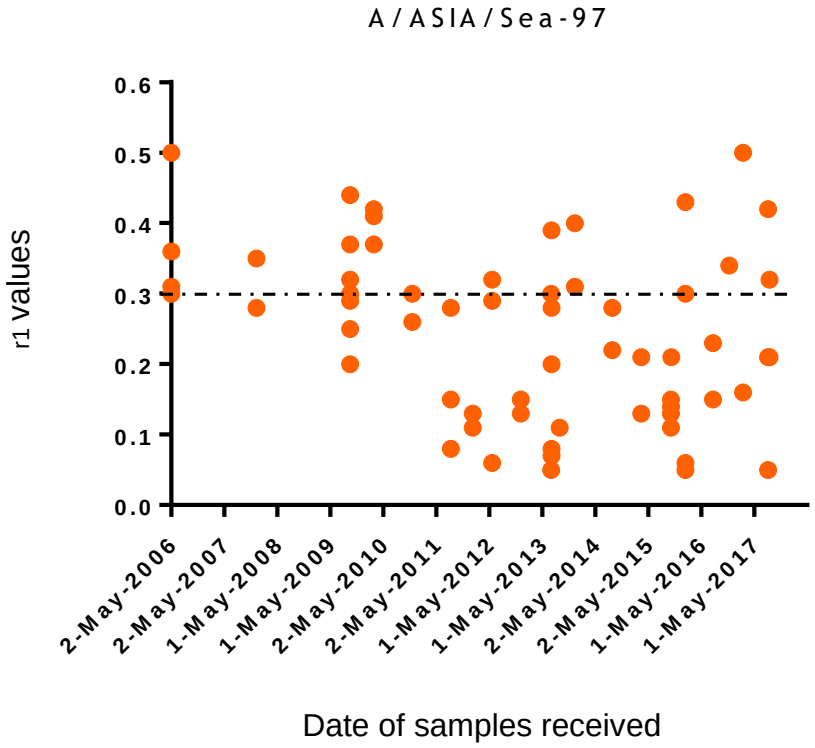

Figure 1.12: The r1 values generated from neutralisation titres of FMD viruses belonging to the A/ASIA/Sea-97 lineage collected in mainland SEA from the year 2006 - 2017. The orange dots represent the individual r1 values and the black dashed line indicate the suggested protective cut-off (0.3) defined by the OIE.

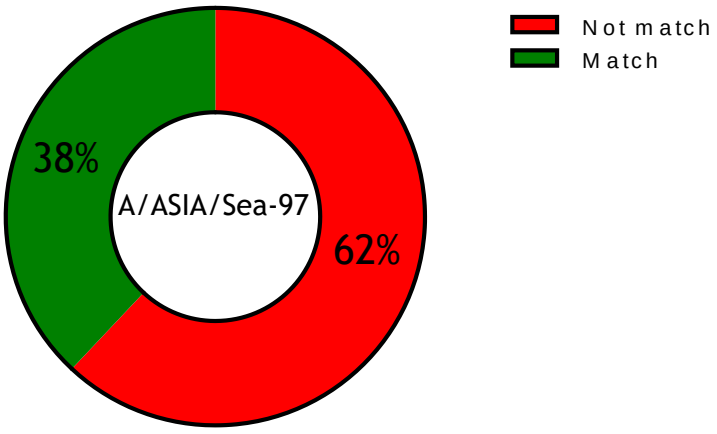

Figure 1.13: The proportion of r1 values generated at WRLFMD that are matched and not matched for viruses collected in mainland SEA from the year 2006 - 2017 for the A/ASIA/Sea-97 lineage. Data represents testing done using neutralisation titres of collected for viruses collected from mainland SEA against the A/May-97 vaccine virus over a 10-year period (2006 to 2017). A cut-off of r1 ≥0.3 defines matched and not-matched field viruses (OIE, 2017).

1.14 Thesis hypothesis and aims

Current vaccines used in P. Malaysia to control FMD include the A/MAY-97 virus. Vaccine matching tests, using virus neutralisation, suggests that this vaccine virus poorly protects against contemporary FMD isolates in the region. In SEA only 32% of field isolates have been shown by *in-vitro* testing to be covered by the A/MAY-97 vaccine virus. However, poor robustness in the current *in-vitro* vaccine matching methods combined with a lack of properly designed field studies of vaccine effectiveness make it difficult to identify the contribution of factors affecting vaccination, including vaccine matching, to incomplete FMD control. It has been widely recognized that the serologically derived relationship coefficients used to determine vaccine matching (r1-values) can produce conflicting results. The high costs of performing cross-virus challenge potency tests has made it difficult to address this problem. This work will help to better characterise the antigenic epidemiology of Malaysian FMD viruses and explore the ability of the current vaccine virus to protect against the contemporary field isolate. This additional knowledge will lead to improved control measures within the region and elsewhere.

The overall hypothesis of this study is that lack of reproducibility in serological vaccine matching methods, particularly the results generated by the virus neutralisation test (VNT) and liquid phase blocking ELISA (LPBE), is caused by intrinsic factors. This is based on the preliminary study (analysis of the retrospective r1 value 10 years data) and situation observed at the field in Peninsular Malaysia. In addition to inherent day-to-day variability, the three main determinants that are proposed to affect the reliability of results are; (i). the variability of FMD virus growth in the cells used in the VNT. Utilising information acquired from the variability of FMD virus growth in the cells used in VNT, (ii) the variability of reference sera used by producing bovine sera (BVS) as standardised sera to assess antigenic relationships between vaccine and field FMD viruses, and (iii) the instability of the FMD virus antigens used in both of these tests. In addition, (iv) results generated after vaccination in a field experiment are used to help understand the relationship of in-vitro test limitations and FMD control in endemic settings.

Therefore, the overall objectives of this thesis are to describe the limitations of the in-vitro vaccine matching methods, to identify the source of variability that is observed when using these tests and to assess the importance and relevance of these findings to FMD control in endemic settings.

Chapter 2:

Influence of cellular factors on *in-vitro* vaccine matching: impact of IB-RS-2 cell division cycle, and the presence of CSFV within the cells.

Acknowledgements:

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2.1 Abstract

Foot-and-mouth disease virus (FMDV) causes an economically important disease that threatens international trade of livestock and livestock production. Virus neutralization test (VNT) is the accepted "gold standard" method used to define serological responses to vaccines and FMDV infection. However, one of the cell lines (IB-RS-2) that is widely used to detect and measure FMDV-specific antibodies is persistently infected with classical swine fever virus (CSFV), which restricts the use of these cells to high-containment laboratories. The aim of this study was to define whether the presence of this virus in these cells impacts upon their ability to propagate FMDV. For ten cell cultures with different passage histories, confluent CSFV positive IB-RS-2 cells supported FMDV replication of field isolates and vaccine virus to higher titres (p=0.015) compared to non-confluent cells (in 7/10 and 8/10 cell cultures, respectively). Moreover, confluent cells yielded lower neutralisation titres in six individual sera tested with significant different in titres, at 100$TCID_{50}$. The presence of CSFV was confirmed using real-time RT-PCR. Data showed that confluent cells generated significantly (p=0.005) higher levels of CSFV genome copies compared to the non-confluent cells. However, no significant correlation was observed between the amount of CSFV and FMDV titres obtained with the respective cell cultures. Further evidence to indicate that CSFV does not influence FMDV replication was obtained using CSFV negative IB-RS-2 cells, where equivalent analytical sensitivity to CSFV positive cells was observed for 25 FMDV isolates. These data reinforce the importance of standardising the cell cycle of cells used for VNT, and indicate that CSFV negative IB-RS-2 cells may provide an alternative to existing cell lines.

2.2 Introduction

Foot-and-mouth disease (FMD) is a highly contagious livestock disease that has negative impacts on a country's economy via effects on livestock productivity and reduced opportunities for trade. The global cost of FMD outbreaks in countries where the disease has been eradicated such as United Kingdom, Netherland, France, Japan and others as listed in OIE FMD status is estimated at US$1.5 billion per year (Knight-Jones and Rushton, 2013), while countries with FMD endemic status face annual loss from livestock production and vaccination alone at an estimated of US$ 6.5 to 21 billion (Knight-Jones and Rushton, 2013; Knight-Jones et al., 2017). Therefore, tools such as vaccination are extremely important to control the disease. One of the steps to achieve an effective vaccination program is to select an appropriate vaccine virus, which antigenically matches the circulating field isolates. FMD is caused by foot-and-mouth disease virus (FMDV) that has seven serotypes; O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3 (Knowles and Samuel, 2003). The FMDV capsid is composed of four structural proteins: VP1, VP2, VP3 and VP4: VP1 contains many of the important epitopes able to elicit virus neutralising and non-neutralising antibodies (Bolwell et al., 1989; Ouldridge et al., 1984; Thomas et al., 1988). FMDV antibodies produced against one FMDV serotype have no cross protection with other serotypes, or in some cases even within serotype (Kitching et al., 1988; Knowles and Samuel, 2003; Robiolo et al., 2010).

The virus neutralisation test (VNT) is the preferred *in-vitro* vaccine-matching method (OIE, 2017) used in FMD Reference Laboratories. This method relies on the use of susceptible cell cultures to measure the ability of bovine reference serum to neutralise standardized doses of vaccines and field viruses. A number of different cells culture systems, such as IB-RS-2 cells, baby hamster kidney cells (BHK 21), and bovine, lamb or pig kidney primary cells have been used for FMDV *in-vitro* VNT methods (OIE, 2017). Of these cells, IB-RS-2 cells are widely used due to their high analytical and diagnostic sensitivity and ease of maintaining the cultures. Despite the fact that the IB-RS-2 cell line was established from primary kidney cells of a physically normal three months old piglet, there was an early indication of the presence of classical swine fever virus (CSFV) in these cells (De Castro, 1964; De Castro, 1970; De Castro, 1973). CSFV is an enveloped, non-segmented, positive strand RNA virus and a member of pestivirus genus within the *Flaviviridae* family. As outbreaks of CSF in pigs can cause major epidemics and are linked with significant economic losses, the virus is classified as the highest tier risk level by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) and classified as a SAPO 3 pathogen by the UK Advisory Committee on

Dangerous Pathogens (ACDP). Thus, the presence of CSFV in IB-RS-2 cells is undesirable as the CSFV positive cell lines can only be maintained in high containment laboratories, limiting their application.

The objectives of this study were to (i) determine cellular factors that influence the *in-vitro* replication of FMDV in IB-RS-2 cells at different cell stages using non-confluent and confluent cultures, (ii) to determine presence and effect of CSFV in IB-RS-2 cells stocks routinely use for *in-vitro* vaccine matching test at The Pirbright Institute. (iii) to explore whether the CSFV negative IB-RS-2 cells have equivalent analytical sensitivity for FMDV replication.

2.3 Materials and methods

2.3.1 Cell lines

Ten vials of frozen CSFV positive IB-RS-2 cells with different passage history were obtained from the cell repository held at The Pirbright Institute (TPI) and the CSFV negative IB-RS-2 cells were received from Friedrich-Loeffler-Institut (FLI), Germany. All the cells were propagated in Glasgow minimum essential media (GMEM) supplemented with 10% adult bovine serum, 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 1% L-Glutamine. Before use, the IB-RS-2 cells were revived, washed and propagated until a fully confluent monolayer was achieved. These were then split on the same day but at different ratio in order to obtain the non-confluent (<60%) and confluent (100%) cells ready to be used 2 days later. The harvested cells were prepared for flow cytometry analysis and for the cell suspension used in VNT. For flow cytometry analysis, the cells were kept on ice prior to fixation. For VNT, a cell suspension was prepared at 1×10^6 cell count per ml for the CSFV positive IB-RS-2 cells.

A preliminary study to determine the optimum cell count of the negative IB-RS-2 cell to be used in the 96 well plates was carried out prior to the FMDV limit of detection experiment. In this preliminary study, four cell concentrations (per ml) of the negative CSFV IB-RS-2 cell were tested; 0.25×10^6, 0.5×10^6, 0.75×10^6 and 1.0×10^6. The result from this experiment showed that 0.5×10^6 cells per ml of the negative CSFV IB-RS-2 cells was the optimum conditions. Therefore, to directly compare the FMDV limit of detection of both the CSFV positive and negative IB-RS-2 cells, the cell cultures were prepared at 0.5×10^6 cells per ml, although this is lower than the cell seeding density used for VNT (described above).

2.3.2 Flow cytometric analysis

The flow cytometric analysis was performed to determine the relationship between the cell division cycle and the cell confluency.

A suspension of 2 × 10^6 CSFV positive IB-RS-2 cells was prepared in two individual wells of a 96 well u-bottom plate before washed twice with 100µl cold PBS buffer. The cells were re-suspended in 100µl per well of 4% paraformaldehyde (PFA) and incubated on ice for 15 minutes for fixation followed by washing with 100µl of PBS and twice with 100µl per well cold flow cytometry (FACS) buffer (PBS with 1% BSA). The fixed cells were permeabilised with 0.1% Triton X100 incubated for 30 minutes at room temperature as the cells were labelled with 1µg/ml 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Darzynkiewicz and Huang, 2004). DAPI is a fluorescent dye that binds selectively to DNA of the cells nuclei to form DNA-DAPI complex to enable the cell cycle analysis. The cell cycle data were collected using a BD LSR Fortessa (Fortessa instrument, BD Biosciences) and analysed in FCS express 5. Debris was excluded based on forward scatter (FSC) and side scatter (SSC) (P1), then gated on singlets based on DAPI Area vs width (P2). Finally, a histogram showing DAPI-(area) FCS multicycle was used to calculate the G1, S phase and G2 values.

2.3.3 FMDV isolates

A vaccine virus (A/May-97, provided by Boehringer Ingelheim, Germany) and 25 different FMDV field isolates and were selected from the repository held at the FAO World Reference Laboratory for FMD (WRLFMD) at TPI (Table 2.1). All the field viruses were treated with stabilised di-ethyl ether to destroy any adventitious lipid-containing organisms before propagation. The FMDV field isolates were previously characterized by phylogenetic analyses based on VP1 coding sequences (Knowles et al., 2016). In order to eliminate the potential influence of adventitious CSFV that might have been carried over with the FMD virus isolates, all viruses in this panel originated from a CSFV negative cell culture (either primary bovine thyroid cells (BTy) or baby hamster kidney cells (BHK).

2.3.4 FMDV titration

Quantification of FMDV was determined by titration of each FMD test virus

Table 2.1 List of FMDV isolates used in the study

Viruses	Serotype/topotype[1]/ lineage	Country of Origin	Passage history (prior to study)
MAY/2/2011	A/ASIA/Sea-97	Malaysia	BTy1
VIT/10/2015	A/ASIA/Sea-97	Vietnam	BTy1
VIT/13/2015	A/ASIA/Sea-97	Vietnam	BTy1
May-97	A/ASIA/Sea-97	Boehringer Ingelheim	BHK(BI)
VIT/9/2015	A/ASIA/Sea-97	Vietnam	BTy1
MOG/2/2016	A/ASIA/Sea-97	Mongolia	BTy1
AFG/6/2017	A/ASIA/Iran-05	Afghanistan	BTy1
MAY/9/2016	O/SEA/Mya-98	Malaysia	BTy1
VIT/4/2016	O/SEA/Mya-98	Vietnam	BTy1
SRL/3/2017	O/ME-SA/Ind-2001	Sri Lanka	BTy1
MOG/1/2017	O/ME-SA/PanAsia	Mongolia	BTy1
NEP/37/2017	Asia1/ASIA	Nepal	BTy1
NEP/45/2017	Asia1/ASIA	Nepal	BTy1
AFG/6/2016	Asia1/ASIA/Sindh-08	Afghanistan	BTy1
ZIM/14/2015	SAT1/II (SEZ)	Zimbabwe	BTy1
MAL/1/2016	SAT1/I	Mali	BTy1
BOT/5/2015	SAT1/III	Botswana	BTy1
ZIM/1/2017	SAT2/II	Zimbabwe	BTy1
BOT/1/2017	SAT2/II	Botswana	BTy1
ZIM/5/2015	SAT2/II	Zimbabwe	BTy1
ZAM/3/2015	SAT3/II	Zambia	BTy1
ZAM/1/2017	SAT3/II	Zambia	BTy1
SAR/1/2006	SAT3/I (SEZ)	South Africa	BTy2
KEN/1/2004	C/ None defined	Kenya	BTy3
NEP/35/1996	C/None defined	Nepal	BTy3
BHU/10/1991	C/None defined	Bhutan	BTy2

[1] Where topotype has been defined

In order to assess the impact of cell cycle on FMDV replication, a titration series of the FMD vaccine virus (A/May-97) and A/MAY/2/2011 FMD field isolate was performed using CSFV positive IB-RS-2 cell cultures derived from different passage histories that each were synchronised (prepared in parallel as confluent and non-confluent cells). These experiments consisted of a four-fold titration carried out in flat-bottom 96 well micro-titration plates using a pre-diluted antigen. Briefly, glycerinated antigen (virus) diluted serially with HEPES Modified Eagles Medium supplemented with 0.2% field antibiotic and 0.4% NaOH (diluent) by making a 10^{-1} step. Dilution were performed in bijou bottles with enough volume needed for titration. Four-fold virus titrations were carried out (in duplicate) in flat-bottom 96 well micro-titration plates (Fisher Scientific) as illustrated in figure 2.1. A total of 50µl of an IB-RS-2 cell suspension at a cell count of 1 X 10^6 cells/ml was dispensed into all wells. The plates were incubated at 37°C for three days before being examined using microscope for cytopathic effect (CPE) indicative of FMD virus replication. The $TCID_{50}$ endpoint titre was calculated as described in figure 2.2 following the Spearman-Kärber method 1931 (Kärber, 1931; Spearman, 1908). The FMDV titres produced by the confluent and non-confluent IB-RS-2 cells were analysed using analysis of variance (ANOVA). A General linear model was built with a random effect for the cells factor (nine different IB-RS-2 cell passages) and a fixed effect for the cell confluency (either confluent or non-confluent cells) and virus (vaccine virus; A/May-97 or field virus; A/MAY/2/2011) factors. Subsequently the data were compared to determine whether different confluency of the IB-RS-2 cells give different FMDV virus titres in both the vaccine and field viruses. (MiniTab 17).

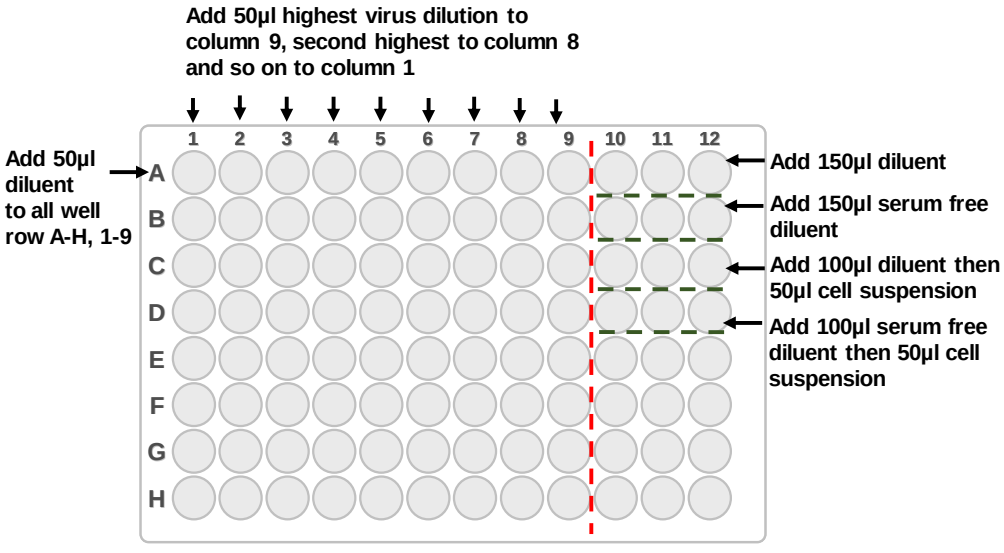
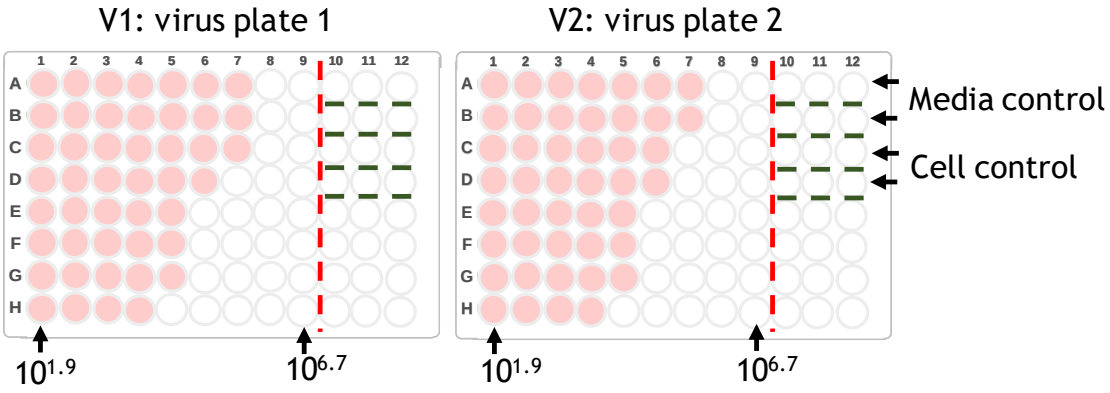


Figure 2.1: Plate layout for virus neutralisation test. Column 1 to 9 of row A to H were for virus titration of one virus. Column 10 to 12 were for control of diluent and cell suspension used.



Virus dilution ($\log_{10}$ 0.6 dilution steps)

Virus doses	$10^{6.7}$	$10^{6.1}$	$10^{5.5}$	$10^{4.9}$	$10^{4.3}$	$10^{3.7}$	$10^{3.1}$	$10^{2.5}$	$10^{1.9}$
NO CPE (V1+V2)	16	16	11	8	2	0	0	0	0
CPE (V1+V2)	0	0	5	8	14	16	16	16	16

z	0	2.5	6.5	11	15	16	16	16
d	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
(z x d)	0	1.5	3.9	6.6	9	9.6	9.6	9.6

Σ(z x d) = 49.6

$TCID_{50}$/ml = Dm + Σ(z x d)/50µl

$TCID_{50}$/ml = Dm + Σ(49.6)/16

5.0 = 1.9 + (3.1)

Recorded as $10^{5.0}$ $TCID_{50}$/50µl

Dm = highest dilution with 100% CPE in all wells

z = ½ of sums of reacting animals of two consecutive sums

d = dilution interval

m = number of wells per dilution

Figure 2.2: Representation of virus titration plates and example of calculation to determine the $TCID_{50}$. Two plates were used as duplicate (V1 and V2). In each plate, the virus was diluted in four-fold dilution from left to right. The control for cell and media is located in column 10, 11 and 12. Pink wells indicate cytopathic effect (CPE) and white wells represent cell monolayer. Virus end point titration ($TCID_{50}$) is calculated following the Spearman-Kärber equation. The example of the stock virus titration is $10^{5.0}$ $TCID_{50}$ (50% of the well have CPE and 50% of the wells contain a monolayer of the cells).

2.3.5 Sera

Inactivated sera collected from six individual animals as described in the next chapter (Chapter 3) material and method section; production of bovine sera (BVS) were used in the virus neutralization (VNT).

2.3.6 Virus neutralisation test (VNT)

Virus neutralisation tests (VNTs) were carried out following the principles outlined in Chapter 2.1.8 Section D for vaccine matching test (OIE, 2017) and according to the WRLFMD protocol. In these VNT experiments both field and vaccine virus were tested simultaneously. The 50% end-point serum neutralization titres at a virus dose of 100$TCID_{50}$ were calculated using linear regression at three virus doses. Inactivated and pre-diluted vaccine antiserum was dispensed on to the plate in two-fold dilutions. Cells were added to all wells and the plates were incubated at 37°C for 72 hours before examination by microscope for cytopathic effect (CPE). VNT assays were carried out using a field isolate (A/MAY/2/2011) and a vaccine virus (A/May-97) with six different vaccine antisera obtained from individual animals collected on 56 days post vaccination (dpv). For the confluent and non-confluent IB-RS-2 cell work, the same dilution of virus was used in all comparison tests, and these tests were carried out on the same day. Virus neutralisation titre data collected for the confluent and non-confluent cells were analysed using analysis of variance; a General linear model with random effect for the serum factor and fixed effect on the cell confluency factor, subsequently tested using Tukey pairwise comparison.

2.3.7 CSFV detection and quantification

CSFV RNA was extracted from a cell suspension containing 1 x 10^6 cells/ml using the RNeasy Mini kit (QIAGEN Ltd.) according to the manufacturer's protocol. The nucleic acid containing the viral RNA was eluted in 50 µl nuclease free water and stored at -80ºC until used. The CSFV RNA was detected and quantified in a real-time RT-PCR assay targets the 5' NTR fragment of the virus using previously described probes (Everett et al., 2010). The reaction mix (One-step SuperScriptTM III/Platinum Taq enzyme mix, Life Technologies) was composed of 5 µl nucleic acid extract, 0.5 µl ROX (1:10), 12.5 µl 2x reaction mix, 2 µl forward and reverse primers (at 10 µM) (CSF100F: 5'-ATG CCC AYA GTA GGA CTA GCA-

3' and CSF192-R: 5'- CTA CTG ACG ACT GTC CTG TAC-3' and 1.5 µl probe (at 5 µM) (5'-TGG CGA GCT CCC TGG GTG GTC TAA GT-3') labelled with BHQ-1 (Black Hole Quencher). All oligonucleotides were synthesized by Sigma-Aldrich (USA). The amplification was carried out using the following cycling conditions: 60°C for 30 min followed by 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec (using an ABI model real-time PCR machine).

The CSFV copy number was quantified using a 10-fold dilution of a CSFV RNA standard prepared using the pCRXLv324-6 plasmid containing the CSFV 5'UTR region of the strain Alfort 187 obtained from Animal and Plant Health Agency, Weybridge, UK. Briefly, after plasmid linearization with Hind III restriction enzyme (New England Biolabs), the RNA was transcribed using the MEGAscript™ T7 Transcription Kit (Ambion) according to the manufacturer's instruction. The quality of the RNA was verified by electrophoresis and quantified using a nanodrop (Thermo Scientific). The RNA was purified using the QIA quick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Serial dilution of the CSFV RNA standards (ranging from 10^1 to 10^8 copies) were prepared based on the Avogadro's constant (number of entities in a mole of substance). One set consist of duplicate of the standard was used for experiment. The CSFV genome copy number was calculated based on the standard curve determined from the ten-fold serial dilution of the standard included in each PCR. Statistical analyses of CSFV genome copy number were performed in MiniTab 17 using the Spearman Rho non-parametric correlation test.

2.3.8 Detection for CSFV positive and negative IB-RS-2 cells

The limit of detection was investigated for all the 26 different field viruses and a vaccine virus (Table 2.1). A virus titration was performed for each virus in a confluent monolayer of CSFV positive IB-RS-2 cells and CSFV negative IB-RS-2 cells at 0.5×10^6 cells/ml. A dilution for all individual viruses were prepared at the same time for tests in CSFV positive IB-RS-2 cells and CSFV negative IB-RS-2 cells in eight replicates on one plate and duplicated on a different plate. Correlation of all the 26 different FMDV strains limit of detection (virus titres) tested with monolayers of the CSFV positive and negative IB-RS-2 cells were assessed with Pearson correlation (r) using MS-Excel.

2.4 Results

2.4.1 The effect of confluent and non-confluent cells on virus titres and serum neutralization titres

Flow cytometric analysis of DNA content was used to assess the stage of the cell cycle on IB-RS-2 cell suspensions obtained from confluent and non-confluent monolayers (Figure 2.3). The cell population of the confluent monolayer was shown to be predominantly at the non-dividing (G1) phase (83.90%) with 11.20% cells entering the DNA replication (S) phase and 4.85% at the division (G2) phase (Figure 2.3a). In contrast for the non-confluent cells, a higher percentage were shown to be entering the DNA replication (S) phase (41.64%) while 10.74% of the cell population was found to be at the division (G2) phase and 47.62% at the non-dividing (G1) phase (Figure 2.3b).

To determine whether the FMDV titre was related to confluency of the IB-RS-2 cells, ten different IB-RS-2 cells passage preparations were tested with FMDV field isolate (A/MAY/2/2011) and FMD vaccine virus (A/May-97). In these experiments, confluent CSFV positive IB-RS-2 cells supported FMDV replication to higher titres compared to non-confluent cells. For the vaccine virus, eight out of ten cell preparations were higher for the confluent cells, while for the field virus this was seven out of ten. The difference of FMDV titres in both field isolate and vaccine virus with confluent and non-confluent cells were statistically significant (p=0.015) with Tukey method at 95% confidence with mean values 4.43 for the confluent cells and 4.23 for the non-confluent cells. (Figure 2.4).

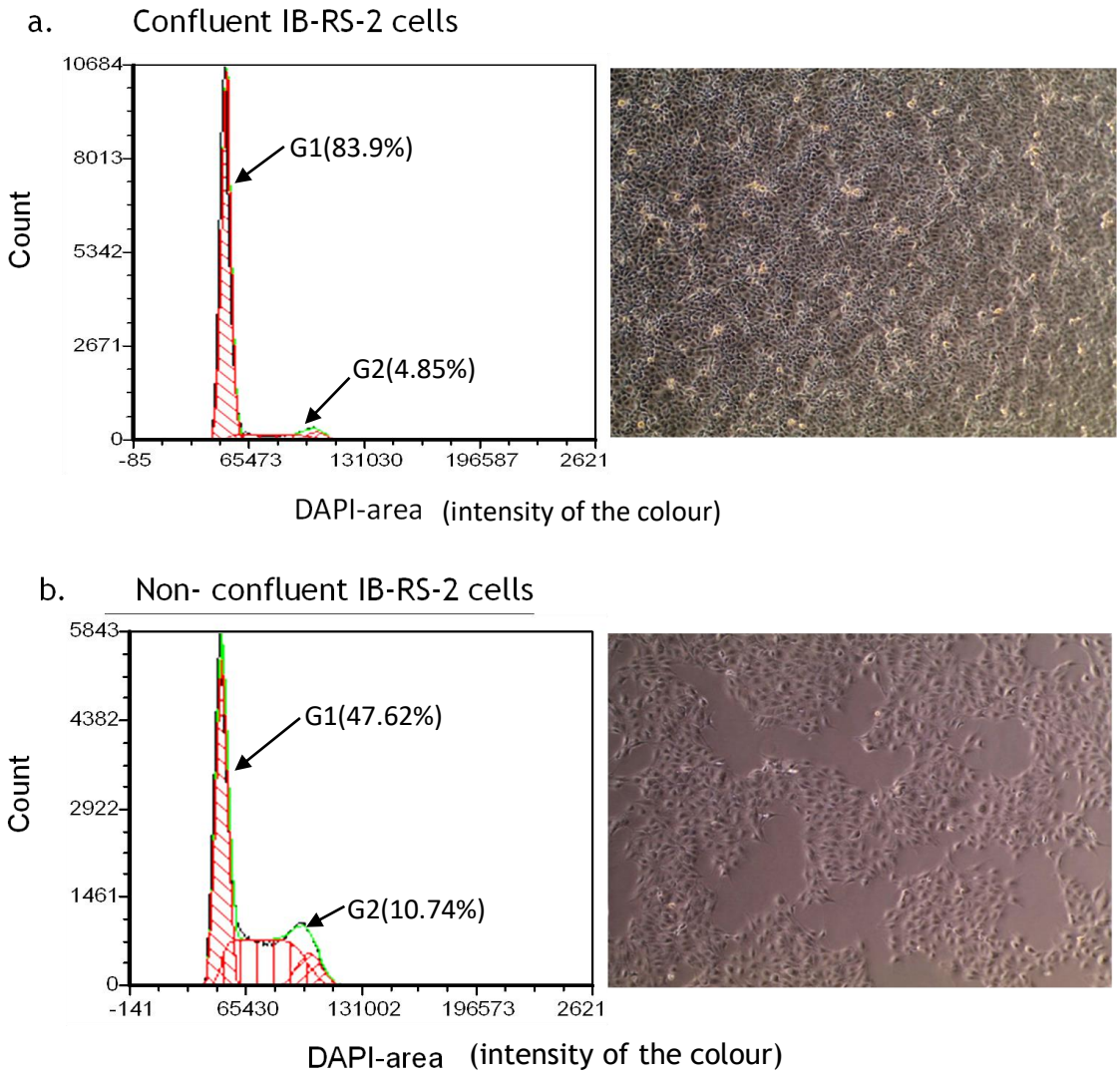


Figure 2.3: Histograms showing the DNA content of confluent and non-confluent IB-RS-2 cell populations.

Cells were trypsinsed, labelled with DAPI and then analysed by flow cytometry. (a) Analyses of cells obtained from the confluent monolayer shows the majority of cells at the non-dividing (G1) phase (83.9%). (b) Analyses of cells obtained from the non-confluent monolayer shows an increased number of cells entering the DNA replication (S) phase (41.6%). Right-hand images are microscope camera-shots showing the status of the cell cultures.

Furthermore, VNT was performed using synchronised cells, under the same experimental conditions. Confluent and non-confluent IB-RS-2 cells with (the same passage history) were used. One FMDV field isolate, A/MAY/2/2011, and six different individual cattle sera were tested. Consistently lower neutralisation titres were obtained for all of the six sera using confluent cells compared with the non-confluent cells (Figure 2.5).

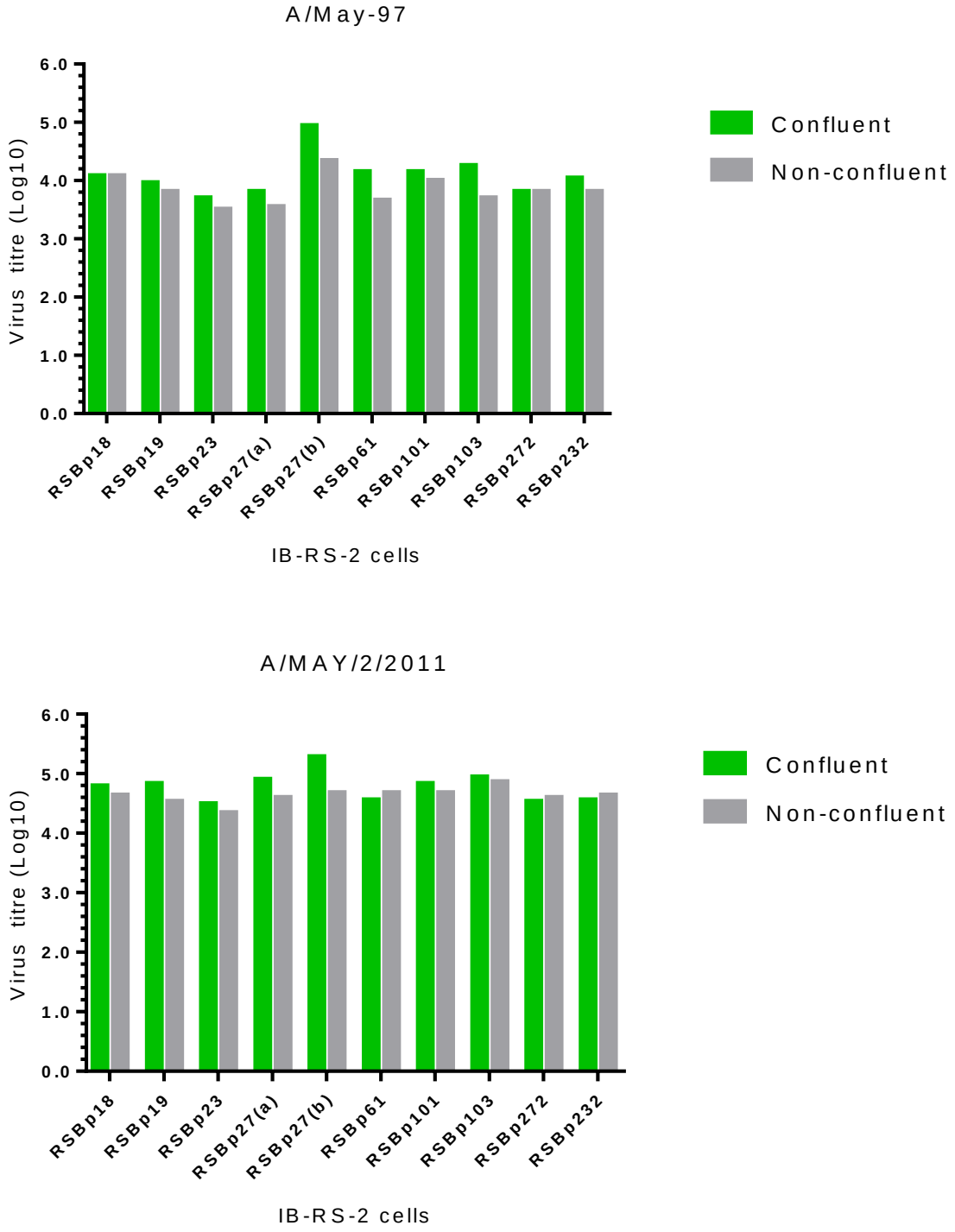


Figure 2.4: FMD virus titres of a vaccine virus (A/May-97) and a field isolate (A/MAY/2/2011) using confluent and non-confluent IB-RS-2 cells. Results for confluent cells are indicated in green and non-confluent cells in grey. The ten IB-RS-2 cell preparations used for each of the FMD viruses are shown on the x-axis (with passage (p) histories indicated. For each pair, the viruses were tested on the same day with the same IB-RS-2 cell preparation for both confluent and non-confluent cells.

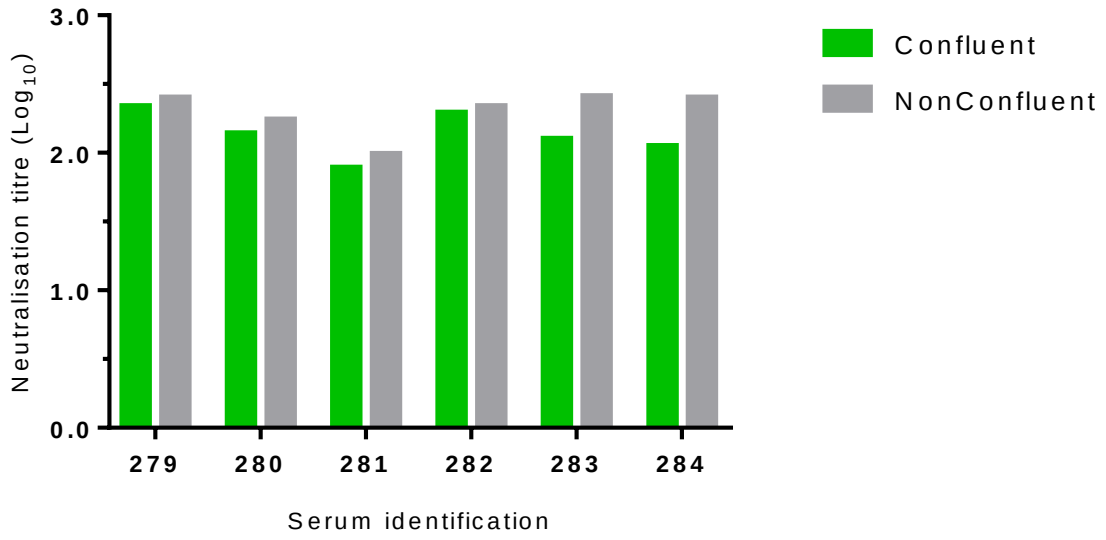
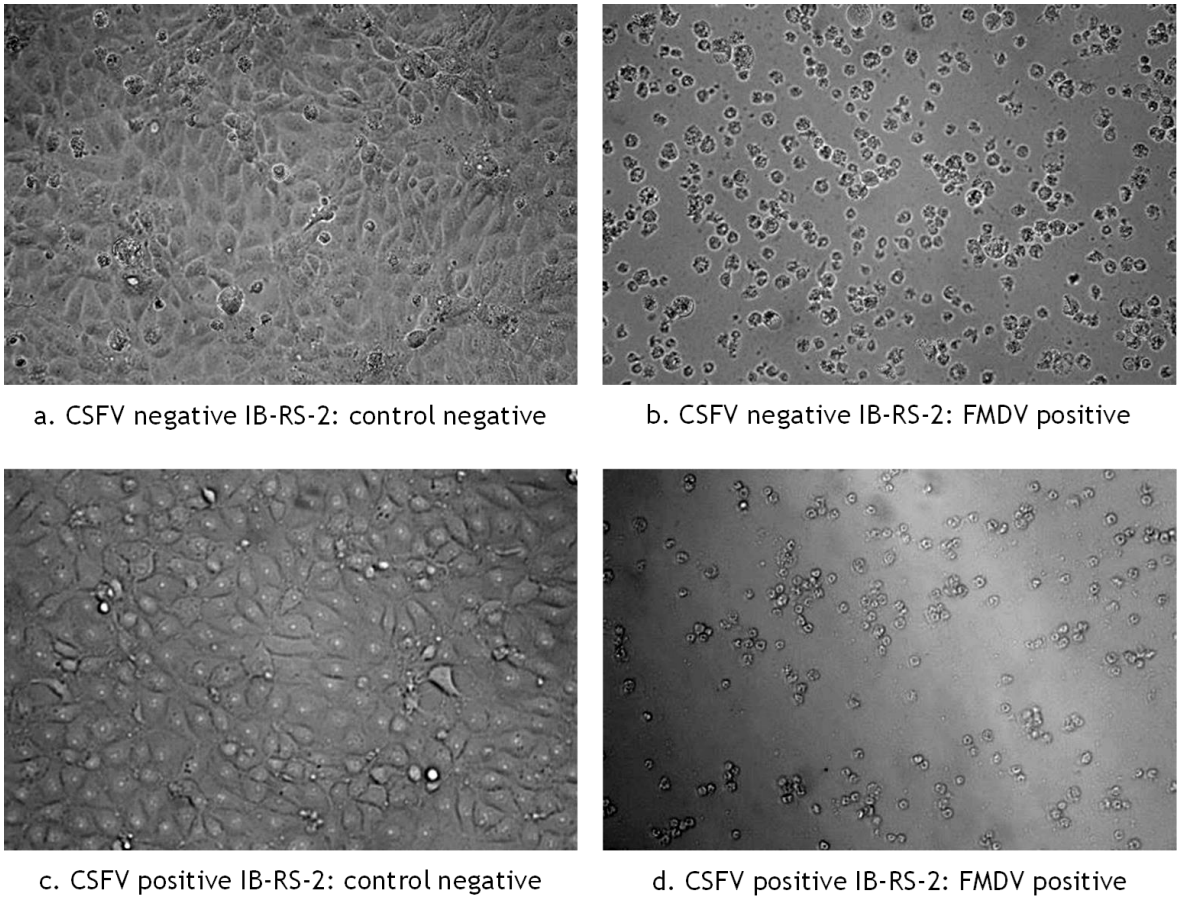


Figure 2.5: VNT of six different individual cattle sera using confluent and non-confluent IB-RS-2 cells. Results for confluent cells are shown in green and non-confluent cells are highlighted in grey. Individual animal numbers from which sera were collected at 56dpv are indicated on the x-axis. The same IB-RS-2 cell preparation were used. VNT was carried out on the field isolate (A/MAY/2/2011) on the same day with the same IB-RS-2 cell preparation for both confluent and non-confluent cells.

2.5 The effect of CSFV on FMDV titres

2.5.1 CSFV positive IB-RS-2 cells verses CSFV negative IB-RS-2 cells

Comparison between the CSFV positive and CSFV negative IB-RS-2 cells seeded at 0.5 X 10^6 cells/ml revealed similar overall morphology after 72 hours incubation (Figure 2.6). However, the confluent monolayer of the CSFV positive IB-RS-2 cells (Figure 2.6c) showed less dense healthy cells compared to the monolayer of the CSFV negative IB-RS-2 cells (Figure 2.6a). Nonetheless, both the CSFV positive and CSFV negative IB-RS-2 cells were sensitive to FMDV infection and showed clear CPE (Figure 2.6b and d), where for both the CSFV positive and negative IB-RS-2 cells, the FMDV infected cells could be clearly distinguished from the negative control.



a. CSFV negative IB-RS-2: control negative

b. CSFV negative IB-RS-2: FMDV positive

c. CSFV positive IB-RS-2: control negative

d. CSFV positive IB-RS-2: FMDV positive

Figure 2.6: Microscopic images of FMDV infection in IB-IB-RS 2 cells. Cell count of 0.5 X 106 cells/ml at magnification of 20X was used in all experiments. Panel (a) negative control: CSFV negative IB-RS-2, panel (b) FMDV infected CSFV negative IB-RS-2, panel (c) negative control: CSFV positive IB-RS-2, panel (d) FMDV infected CSFV positive IB-RS-2.

2.6 Relationship of the CSFV negative and CSFV positive IB-RS-2 cells in FMDV detection.

Twenty-five FMDV field viruses and one FMDV vaccine virus were tested to determine the limit of detection using the CSFV negative and CSFV positive IB-RS-2 cells. Pearson correlation analysis of all the 26 FMDV's limit of detection using CSFV positive and CSFV negative IB-RS-2 cells showed moderate positive relationship with the *r square* value of 0.56 (Figure 2.7).

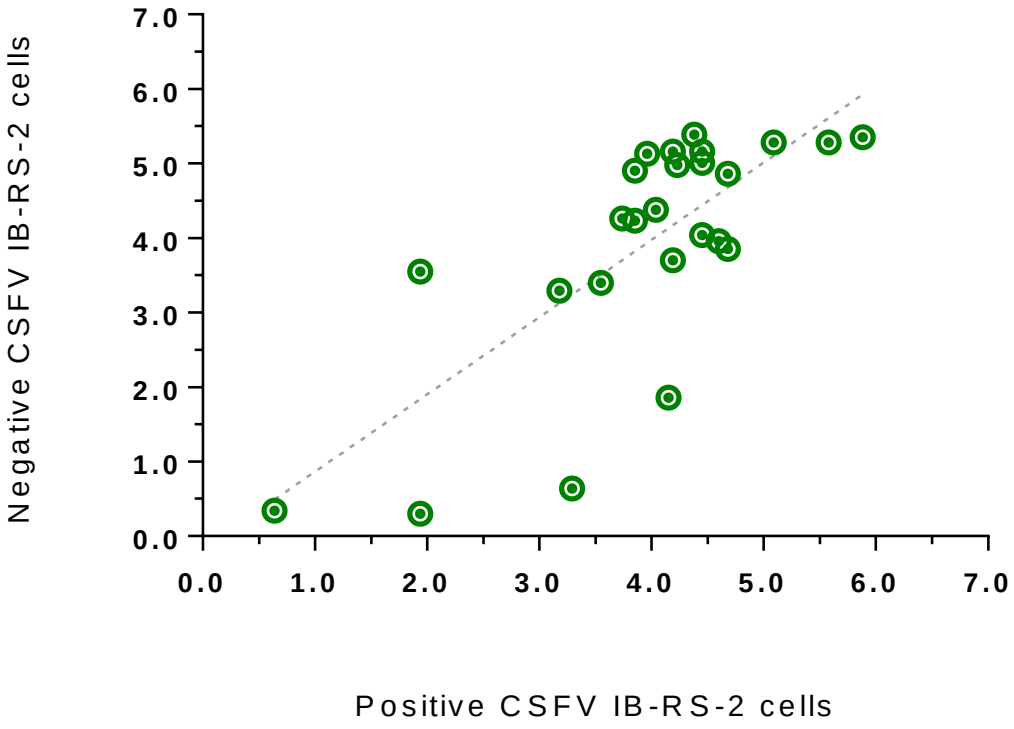


Figure 2.7: Relationship of FMD viruses limit of detection in CSFV positive and negative IB-RS-2 cells. All experiments were performed in duplicates for both the CSFV positive and negative IB-RS-2 cells. The number of infected wells with cytopathic effect (CPE) was used to calculate virus titre. On the y-axis are the FMDV titre with CSFV positive IB-RS-2 cells in log10. On the x-axis are the same FMDV titre with CSFV negative IB-RS-2 cells in log10.

2.7 CSFV genome copies contained in different confluency of CSFV positive IB-RS-2 cells

The CSFV genome copies contained in the confluent and non-confluent CSFV positive IB-RS-2 cells was determined and compared.

When using eight different passages of the CSFV positive IB-RS-2 cells at the same cell count (1 X 10^6 cells/ml), there were different number of CSFV genome copies determined in the confluent and non-confluent cultures (Figure 2.8). The actively dividing, non-confluent cells contained significantly higher CSFV genome copy numbers/cell as compared to the confluent cells which were at stationary state ($U_{\text{confluent IB-RS-2 cell}}$ = 1.803, $U_{\text{non-confluent IB-RS-2 cells}}$ = 4.379; p = 0.0054). In non-confluent cells the highest number of CSFV genome copies was determined as 16.39 copies per cell while the lowest as 2.22 copies

per cell. Whereas, in confluent cells, the highest and lowest number of CSFV genome copies was determined as 3.22 and 0.19 copies per cell, respectively.

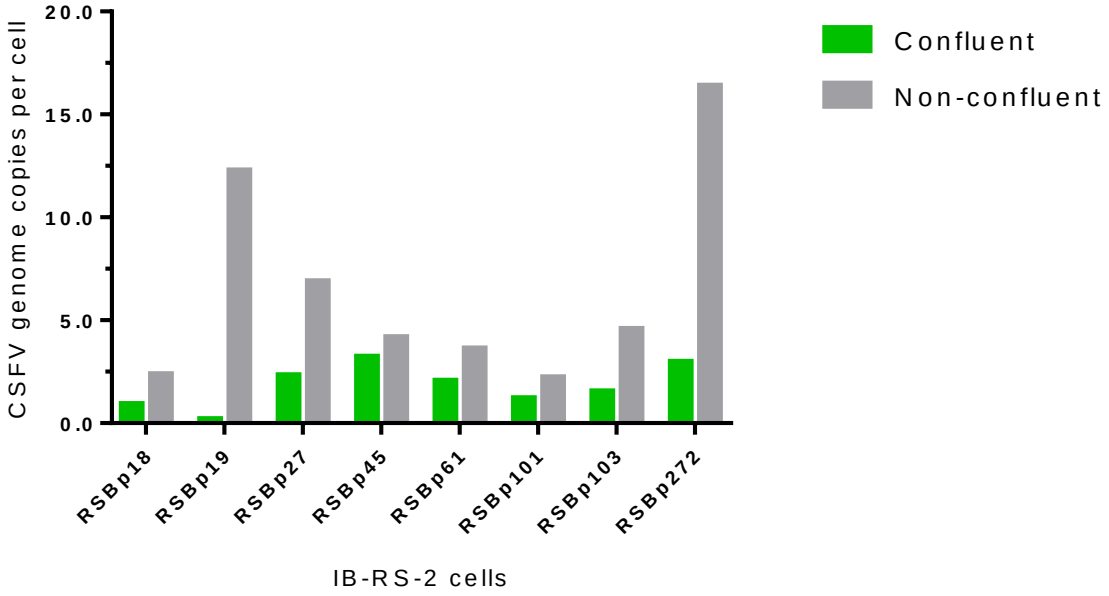


Figure 2.8: CSFV genome copy numbers per cell determined in the confluent and non-confluent IB-RS-2 cell preparations by real-time RT-PCR. Results for confluent cells are indicated in green and non-confluent cells are indicated in grey. The x-axis shows the results for eight different IB-RS-2 cell preparations with different passage histories. The y-axis shows the CSFV genome copy number per cell.

The relationship between FMDV titre and the CSFV genome copies was determined for the A/May-97 vaccine virus, as well as for the A/MAY/2/2011 field isolate in eight different confluent and non-confluent CSFV positive IB-RS-2 cell preparations at the cell count of 1 x 10^6 cells per millilitre. As shown in Figure 2.9, no correlation was observed between the amount of CSFV genome copies in all the confluent and non-confluent eight CSFV positive IB-RS-2 cells and FMDV titre for both vaccine and field isolate (R_2 = -0.171; P = 0.320). This indicates that there is no significant influence of the amount of CSFV genome copies on the growth of field and vaccine FMD viruses.

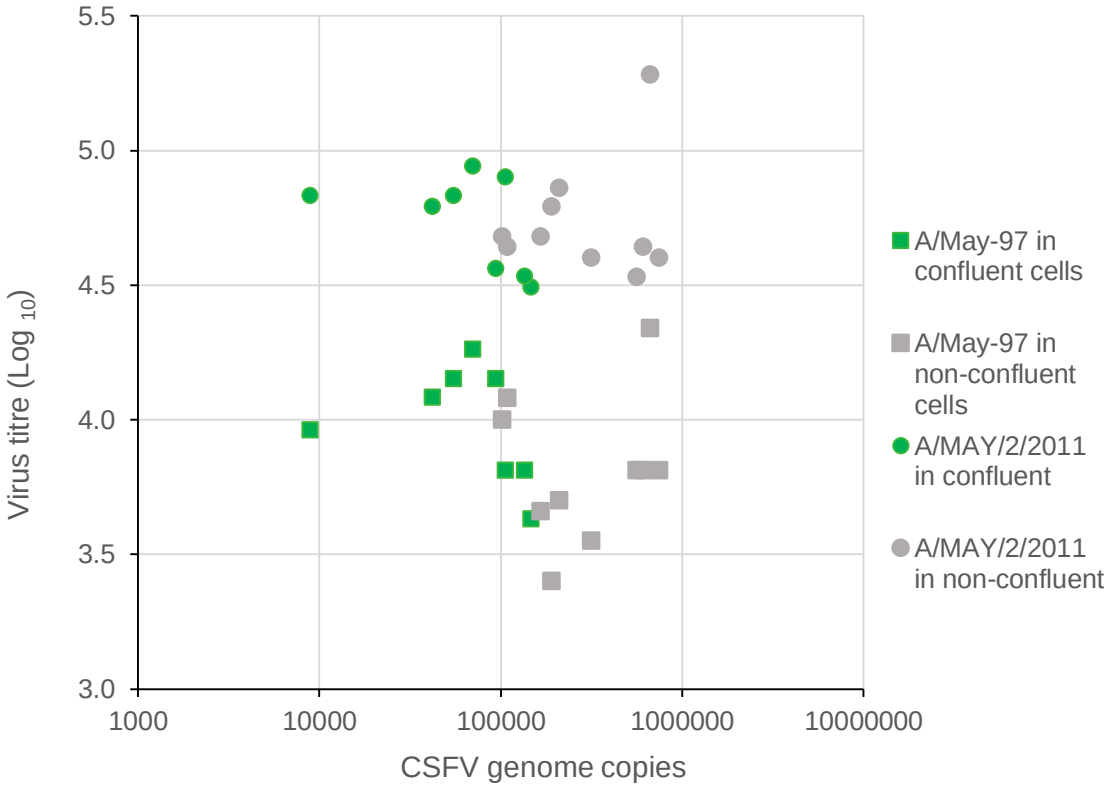


Figure 2.9: Relationship between FMDV titre and the quantity of CSFV genome copies present in cells. The relationship between the A/May-97 vaccine virus and the A/MAY/2/2011 field isolate with the number of CSFV genome copies was plotted. Results for confluent cells are shown in green while non-confluent cells are shown in grey. The data for the FMDV field isolate is marked by the circular (○) symbol while the FMD vaccine virus is represented by the rectangular (□) symbol. The x-axis shows the CSFV genome copy number detected in a real-time RT-PCR reaction. The y-axis shows the log10 FMDV titres of field isolate and vaccine virus using confluent and non-confluent cells. The viruses were tested on the same day with the same IB-RS-2 cell preparation for both confluent and non-confluent cells.

2.8 Discussion

Virus neutralisation test (VNT) is an important laboratory method used to measure FMD serological responses. The OIE terrestrial animal manual (OIE, 2017) defines 4 main purposes for this test: (1) to certify animals prior to import or export, (2) to confirm suspect cases of FMD, (3) to substantiate freedom from infection and (4) to assess post-vaccination performance including the use of vaccines in endemic countries.

The method is also recommended for use in FMD vaccine matching tests in order to select the most suitable FMD virus for the production of a new FMD vaccine. This chapter studied the impact of IB-RS-2 cells at different stages of the cell cycle on the FMDV virus titres and virus neutralization titres that are measured. Since the routinely used cultures contain CSFV, further experiments explored the possibility to use the CSFV negative IB-RS-2 cells as alternative cell culture systems in FMD virus titration.

Many FMD Reference Laboratories use IB-RS-2 cells for routine VNT work to measure antibody responses. However, there is no existing knowledge on about how IB-RS-2 cell growth affects the neutralisation titres that are generated with this system, while it has been reported that IB-RS-2 cells used in different FMD Reference laboratories have different sensitivity to propagate FMD virus from clinical samples (Ferris et al., 2006). Therefore, here the effect of different extremes in the cell cycle (indicated by the use of confluent and non-confluent monolayers) of IB-RS-2 cells in FMD virus titre and VNT was studied. In FMD virus propagation studies, the viral titres were consistently lower in the non-confluent IB-RS-2 cells compared to FMD virus titres generated from the confluent IB-RS-2 cells. Consistently lower serum neutralisation titres were also obtained with the confluent cells compared to the results in VNT with the same sera and FMD viruses with non-confluent cells. This information highlights the variability that might be introduced into *in-vitro* serological methods when cells are not standardized prior to use in VNTs. This factor needs to be carefully considered by FMD diagnostic laboratories and the results from this study suggest that the same confluency of cells should be used in FMD virus titrations and VNT methods to reduce the inherent variability of the test.

Despite the widespread use of the IB-RS-2 cell line, earlier extensive chromosome analysis by karyotype homogeneity of IB-RS-2 cell clones reported the manifestation of certain chromosome pattern ('spontaneous' degeneration) that at the time were thought to underlie the increased the cell sensitivity to FMD virus (De Castro, 1970). Subsequent studies undertaken at TPI (and elsewhere) have shown that these cells are persistently infected with CSFV. Earlier findings showed the presence of CSFV in IB-RS-2 cells by direct fluorescent antibody staining (House et al., 1988). The open reading frame of the CSFV

genome contains a protein known as N^{pro} which plays a role in limiting the IFN-α induction (Ruggli et al., 2003), and it has even been suggested that the presence of CSFV in these cells may contribute to the high sensitivity to FMD virus due to innate antivirus effects. This present study confirmed the earlier finding and quantified the genome copy numbers present in the cell by quantitative real time RT-PCR.

The quantitation of CSFV copy numbers of the CSFV present in the IB-RS-2 cultures in this study revealed that the IB-RS-2 cultures maintained the CSFV even though the number of copies/cells appears to be relatively low (on average, close to 1 or 10 virus copies/cell). Further tests demonstrated that the actively dividing, non-confluent IB-RS-2 cells contained significantly higher copy numbers of CSFV genome compared to the confluent cells, which were at stationary stage. In addition, this study also shows that there was no relationship between the CSFV genome copy number and the FMD virus titre.

The potential use of the CSFV negative IB-RS-2 cells for FMD virus serological testing was also explored. In this study when the sensitivity to FMDV infection was measured using CSFV positive IB-RS-2 and CSFV negative IB-RS-2 cells, no significant difference was observed. This supports an earlier study by House who claimed that the CSFV free clone of IB-RS-2 cells (IB-RS-2D10), showed similar sensitivity to FMD virus as the parent CSFV positive IB-RS-2 cells (House et al., 1988). Furthermore, this present study also shows no physical difference between CSFV positive and CSFV negative IB-RS-2 cells, which indicates the suitability of CSFV negative IB-RS-2 cells for FMD virus titration and VNT. The current VNT standard method requires 50 µl of CSFV positive IB-RS-2 cells 1 X 10^6 cell count per ml. This study revealed that these tests performed with the CSFV negative IB-RS-2 cells require only 50 µl of 0.5 X 10^6 cells count per ml. The reduction in the number of cells is likely because the CSFV negative IB-RS-2 cells achieve higher growth rate than the CSFV positive IB-RS-2 cells, however, this hypothesis needs to be further explored. The 50% reduction of cell count per ml needed in the *in-vitro* methods leads to cost, time and resources reduction. Another significant advantage of the CSFV negative IB-RS-2 cells is the ability of handling outside high containment facility. This subsequently widens the application of the usage of these cells such as potential use for FMD virus amplification during vaccine production where the use of “clean” cells without adventitious viruses is essential. The results in this study emphasize the importance of IB-RS-2 cell stage (confluent cell) to be utilised in serological methods of FMD. Furthermore, the study indicated that CSFV present in the IB-RS-2 cultures does not have any significant impact upon their sensitivity to FMD virus, findings that are supported by the use of the CSFV negative IB-RS-2 cells for the propagation of FMD virus.

Chapter 3:

In-vitro vaccine-matching for foot-and-mouth disease virus: does bovine vaccinal sera (BVS) impact upon the reliability of serological immune responses?

Acknowledgements:

Jamaliah Senawi generated all laboratory data presented in this chapter. Animal work was managed by Dr Emma Fishbourne and undertaken by animal staff at TPI. Jamaliah Senawi acknowledges assistance from Dr Simon Gubbins for the analysis of the neutralization and total antibody titre data to assess the variability of the in-vitro vaccine matching results.

3.1 Abstract

Systematic vaccination is a proven tool to control foot-and-mouth disease (FMD) particularly in endemic areas. Effective vaccination programmes are usually reliant upon selecting a vaccine that is antigenically matched to the circulating FMDV field strains. *In-vitro* vaccine matching tests provide important evidence for the selection of these vaccines; however, the virus neutralisation test (VNT) which is considered to be the gold standard method lacks in reproducibility which sometimes causes uncertainty in the vaccine virus selection process. This uncertainty becomes apparent when repeated testing for the same virus strains and the same vaccine sera generates relationship coefficient (r1 values) that are above and below the suggested antigenic-match cut-off (0.3). Therefore, the aim of this study was to investigate the impact of using different bovine vaccinal sera (BVS) on the test results. In this study, BVS was produced in six cattle vaccinated and boosted with a high potency (at least $6PD_{50}$) A/May-97 monovalent vaccine. Serological testing used the vaccine virus A/May-97 (homologous) and a representative Malaysian field isolate (A/MAY/2/2011; heterologous) from a viral lineage with a history of low r1 values. VNT and liquid phase blocking ELISA (LPBE) were performed on individual and pooled BVS at different time points to determine antibody titres which were then used to calculate the relationship coefficient (r1 values). Generally, sera from all six animals showed a similar pattern of increased antibody titres measured by VNT and LPBE after vaccination against both homologous and heterologous viruses. For the LPBE, antibody titres for individual sera were repeatable when measured within the same day and between different days. For this assay, only animal-to-animal variability was significant, with this contributing 49.5% and 57.2% of the variance in titres for sera collected at 21 dpv and 56 dpv, respectively. However, the data for the VNT were more variable, and the day-to-day variability contributed 47.4% of the variance for sera at 21dpv whereas, sera at 56dpv contributed 17.7% of the variance of the day-to-day variability. There was also significant animal-to-animal variability for sera tested by VNT. Pooling sera from different animals helped reduce the between-day variability of VNT. The inherent variability of these two assays was reflected in the r1 values that were measured, where the LPBE showed consistent values for individual and pooled sera. In contrast, r1 values determined using VNT were more variable above and below the suggested antigenic-match cut-off. This study revealed the inherent variability of the *in-vitro* vaccine matching methods, the effect of sera collected at different sampling times, booster vaccination and pooling on serological immune response and the r1 values calculated. These findings highlight the importance of using standardised BVS to reduce some of this variation.

3.2 Introduction

Foot-and-mouth disease virus (FMDV) serotype A is often considered to be the most antigenically variable among the seven FMDV serotypes (Knowles and Samuel, 2003). This high antigenic variability contributes to the complexity of FMD control, since antibodies induced by vaccination with one serotype A vaccine virus may have little or no cross-protection against unrelated serotype A field isolates (Kitching et al., 1988; Knowles and Samuel, 2003). Therefore, in order for an FMD vaccine to be effective, the vaccine strain selected must be antigenically matched with the current field virus (Paton et al., 2005).

Vaccine performance is ideally assessed using *in-vivo* methods in susceptible animal species that are vaccinated and challenged according to the European Pharmacopeia and OIE guidelines, but this approach is expensive, time consuming and also raises animal welfare concerns. Other approaches such as modelling techniques utilising FMDV capsid genetic sequence data along with antigenic cartography cannot yet accurately predict the suitability of a vaccine virus for a specific field isolate (Ludi et al., 2014). However, other studies have shown that phylogenetic distance between serotypes correlates reasonably well with antigenic distance measured by cross-reactivity to polyclonal antisera (Mateu, 1995). These insights have led to the development of *in silico* mathematical modelling tools that combine structural information to the amino acid sequence data, but these are not yet used for routine vaccine-matching (Reeve et al., 2010).

Virus neutralisation test (VNT) and the liquid phase blocking ELISA (LPBE) are *in*-vitro tests used to measure FMDV-specific antibodies that work in different ways. VNT utilises susceptible cell cultures to measure the ability of a serum to neutralise a virus dose of a $100TCID_{50}$, where cytopathic effect (CPE) is used as the indicator for neutralisation. On the other hand, for the LPBE, rabbit polyclonal antibody raised to strain specific FMDV is used as trapping antibody to bind a fixed dose of virus. A detector antibody (guinea pig polyclonal antibody raised to strain specific FMDV) is unable to bind if an FMDV antigen complexes with the test bovine antisera. The inhibition due to the formation of antigen-bovine antibodies complexes can be measured to calculate a percent of inhibition (PI) value for the test. In summary, VNT only measures neutralising antibodies, whereas LPBE measures all antibodies (neutralising and non-neutralising) that bind to external epitopes on the virus capsid. It is thought that neutralising antibodies are more closely related to protection (McCullough et al., 1992; Reading and Dimmock, 2007), and are a better predictor of protection (Mattion et al., 2009; Robiolo et al., 2010).

For vaccine-matching using both the VNT and LPBE, the serological relationship is expressed as a relationship coefficient (r1 value) of the vaccine virus and field isolate.

The r1 value indicating antigenic cross-reactivity between the two FMD viruses can be calculated by looking at the ratio of the heterologous and homologous virus neutralisation titres (Ferris and Donaldson, 1992; Rweyemamu, 1984). It is assumed that the closer the r1 value is to 1 the more antigenically similar the two FMD viruses are.

There are reports indicated that r1 values suffer from high variability and do not always reflect the performance of a vaccine in host species. For example, high-potency vaccines were able to induce protection even though low r1 values were observed (Brehm et al., 2008). Conversely, other studies concluded that only partial protection of the animals occurred despite high r1 value (European Commission, 2013). It has also been noted that r1 values generated from VNT are not reproducible (Tekleghiorghis et al., 2014). Variation between batches of sera can also lead to inconsistent results (Kitching et al., 1988).

In this study, a pair of serotype A (lineage A/ASIA/Sea-97) FMD viruses were used; A/May-97 vaccine virus (homologous) and A/MAY/2/2011 field isolate (heterologous). Focus was given on these viruses because retrospective vaccine matching data (2006-2017) from mainland Southeast Asia indicated that only 38% of A/ASIA/Sea-97 field isolates were antigenically matched to the Sea-97 vaccine virus A/May-97 (as described in Chapter 1 section 1.13.2 of this study). However, in contrast to these poor r1 values from the laboratory tests, field observations on a dairy cattle farm in Malaysia showed no clinical FMD after vaccination with A/May-97, with antibody responses in individual animals that were indicative of protection (as described in Chapter 5 of this study); observations that indicate that the vaccine is effective. Therefore, the objectives of the work in this chapter were (i) to identify the inherent variability of the *in-vitro* vaccine matching methods, (ii) to investigate the effect of different sampling time on the serological immune response and r1 values, (iii) to determine the effect of booster vaccination on r1 values and (iv) to determine the effect of pooling sera on r1 value.

3.3 Materials and methods

3.3.1 Selection and propagation of viruses (in addition, see Appendix i Phylogenetic tree for lineage A/ASIA/Sea-97 viruses)

The FMDV serotype A vaccine strain (A/May-97) was provided by Boehringer Ingelheim. A representative serotype field isolate from Malaysia; A/MAY/2/2011, from the A/ASIA/Sea-97 lineage with a history of poor vaccine matching (r1 value), was selected as the heterologous virus from the WRLFMD archive. This virus was collected in Kg Sawah, Port Dickson, Negeri Sembilan, Malaysia from Cattle on 3rd May 2011 and has been previously characterised by phylogenetic analyses based on the VP1 coding sequence (Knowles NJ, 2016). In preparation for VNT, both viruses were passaged twice in the IB-RS-2 cell line (De Castro, 1964) to achieve virus titres of more than 3.0 $\log_{10}$. HEPES Modified Eagles Medium supplemented with 0.2% field antibiotic; mixture of penicillin (10MU), neomycin (25,000µg/ml), polymyxin B (100,000U/ml) and amphotericin B (field antibiotic) from central services unit (CSU), TPI and 0.4% sodium hydroxide solution (NaOH) from Sigma-Aldrich was used for passaging cells. Viruses were propagated in 175mls flasks (Greiner Bio-One, Greiner Bio-One International GmbH) on a 100% confluent monolayer of IB-RS-2 cells. For long term storage, glycerol was added to the viruses at ratio of 1:1 after the viruses were harvested. The viruses were titrated, aliquoted and stored at -80°C until used for the VNT or LPBE that is compared with cut-off values of Barnett et al (Barnett et al, 2003).

3.3.2 Production of bovine vaccinal sera (BVS)

Six Holstein-Friesian calves aged 6 months were kept in a disease-free isolation experimental unit (Greenfields) at The Pirbright Institute, Compton Campus, UK. The calves were acclimatised for one week prior to the start of the experiment. At day 0, two millilitres (ml) of inactivated monovalent FMDV A/MAY-97 vaccine, with a minimum protective dose of $6PD_{50}$, was administered intramuscularly (kindly provided by MERIAL/Boehringer Ingelheim). Booster vaccination was carried out using the same dose and route of the vaccine at 21 days post vaccination (dpv). Fifty ml blood samples were collected at 0, 7, 14, 21, 28, 35, 42, 49 and 56 dpv from coccygeal vein of each calf into five plain (red top) 10ml vacutainers. These samples included collections on the day of initial vaccination and booster (days 0 and 21). After collection the tubes were brought

in a cool box to The Pirbright Institute, Pirbright, UK, and placed in a refrigerator at 4°C overnight. The tubes were then centrifuged at 2000g for 5 minutes using a bench centrifuge (model: Hettich Rotanta 460R) after which the sera were decanted. The sera were placed in a water bath at 56°C for 30 minutes to inactivate any viruses and complement factor that might have been present (according to Pirbright Institute biosecurity regulations) before aliquoted in 4 ml cryo-vials and stored at -20°C until use.

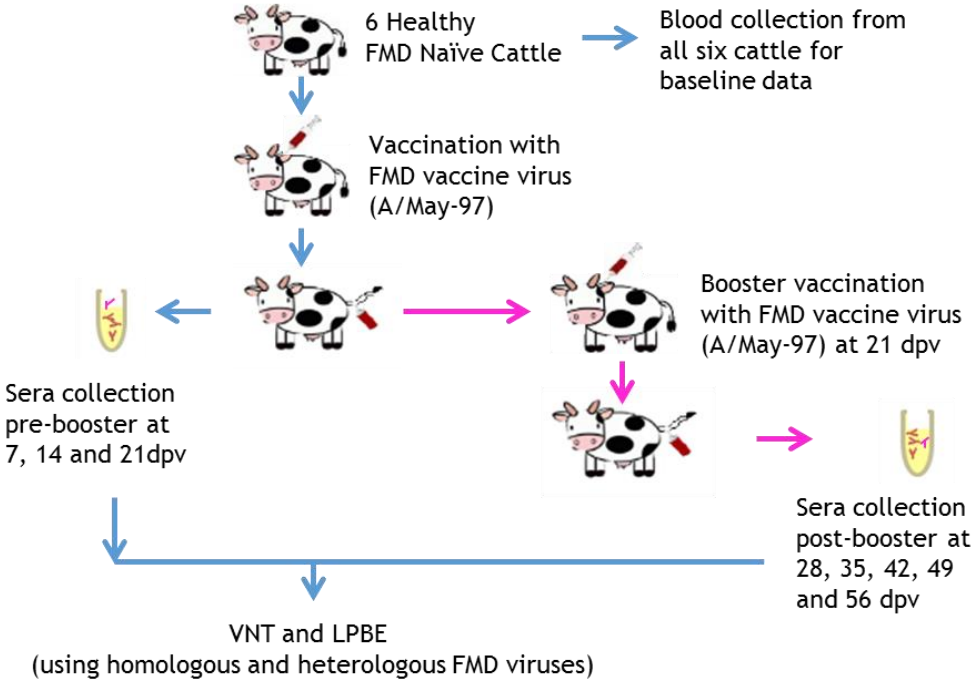


Figure 3.1: An outline of the animal experiment designed to evaluate the effect of sampling interval and booster vaccination on vaccine-matching r1 values. Bovine vaccinal sera (BVS) was collected from six calves at regular intervals (0, 7, 14, 21, 28, 35, 42, 49 and 56dpv) pre and post-vaccination, to evaluate the effect of sampling interval and booster vaccination on r1 values.

3.3.3 Production of pooled post-vaccination bovine sera

Inactivated sera were tested individually and as pools. Three types of pooled sera were prepared by mixing an equal amount of individual animal sera (500µl) based on the initial neutralisation titre results: (i) two pools of sera from two animals; comprising either two individual animal sera with similar neutralisation titres or two individual animal sera with different neutralisation titres, (ii) three pools containing five individual animal sera: with

highest neutralisation titres, lowest neutralization titres and a mixture of low and high neutralisation titres and (ii) pools of all six individual animal sera collected at the same time-point after vaccination (Figure 3.2).

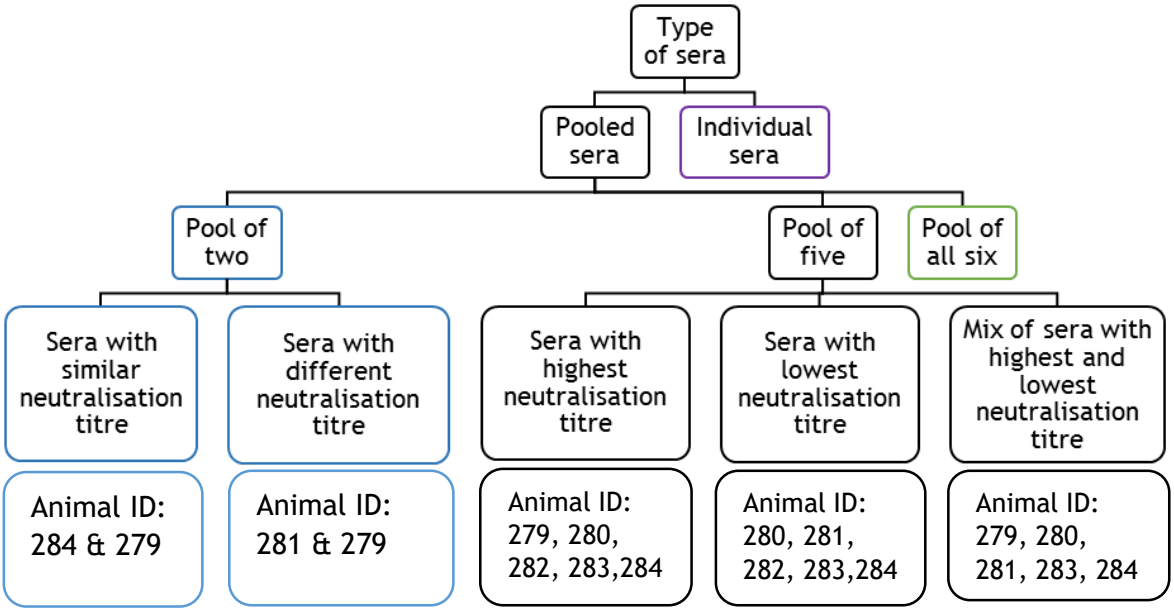


Figure 3.2: Summary of different types of sera (individual and pools) used for the study. The approaches to prepare individual and pooled sera are highlighted.

3.3.4 Virus titration

Dilutions of the stock virus were performed in bijou bottles containing HEPES Modified Eagles Medium supplemented with 0.2% field antibiotic and 0.4% NaOH. Four-fold virus titrations were carried out (in duplicate) in flat-bottom 96 well micro-titration plates (Fisher Scientific). A total of 50µl of an IB-RS-2 cell suspension at a cell count of 1 X 10^6 cells/ml was dispensed into all wells. The plates were incubated at 37°C for three days before being examined by microscope for cytopathic effect (CPE) indicative of FMD virus replication. The $TCID_{50}$ endpoint titre was calculated following the Spearman-Kärber method 1931 (Kärber, 1931; Spearman, 1908). The virus titre information was then used to determine the correct virus dilutions to generate $100TCID_{50}$ used for the VNT (described below). This study used a method similar to the WRLFMD protocol where titrations carried out must be within 0.3 $\log_{10}$ (two-fold) of the running mean for that virus. A virus titration plate was also run every time a neutralization test was carried out.

3.3.5 Virus neutralisation test (VNT)

All VNTs were performed in 96 well plates (Fisher Scientific). Both the vaccine and field viruses were tested simultaneously under the same conditions following the principles outlined in Chapter 2.1.8 Section D for vaccine matching test (OIE, 2017) and according to the WRLFMD protocol (SAU-SOP-4 and SAU-SOP-8). An additional serum control using FMDV A_{22} Iraq with the same BVS was included in every new experiment to monitor the serum control running mean of the experimental system. In short, HEPES Modified Eagles Medium (HMEM) supplemented with 0.2% field antibiotic (penicillin-streptomycin) and 0.4% NaOH was used as media to dilute the sera and viruses. Viruses were pre-diluted in bijou bottles and three virus doses were set-up so that a four-fold dilution fell on either side of the estimated $100TCID_{50}$ (as described above). A total of 50µl of media were dispensed into each well of the plates except wells of top row (A). All sera were tested in duplicate wells. A total of 50µl of the tested sera were placed in the first two rows (row A and row B). Sera were serially diluted in two-fold down the plate starting from row B. A total of 50µl of the three virus doses were dispensed to designated wells containing the sera, after which the plates were then incubated at room temperature. After approximately 45 minutes, 50µl of IB-RS-2 cell suspension containing $1x10^6$ cells/ml were dispensed to all wells. A sticky plastic seal was used to cover each plate, and the plates were incubated at 37ºC for 72 hours before examined for neutralization (no CPE) under a microscope. Serum neutralisation titres at 50% for each virus doses were calculated using the Spearman-Kärber method (Kärber, 1931; Spearman, 1908). Finally, the $TCID_{50}$ end point serum neutralization titre at a virus dose of $100TCID_{50}$ ($2log_{10}$) was estimated using linear regression line of the three virus doses. The suggested protective cut-off used was at $log_{10}1.4$ (Barnett, 2003).

3.3.6 Liquid phase blocking ELISA (LPBE)

Field FMDV isolate (A/MAY/2/2011) and vaccine virus (A MAY-97) were propagated in 800ml roller bottles on an IB-RS-2 cell monolayer. The viruses were inactivated with 0.1M bromoethlyeneimine (BEI) twice followed by innocuity testing to ensure complete inactivation. For the innocuity test, a total of 5 bung tubes were prepared with confluent monolayer of primary bovine thyroid (BTy) cells for each virus. Four bung tubes were inoculated with 0.2 ml of the BEI inactivated virus and one with PBS as control. The tubes were then examined for CPE daily for 3 days. The absence of CPE of all the 5 bung tubes

indicated that the virus was completely inactivated. Each virus was harvested from the four bung tubes (excluding the control bung tube) and were pooled and used in an antigen detection sandwich ELISA. An antigen detection sandwich ELISA was performed in accordance to the method described by Ferris et al. (Ferris and Dawson, 1988) to confirm the integrity and determine the titre of the FMDV antigens. The FMDV antigens then aliquoted and kept at -80°C until used. The LPBE was performed as described by Hamblin et al (C. Hamblin et al., 1986) according to the principle of LPBE illustrated in Figure 3.3.

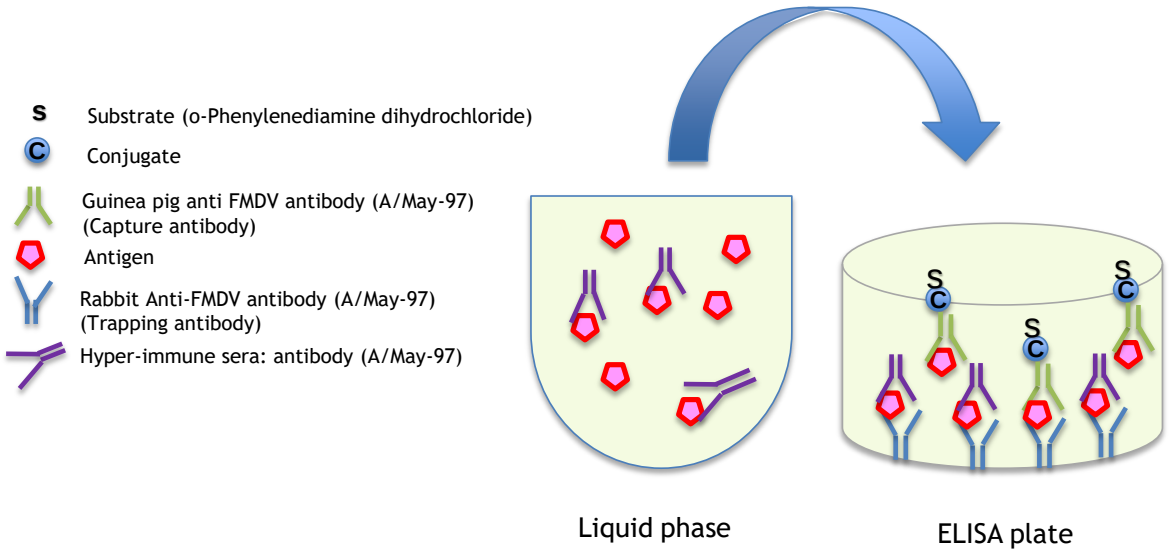


Figure 3.3: The principle of LPBE. In this assay a liquid phase was performed in a carrier plate and incubated overnight where the antigen antibody complex ( ) will form (between the hyper immune (test) sera and the FMDV antigen). The liquid phase was then transferred to the ELISA plate. The well of the ELISA contained trapping antibody (rabbit anti FMDV antibody for A/May-97) that trapped the antigen antibody complexes ( ) and any free FMDV present in the liquid phase. Subsequently, a guinea pig anti-FMDV antibody (capture antibody) is used to detect free FMDV antigen ( ). The capture antibody is then bound to the conjugate (Horseradish peroxidase conjugated rabbit anti-guinea pig immunoglobulin (DAKO- P0141)) and the chromogen substrate solution (O-phenylenediamine dihydrochloride (OPD)) that generates the colour in the test. Thus, more free antigens in the liquid-phase will give more colour, and hence a higher optical density (OD) will be read, which is inversely proportional to the FMDV-specific antibody titre.

Briefly, for both the field isolate and the vaccine virus, flat bottom ELISA plates were coated with a pre-determined dilution of 100ul of rabbit anti FMDV A/May-97 sera [provided by the Serum Assay Unit, The Pirbright Institute (SAU, TPI)] as trapping antibody.

The coated ELISA plates were incubated overnight at room temperature. The liquid phase consisted of serial diluting the test sera using two-fold dilutions in duplicate wells with the respective FMD viruses. The volumes used were 50µl of sera and 50µl of pre-diluted FMDV virus suspension in U bottom 96 well plates (carrier plate). The plates were incubated in 1°C - 8°C overnight on an orbital shaker. The coated ELISA plates were washed with phosphate-buffered saline containing 0.05% Tween 80, 0.5M NaCl (PBST) three times and dried manually by tapping the plate on non-linen cloth (blue roll). Then, 50µl of the liquid phase were transferred from the carrier plates onto the ELISA plates containing the trapping antibody. The ELISA plates were then incubated at 37°C for an hour before being washed again three times with PBST and dried manually. Fifty microliters of predetermined dilution guinea pig anti-serum (capture antibody) was added to each well of the ELISA plate and the plate was again incubated at 37°C. After one hour, the plates were washed three times and dried, after which 50ul of pre-diluted horseradish peroxidase conjugated rabbit anti-guinea pig immunoglobulin (DAKO – PO 141) in an equal volume of sterile glycerol was added to all the wells. Plates were incubated at 37°C for one hour before being washed four times and dried. A total of 50µl of substrate chromogen solution, o-phenylenediamine dihydrochloride (OPD) (Sigma, P-8412) mixed with 30% hydrogen peroxidase was added to all the wells and left at room temperature for 15 minutes for colour to develop. A total of 50µl of 1.25M sulphuric acid was added to stop the reaction. Finally, the plates were read with an ELISA reader (V-Max model) at 490 nm.

The percentage of inhibition was calculated using the following formula:

$$\text{Percentage of Inhibition (PI\%)} = 100\% - \frac{\text{(Optical density (OD) of test serum well)} \times 100\%}{\text{(Median OD of antigen control well)}}$$

The antibody titres were expressed as the reciprocal of the final dilution of serum with 50% percentage of inhibition sera were tested three times per day and at two different days and that with these results within and between day was tested.

3.3.7 Determination of r1 value

The r1 value measures the antigenic cross-reactivity (matching) between a vaccine virus (homologous) and a field isolate (heterologous). The r1 value is calculated by dividing the neutralisation/total antibody titre against the heterologous with the neutralisation/total antibody titre of the homologous virus for VNT and LPBE. The closer the r1 value is to 1

the more antigenically similar the field isolate is to the vaccine virus. The OIE recommends the minimum suggested antigenic-match cut-off at 0.3 for VNT. Whereas, for the LPBE two level of minimum suggested antigenic-match cut-off; r1 = 0.2 – 1.0 and r1 = 0.4 – 1.0. With the higher level of r1 values (r1 = 0.4 – 1.0) suggested close relationship between field isolate and vaccine strain, where a potent vaccine containing the vaccine virus is likely to confer protection. An r1 value between 0.2-0.39 suggests that the field isolate is antigenically related to the vaccine virus. Where the vaccine virus might be suitable for use if no closer match can be found, provided that a potent vaccine is used and booster vaccination applied (OIE, 2017). The r1 value is calculated using the formula below.

$$\frac{\text{reciprocal arithmetic antibody titre of BVS against field virus}}{\text{reciprocal arithmetic antibody titre of BVS against vaccine virus}}$$

Throughout this study, the r1 values were calculated independently and recorded for each test performed.

3.3.8 Statistical analysis

Independent-t test was used to assess whether VNT performed by two different operators produce same or different results. Paired-t tests using Minitab (version 17) were carried out to determine whether the antibody titres after primary vaccination were different from the antibody titres after booster vaccination.

In order to assess the within day and between days variability of the two *in-vitro* vaccine matching methods, linear mixed models with backward selection were constructed for the individual VNT and LPBE data at 21dpv and 56dpv. This is to determine the sources of the variation that may influence the test. Specifically, three models were considered for each analysis. These all included whether the sera were tested with homologous or heterologous virus (as a fixed effect), but differed in the sources of variation which may influence the test (as nested random effects). The first includes all the three parameters that may influence the test; different animals, test carried out at different days, and test repeats within a day. In the second, the three parameters were reduced to two: different animals and test carried out at different days. Finally, the third model only includes one parameter; different animals. The models were compared by considering the change in residual deviance to determine which of these sources of variation is significant ($P<0.05$). The statistical analysis of the VNT and LPBE data was carried out by Dr Simon Gubbins

(The Pirbright Institute) using the lme4 package (Bates, 2015) in R version 3.4.3 (RCoreTeam, 2017)

For the pooled sera general linear models were performed on VNT and LPBE data at 56dpv to assess within-day and between-days variability using Minitab (version 17). For each VNT and LPBE analysis, six different pooled sera as described in section 3.3.3 were included. The different pooled sera and virus (homologous or heterologous) factors as fixed effect whereas the test carried out on different days and repeats on the same days as random effects. The first model includes all the four factors that may influence the test; test carried out at different days, all the six different pooled sera, both homologous and heterologous virus and test repeated on the same day. In the second model, the four factors were reduced to three factors which includes test carried out at different days, all the six different pooled sera, both homologous and heterologous virus. Finally, the third model includes only two factors namely all the six different pooled sera, and homologous vs heterologous virus. The models were compared by considering the change in residual deviance to determine which of these sources of variation is significant ($P<0.05$).

3.4 Results

3.4.1 Effect of booster vaccination on individual animal sera using VNT

Post-vaccination sera from individual cattle collected over a period of 56 days were tested at seven-day intervals to assess the effect of primary vaccination and booster vaccination on the serological immune responses measured against the homologous (vaccine virus: A/May-97) and heterologous (field isolate: A/MAY/2/2011) viruses, as well as the derived vaccine-matching (r1) values for each of these measurements.

The neutralisation titres against the homologous virus were significantly higher ($P<0.05$) than those against the heterologous virus. However, one animal (Animal 281) showed a different pattern of homologous virus neutralisation titres compared to other animals after primary vaccination (resulting in a higher heterologous titre than homologous titre on day seven). In this animal, the neutralisation titres increased in a more gradual manner and were lower than for the other animals, and without a marked peak response of the booster vaccination (Figure 3.4a and 3.4b). The homologous neutralisation titres of the other five individual animals increased after primary vaccination to reach the suggestive protective cut-off (1.4 $\log_{10}$) at 21dpv (Barnett et al., 2003). A further increase of the

neutralising antibody against the homologous virus was measured after booster to reach a peak in all animals at 35dpv. At 42dpv the neutralisation titres started to plateau until the last sampling point at 56dpv (Figure 3.4a). On the contrary, the heterologous neutralisation titres increased to a value of 1.28 $\log_{10}$ at 7dpv but then a plateau persisted until after the booster vaccination. The heterologous neutralisation titres took more than two weeks to increase beyond the suggestive protective cut-off (1.4 $\log_{10}$) and peaked at 42dpv before starting to decrease (Figure 3.4b).

Despite the increase in neutralisation titres in all animals, calculated r1 values for each homologous/heterologous virus pair were variable (Figure 3.5). Analysis on individual sera of all the six animals at different sampling times (7, 14, 21, 28, 35, 42, 49 and 56dpv) generated highly variable r1 values, both below and above the suggested antigenic-match cut-off (0.3). The r1 values for Animal 279 ranged from 0.18 to 0.71, Animal 280 from 0.13 to 0.41, Animal 281 from 0.16 to 0.74, Animal 282 from 0.20 to 0.96, Animal 283 from 0.32 to 0.49 and Animal 284 from 0.15 to 0.62. Only one individual sera (Animal 283) showed r1 values above the cut-off of 0.3 for all sampling time points, while sera from some animals (such as Animal 280) generated r1 values below 0.3 for the majority (five of eight) of the time points (Figure 3.5).

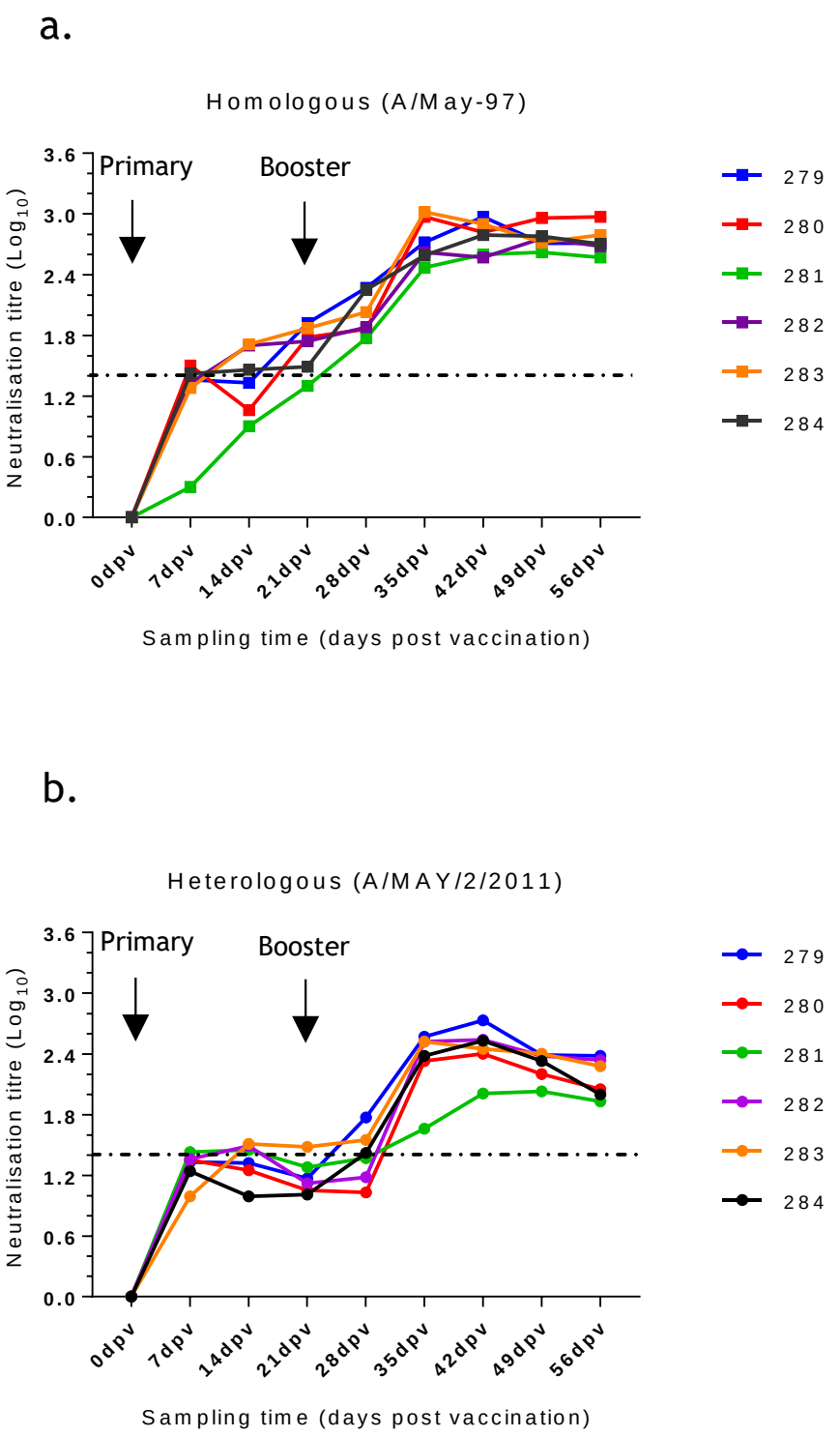


Figure 3.4: A time course of log10 neutralisation titre of specific antibodies against FMD viruses after primary and booster vaccination. The neutralisation titre against homologous virus (panel a) and heterologous virus (b) measured by VNT. The dashed line highlights 1.4 log10, the suggested protective cut-off for FMDV serotype A (Barnett et al., 2003).

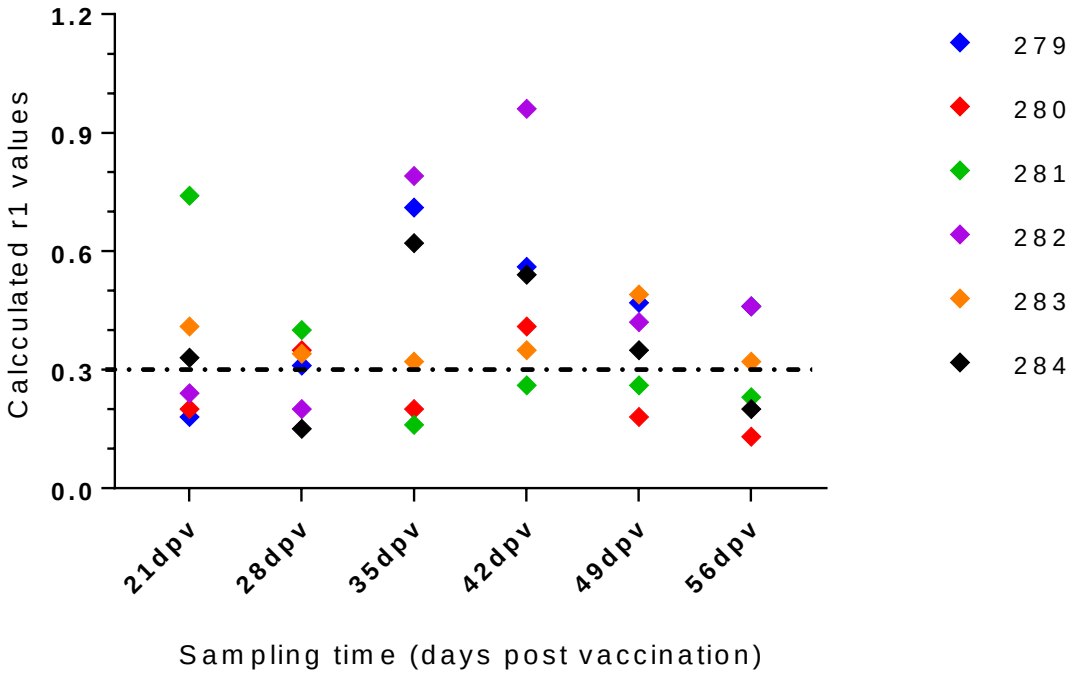


Figure 3.5: The r1 values generated from neutralisation titres of six individual animals at the six sampling time points. The six individual animals represented by coloured diamonds. The black dashed line highlights 0.3, the minimum acceptable cut-off indicative of an adequate antigenic match (O.I.E, 2018).

3.4.2 Results for virus neutralisation test versus liquid phase blocking ELISA

The serological immune responses and corresponding r1 values measured by VNT and LPBE were compared.

Serological immune responses of sera collected at 21dpv and 56dpv had consistently higher antibody titres measured by LPBE compared to VNT (Figure 3.6). Both VNT and LPBE showed higher homologous serological responses than the heterologous responses for sera collected at 21dpv and 56dpv (Figure 3.6a, b, c and d). At 21dpv, three out of six animals had heterologous mean neutralisation titres that were lower than the suggestive protective cut-off (Figure 3.6a). By contrast, the total antibody titres measured by LPBE of all the six animals at 21 and 56dpv were above the suggestive protective cut-off (Figure 3.6c and d). The r1 values of neutralisation titres and the total antibody titres were calculated for all individual animals (Figure 3.7a and b). For the neutralisation data three out of six animals showed similar mean r1 values for both 21dpv and 56dpv. Whereas, the other three of the six animals (Animals 279, 281 and 282) showed different mean r1 values at 21dpv and 56dpv with values below and above the antigenic-match cut-off for both

days. (Figure 3.7a). The r1 values generated using LPBE were less variable than those generated by VNT (Figures 3.7b). Nonetheless, different r1 values were observed using sera collected at 21dpv and 56dpv with the majority of mean r1 values for the 56dpv sera being below the suggested antigenic-match cut-of, while the majority of the r1 values using 21dpv sera were above the suggested antigenic-match cut-off (0.2) as in Figure 3.7b.

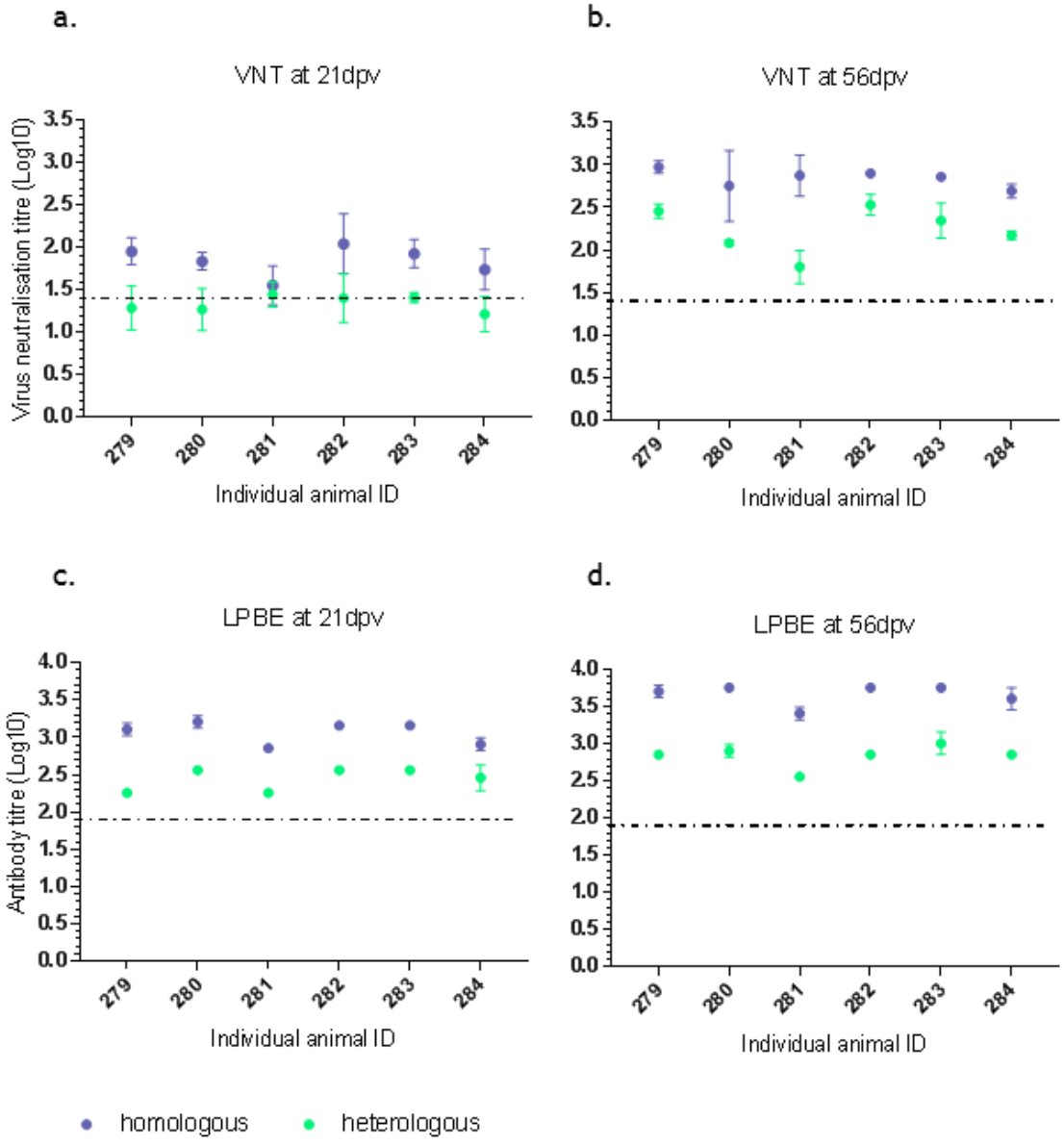


Figure 3.6: The mean log10 neutralisation and total antibody titres for six individual animals against the homologous and heterologous viruses at 21 and 56dpv. Every individual animal serum was tested in duplicate, replicated for three times and repeated at three different days; (a) mean neutralisation titres at 21dpv, (b) mean neutralisation titres at 56dpv, (c) mean total antibody titre at 21dpv and (d) mean total antibody titre at 56dpv. For VNT, the black-dashed line highlights log10 1.4, the protective cut-off for minimum neutralisation titres (Barnett et al., 2003), while for LPBE, log10 1.9 indicates the protective cut-off for the total antibody titres (Maradei et al., 2008).

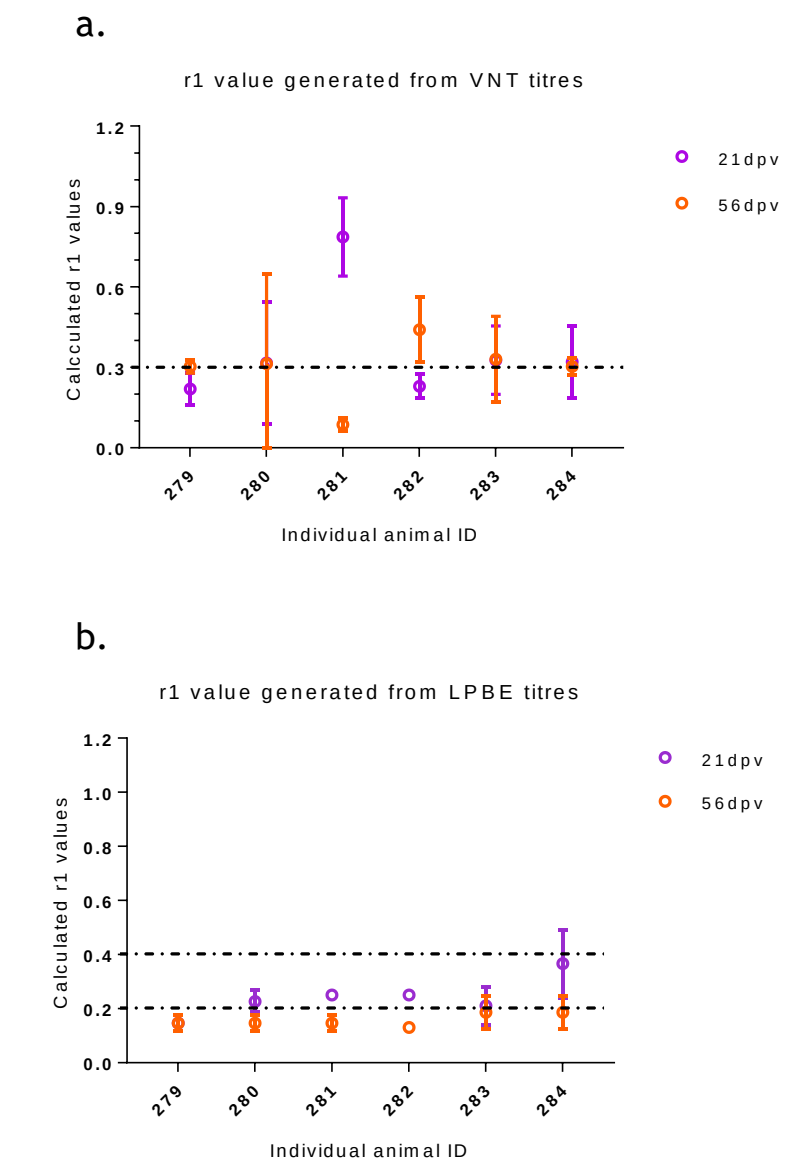


Figure 3.7: The r1 values of individual animals at 21dpv and 56dpv generated from VNT titres and LPBE titres. The r1 value were calculated for each three replicate and three repeats. Panel (a) shows r1 values generated from VNT titres and panel (b) showed r1 values generated from LPBE titres. The cut-off(s) for the r1 value are highlighted on the y-axis. The black dashed line highlights 0.3; the minimum acceptable cut-off point for r1 values generated by VNT (OIE, 2017). Two back dashed line highlight 0.2 the minimum and 0.4 the preferred acceptable cut-off point for r1 values generated using LPBE (Ferris and Donaldson, 1992).

3.4.3 Influence of pooled sera after vaccination

Pooled sera of multiple different combinations were tested to assess the influence of pooling on the variability of the serological immune responses measured by VNT and LPBE and corresponding vaccine-matching results (r1-values).

To assess the effect of pooling on serological immune responds, pooled sera were tested alongside the sera from the component individual animals. Both pools that comprised sera from two animals (Animals 279 + 283 that showed similar neutralisation titres, and Animals 282 + 284 that had very different neutralisation titres) showed similar patterns with the VNT and LPBE. Both neutralisation and total antibody titre against the homologous virus were higher than the titre against the heterologous virus for individual and pooled sera of (the individuals and pooled sera of the two). Further investigation on the effect of pooled sera and the corresponding calculated r1 values were carried out using four different pools constructed using sera from multiple different animals. These pooled sera were prepared based on the level of neutralisation titres measured for the individual animals (see section 3.3.3 for details). In general, these results had a similar pattern to the data generated for individual sera: both the neutralising and the total antibody titres of the homologous were higher compared to the heterologous titres (Figure 3.8a and b), and the titres measured using LPBE were consistently higher than the neutralizing antibody titres measured by VNT (Figure 3.8a and b). The r1 values for the pooled sera showed less variability with the LPBE than with the VNT, as was also seen with the previous analysis on individual sera.

The range of the r1 values generated using VNT titres of different the pooled sera were varied; pooled of high: 0.22 to 0.36, pooled of low: 0.23 to 0.63, pooled of mix: 0.17 to 0.56 and pooled of all the six sera: 0.16 to 0.44. On the other hand, a tighter range of r1 values was observed when using LPBE titres. For these LPBE data the ranges were 0.18 – 0.25 for pooled of high, for pooled of low 0.13 – 0.25, pooled of mix 0.18 – 0.25 and for the pooled of all the six sera 0.18 – 0.25 (Figure 3.9a and b). These analyses indicated that the r1 values of the pooled sera generated from VNT titres were more variable than the r1 values generated from the LPBE titre.

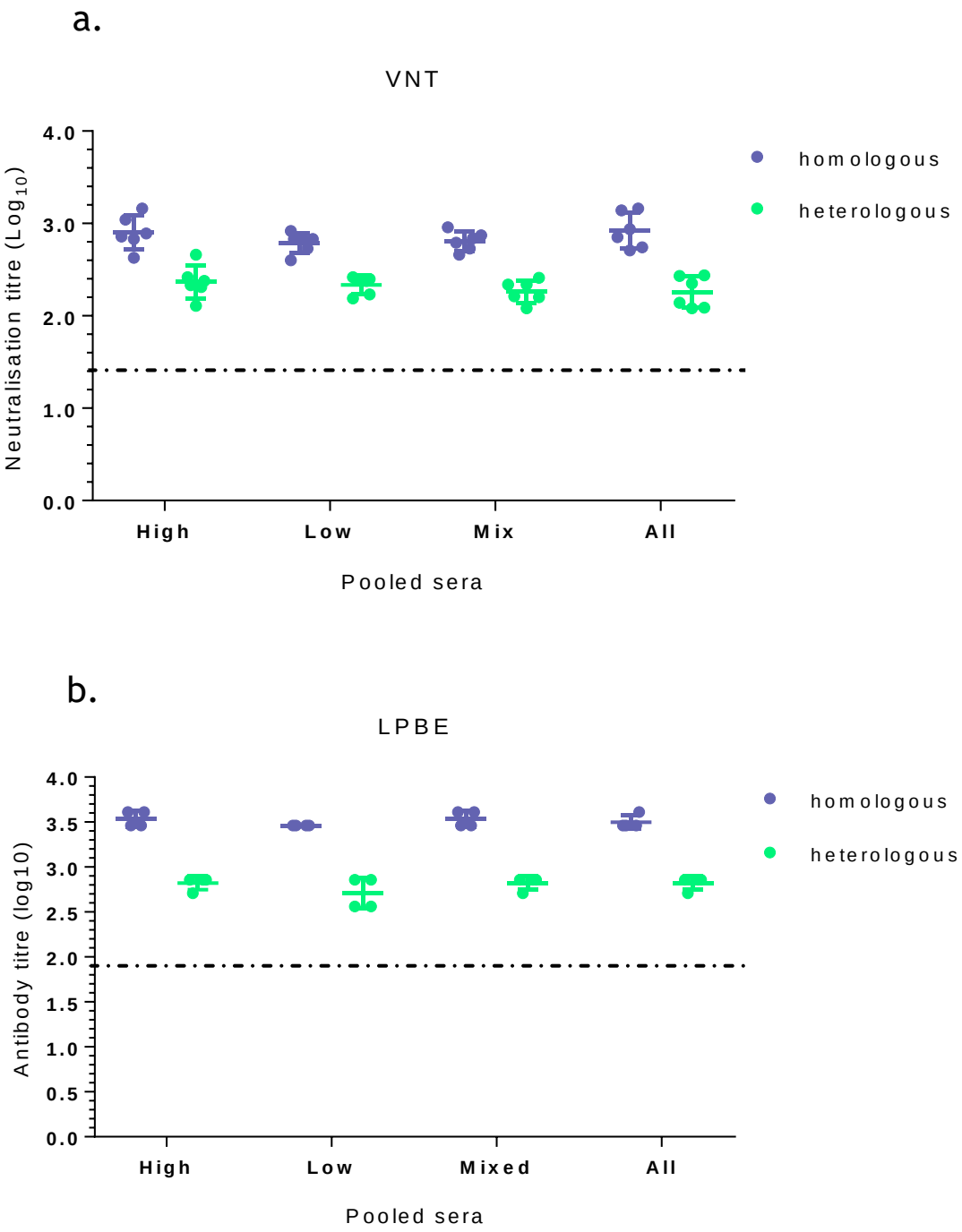


Figure 3.8: Pooled sera at 56dpv neuralisation and total antibody titres measured for four different sera pools using VNT and LPBE. Every sera pool was tested using (a) VNT and (b) in duplicate, replicated for three times and repeated at three different days against the homologous and heterologous. Both the mean neutralisation titres and mean total antibody titres were measured in log10 with the mean indicated by the bar and the line indicating the standard deviation. The cut-off for the mean neutralisation and total antibody titres is highlighted on the y-axis. The black dashed line highlights log10 1.4; the protective cut-off for the VNT (3.8a) (Barnett et al., 2003) and log10 1.9; the suggestive protective cut-off for LPBE (3.8b) (Maradei et al., 2008).

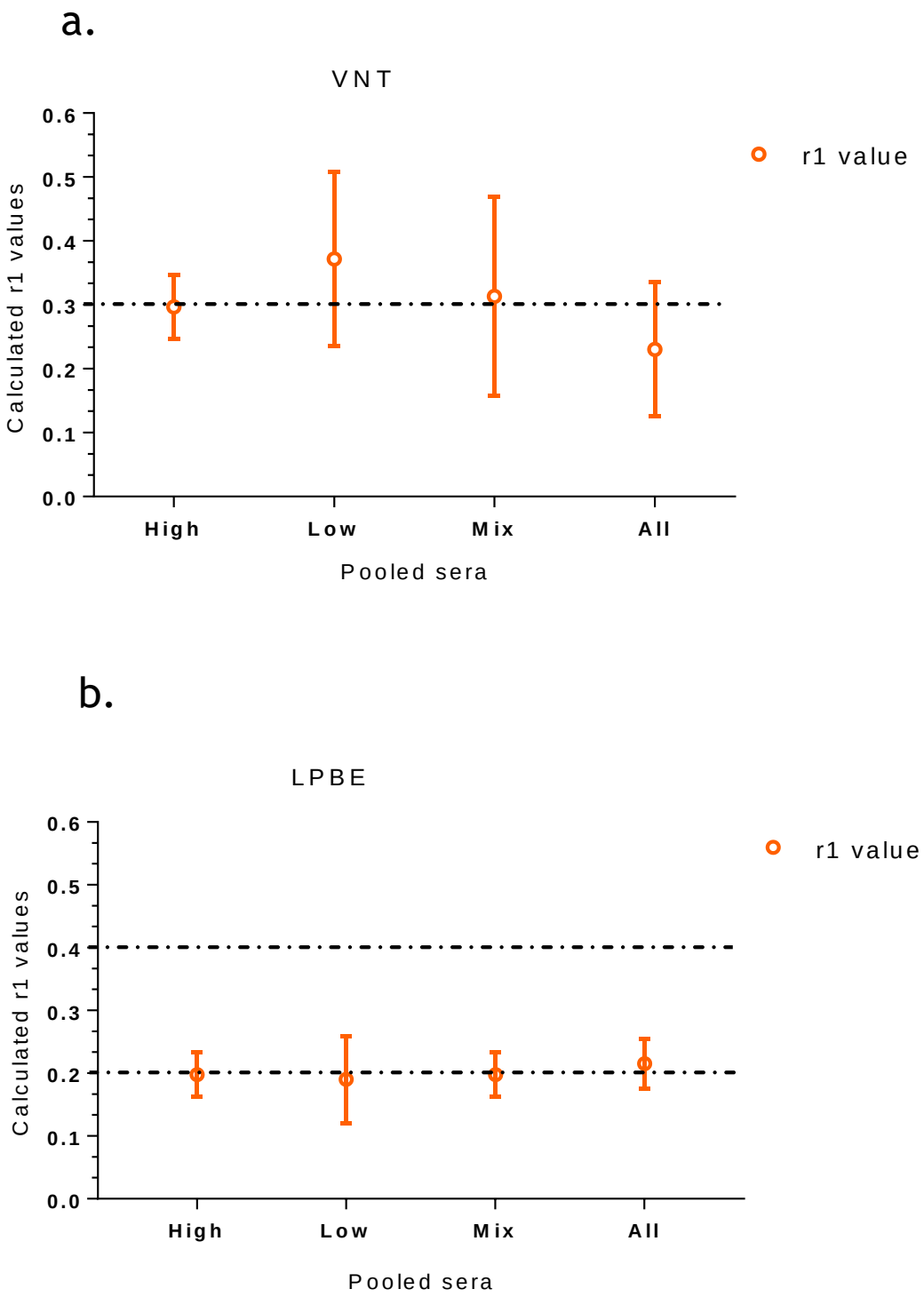


Figure 3.9: The r1 values generated for four different sera pools at 56dpv.

(a) r1 value generated from VNT titres and (b) r1 value generated from LPBE titres. On the x-axis are the animal identification numbers. The cut-offs for the r1 value are highlighted (as dashed lines) on the y-axis: 0.3, the antigenic match cut-off point for VNT (3.9a) (O.I.E, 2018), and 0.2, the minimum cut-off point, or 0.4, the preferred acceptable cut-off point for LPBE (3.9b) (Ferris and Donaldson, 1992).

3.4.4 Repeatability of virus neutralisation test and liquid phase blocking ELISA results

3.4.4.1 Comparison of virus neutralisation test by two different operators

Neutralisation titres of BVS collected at 21dpv and 56dpv against the homologous virus were generated by two different operators on two different days. The results are shown below (Table 2.1), with Operator 1 producing consistently lower titres compared to Operator 2. Although the results were not identical, these differences were not statistically significant. Furthermore, 7/12 of the results were within a two-fold dilution. As expected, for both operators, all boosted individual sera (56dpv) showed higher serum neutralisation titres compared to sera from cattle receiving only one vaccination.

Table 3.1: The log_{10} virus neutralisation titres of the homologous virus (A/MAY-97) against the same six BVS by two different operators on different days.

Animal identification (ID) (serum)	OPERATOR 1		OPERATOR 2	
	21dpv	56dpv	21dpv	56dpv
279	1.80	2.85	1.95	3.15
280	1.95	2.55	1.95	3.15
281	1.35	2.55	1.95	2.85
282	1.50	2.70	2.10	3.00
283	1.35	2.85	1.80	3.00
284	1.50	2.40	1.80	2.85

3.4.4.2 Within day and between day variability

3.4.4.2.1 Individual sera

Post primary and booster vaccination of individual and pooled sera were tested repeatedly within the same day and across different days to determine the source of the inherent variability of the *in-vitro* vaccine-matching methods (VNT and LPBE).

The result for the model built and compared to determine the source of the inherent variability are shown in table 3.2. For VNT applied to individual sera at 21dpv and 56dpv, there was no significant (21dpv: p=1.0 and 56dpv: p= 1.0) variation in titres within a day, but there was significant (21dpv: p=0.004 and 56dpv: p= 0.0007) variation both (i) between days and (ii) between animals. However, the contribution of the sources of

variation differed between the time points. At 21 dpv, day-to-day variability contributed 47.4% of the variance, while between animal variability contributed only 0.4% of the variance. By contrast, at 56 dpv, day-to-day variability contributed 17.7% of the variance, while between animal variability contributed 25.7% of the variance. For LPBE applied to individual sera at either 21 dpv or 56 dpv, there was no significant (21dpv: p=0.4 and 56dpv: p= 0.14) contribution of within day or between-day variability to the variance in titre. Only animal-to-animal variability contributing 49.5% and 57.2% of the variance in titres for sera collected at 21 dpv and 56 dpv, respectively. The estimated R1 values based on VNT were 0.32 (95% confidence interval (CI): 0.27 to 0.38) at 21 dpv and 0.27 (95% CI: 0.23 to 0.33) at 56 dpv. The estimated R1 values based on LPBE were 0.25 (95% confidence interval (CI): 0.23 to 0.28) at 21 dpv and 0.15 (95% CI: 0.13 to 0.17) at 56 dpv (Figure 3.10).

Table 3.2: Three models were built namely Model 1: Individual animal/different days/replicates in a day, Model 2: Individual animal/different days and Model 3: Individual animal. The models were compared for both in-vitro methods (VNT and LPBE) and sera collected at 21dpv and 56dpv."

Method	Sera	Models	AIC	p value
VNT	21dpv	Model 1: Individual animal/different days/replicates in a day Model 2: Individual animal/different days	-27.34 -29.34	1.0
		Model 2: Individual animal/different days Model 3: Individual animal	-29.34 -23.00	0.004**
	56dpv	Model 1: Individual animal/different days/replicates in a day Model 2: Individual animal/different days	41.80 39.80	1.0
		Model 2: Individual animal/different days Model 3: Individual animal	39.80 49.24	0.0007***
LPBE	21dpv	Model 1: Individual animal/different days/replicates in a day Model 2: Individual animal/different days	-108.45 -110.45	1.0
		Model 2: Individual animal/different days Model 3: Individual animal	-110.45 -111.68	0.4
	56dpv	Model 1: Individual animal/different days/replicates in a day Model 2: Individual animal/different days	-113.40 -115.40	1.0
		Model 2: Individual animal/different days Model 3: Individual animal	-115.40 -115.22	0.140

3.4.4.2.2 Pooled sera

Analysis of VNT and LPBE on different combinations of pooled sera collected at 56dpv showed no significant differences within and between days test for VNT (p=0.70 and p=0.10, respectively) and LPBE (p=0.33 and p=0.44, respectively). These results indicated that pooling sera at 56dpv reduces the day-to-day variability of the neutralising antibody measured by VNT.

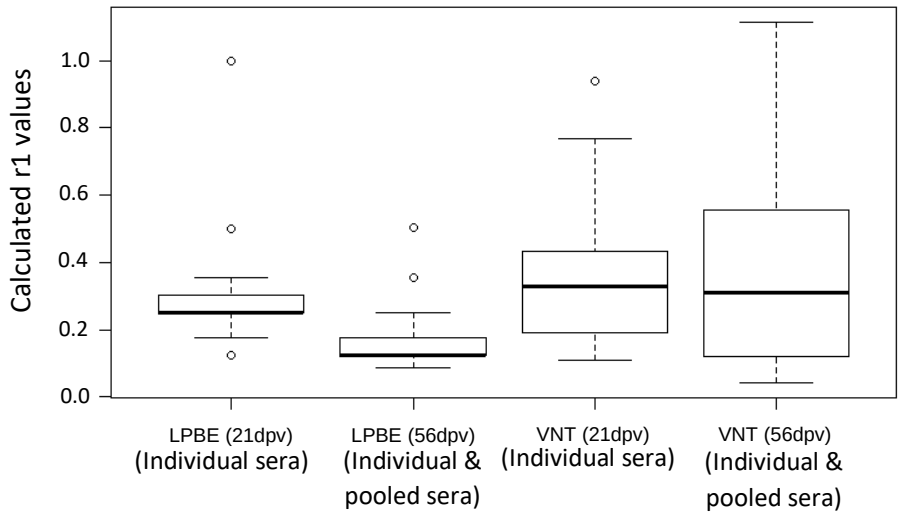


Figure 3.10: Box plots representing the r1 values of the individual and pooled sera tested using LPBE and VNT at 21dpv and 56dpv. On the x-axis are VNT and LPBE carried out on sera collected on 21dpv and 56dpv. The dense black line in the box indicated the mean while the upper and lower whiskers represented standard deviation at 95% confidence interval.

3.5 Discussion

The overall aim of this chapter was to determine how the origin of bovine vaccinal sera (BVS) impacts upon results generated by *in-vitro* vaccine matching methods. The experiments focused on serotype A which is considered to be the most genetically and antigenically diverse among the FMDV serotypes (Knowles and Samuel, 2003). VNT and LPBE were performed on the A/May-97 (homologous) and A/MAY/2/2011 (heterologous) viruses using individual and pooled BVS. These viruses were selected based on previous poor r1 values and the retrospective vaccine matching data collected from 2006 to 2017 at WRLFMD. In contrast to experiences from the field, these data indicated that the

A/May-97 vaccine virus is not likely to confer protection against the serotype A FMDVs (from the A/ASIA/Sea-97 lineage) circulating in Malaysia.

Cattle were vaccinated using a vaccine produced commercially to ensure the quality, purity and safety of the vaccine. This particular high-potency vaccine (of at least $6PD_{50}$) and formulation mirrored the field situation in Malaysia. The BVS was produced from FMD naïve cattle to ensure no pre-existing FMDV antibodies could interfere with the results. As expected, both VNT and LPBE revealed that the neutralising and total antibody titres of the BVS against the homologous virus were consistently higher than antibody titres against the heterologous virus (Oh et al., 2012; Pay and Hingley, 1987).

Neutralising antibodies measured by VNT correlate with protection (Barnett et al., 2003; Maradei et al., 2008; Pay and Hingley, 1987; Van Maanen and Terpstra, 1989). "Protective" cut-off values have been established to measure post-vaccination responses using VNT (Barnett et al., 2003) and LPBE ($\log_{10}$ 1.9: (Maradei et al., 2008). In this study, all samples tested by LPBE at 21 dpv were above the protective cut-off, while all corresponding values for the VNT were below the neutralisation cut-off. Although LPBE titres indicated that all animal reached "protective" heterologous titres after a single dose of vaccine, these findings indicated that single vaccination did not necessarily elicit high enough neutralising antibody titres to protect the cattle against the heterologous virus (A/MAY/2/2011). However, conclusions regarding the level of protection of individual animals based solely on serological cut-off values are not always reliable. Indeed, other researchers claim that the serological immune responses can only be used as a guide (McCullough et al., 1992) and that protective humoral responses are dependent on other factors such as antibody avidity and spectrum of the antibody response (Siegrist, 2013). For the purpose of assessing immunity at population level, the FMD Post Vaccination Monitoring (PVM) guide suggests that protective cut-offs should be adjusted depending on the size and dynamic of the population (Ferrari et al., 2016). It is also important to recognise that there are different methods used to determine protective responses in animals. In South America, researchers commonly use 75% expected percentage of protection (Maradei et al., 2008), while others use titres at which animals are protected with probability of 50% (Barnett et al., 2003). Thus, currently there is no clear and harmonised approach used to define serological cut-off values that correlate with protection. The effect of booster vaccination was measured at 56dpv where both VNT and LPBE antibody titres (for the homologous and heterologous viruses) were above the suggestive protective cut-off. These findings support importance of a booster dose particularly to increase the heterologous neutralisation antibody titres beyond the suggested protective cut-off for both VNT and LPBE (Knight-Jones et al., 2016).

Sera collected from these vaccinated animals was used for vaccine-matching. The r1 values generated from VNT titres showed variable results for sera collected at 21 dpv (above and below the suggested antigenic match cut-off point of 0.3), whereas LPBE generated more consistent r1 values (above the lower level antigenic match cut-off of 0.2) for 5 out of 6 animals. These conflicting results are representative of the difficulties that are often experienced by FMD Reference laboratories (such as WRLFMD, at TPI), where variability in vaccine matching data can lead to uncertainty in the selection of an appropriate vaccine. Analysis undertaken in this study, indicated that for VNT there were two main sources of this inherent variability: one factor was day-to-day variability, and the other was differences between sera collected from different individual animals. Based on this knowledge, it can be concluded that in order to reduce the inherent variability of VNT, both the homologous and heterologous viruses need to be tested simultaneously on the same day. It can be expected that repeating the vaccine matching test by VNT on different days will provide a range of r1 values for the particular viruses tested. Furthermore, VNT method requires personnel to read the CPE by eye using the microscope. Therefore, the outcome of the results may vary between personnel depending on the personnel's sensitivity to recognise the CPE, although operator-to-operator differences measured in this study were not significant.

The other source of the inherent variability affecting both the VNT and the LPBE was identified as the individual animal sera used. Fortunately, this source of inherent variability can be reduced by pooling sera irrespective of the number of sera (ranging from 2 to 6) and the type of sera (highest, lowest, mix and all). Other researchers have reported similar observations about the impact of pooling sera to reduce inter-animal and inter-trial variation (Mattion et al., 2009). However, to limit the influence of outliers, Brehm et al. advised to make pools comprising of five sera from different individual animals (Brehm et al., 2008) which is also an approach recommended in the OIE Manual of Diagnostic Test and Vaccine for Terrestrial (OIE, 2017). The reason that pooling decreases variability is unknown. However, it might be due to the different quality of the antibodies such as the avidity, specificity or neutralising capacity in the serum produced by different individual animals. Therefore, pooling serum from different animals lowers the variability in the total antigen antibody complexes exhibit by LPBE titre or neutralising antibody as in VNT titres.

Although some sources of inherent variability of VNT and LPBE have been identified in this Chapter, and mitigation measures have been laid out to achieve more precise results, the issue with the variability of r1 values above and below the cut-off still persist. However, since the suggested antigenic-match cut-off is a fixed value for VNT and LPBE, regardless of the FMDV serotypes or strains, r1 values for the same viruses may include

determination above and below the expected matched threshold. For the VNT, these range of r1 values are probably due to the fact that the r1 values were calculated from neutralisation titres which allow $\log_{10}$ 0.3 (two-fold dilution) more or less than the running mean. Since r1 values are derived by dividing the heterologous and homologous titres, the differences between repeated values can be widened by the variability of the test. This high variability for VNT is not taken into account in the r1 value cut-off. In contrast, *in-vitro* vaccine matching using LPBE method where the day-to-day variability has no significant influence on the result the cut-off point for the r1 value is in a range that cater for some variability.

In summary, this work shows that LPBE is a more reproducible *in-vitro* method to measure post FMD vaccination serological immune responses compared to VNT as mentioned by other researchers (Robiolo et al., 2010). This finding is also in agreement with other reports that LPBE showed less variation than VNT (Tekleghiorghis et al., 2014; Van Maanen and Terpstra, 1989). The present study observed precision in LPBE for measuring the total antibody titres and r1 values within the group of sera at 21dpv or 56dpv. However, the degree to which these r1 matching values relate with protection was not assessed in this study since there was no challenge experiment involved. This is an important deficiency of the current *in-vitro* vaccine matching result since they do not take into account neutralisation or total antibody titres in determining the antigenic relationship. Hence, future work to assess the accuracy of the *in-vitro* vaccine matching test is suggested. This could include *in-vivo* challenge experiments and humoral antibody measurements (including isotyping and avidity ELISAs (Brito et al., 2014; Capozzo et al., 1997; Lavoria et al., 2012).

Chapter 4:

Influence of viral antigen factors in *in-vitro* vaccine matching: does FMDV capsid integrity have an impact upon the reliability of serological immune responses?

Acknowledgements:

Jamaliah Senawi generated all laboratory data presented in this chapter. Jamaliah Senawi acknowledges assistance from Dr Julian Seago to perform the FMDV purification using the sucrose cushion method and the subsequent PaSTRy analysis to determine the FMD virus capsid dissociation points. Dr Eva Perez kindly provided the M3ggsVI-4Q6E antibody and M3ggsVI-4Q6E antibody in biotinylated form.

4.1 Abstract

Foot-and-mouth Disease (FMD) virus is sensitive to high temperature and pH < 6.5 resulting in dissociation of the virus capsid that contains the determinants that define virus antigenicity. Therefore, it is vital to understand the impact of FMD virus integrity on *in-vitro* vaccine matching assay particularly virus neutralisation test (VNT), which is currently recognised as the gold standard to determine the suitability of vaccine virus. Without excipient (glycerol) to stabilise the virus, mild heat treatment at 45°C of FMD viruses indicated that the capsid integrity of the field isolate is more heat stable compared to vaccine virus as determined by Llama single-domain antibody fragments 12S double antibody sandwich ELISA (VHHs DAS ELISA) and virus titration methods. A similar result was seen using the particle stability thermal release assay (PaSTRy) that the relative stability of FMD vaccine virus (A/May-97 at 53.3°C) was lower compared to two field isolates (A/MAY/2/2011 at 56.5°C and A/VIT/13/2015 at 56°C). Subsequently a stabilised vaccine virus and field isolate were tested with VNT and liquid phase blocking ELISA (LPBE) to assess the impact of dissociated virus particles (DVPs) contained in FMD virus preparation upon *in-vitro* vaccine matching assays. These studies identified that 12S capsid particles compete the intact capsid (146S) to bind antibodies that would otherwise be available in *in-vitro* vaccine matching methods resulting in an artificial decrease in neutralisation titres (measured by VNT) and FMDV total antibody titres (measured by LPBE). Additionally, variable 12S amount were detected in 10 FMD viruses of the same lineage (A/ASIA/Sea-97) and eight FMD viruses of the same lineage (A/ASIA/Iran-05).

4.2 Introduction

Success of foot-and-mouth disease (FMD) control campaigns by vaccination is influenced by multiple factors particularly the quality and stability of the selected vaccine virus (antigen). It is well established that antigen integrity is imperative for effective FMD vaccine performance (Cartwright et al., 1980; Cartwright et al., 1982; Hingley and Pay, 1987; Meloen and Briaire, 1980). However, the direct impact of FMD antigen integrity on the ability of *in-vitro* assays to measure specific antibody responses is yet to be fully understood.

FMD virus is a small, non-enveloped, positive-sense single stranded RNA virus that belongs to the *Picornaviridae family*. Similar to other picornaviruses, the protein capsid of FMDV is made of 60 copies each of the four structural proteins (VP1, VP2, VP3, and VP4) arranged in an icosahedral lattice of 12 pentameric building blocks (Rueckert and Wimmer, 1984). Capsid proteins VP1, VP2 and VP3 are positioned externally, whereas VP4 is concealed within the capsid and is predicted to be in contact with the viral RNA (Mateu, 2017). The antigenic and immunogenic properties of FMD virus are mainly located in VP1 of the capsid, complemented by other sites present in VP2 and VP3 structural proteins (Reeve et al., 2016; Thomas et al., 1988). However, FMDV capsid are particularly fragile compared to other members in the *Picornaviridae family* such as the enteroviruses.

The established method to quantify FMD virus capsid particles uses sucrose density gradient (SDG) centrifugation developed by Barteling and Meloen (Barteling and Meloen, 1974). Intact FMD virus capsids have a sedimentation coefficient of 146 in a sucrose gradient (146S). It is generally accepted that 146S is the antigenic component which stimulates protective antibody responses (Brown and Cartwright, 1961; Doel and Chong, 1982a; Kotecha et al., 2015; Randrup, 1954). The 146S capsid dissociates under mildly acidic conditions at pH below 6.5 (Brown and Cartwright, 1961; Caridi et al., 2015), and at temperatures above 56^{o}C (Brown and Crick, 1959). These conditions cause the irreversible dissociation of FMD virus capsid into twelve pentameric subunits, each with a sedimentation coefficient of 12S (12S capsid particles). It has also been reported that at temperatures above 37°C, the commercial inactivated FMDV vaccine can rapidly convert into these immunogenically incompetent 12S capsid particles (Doel and Chong, 1982b). SDG method is laborious, time consuming and highly operator dependent. As alternatives to SDG centrifugation, the proportion of 12S can also be determined based on size-exclusion using high performance chromatography (HPLC) (Spitteler et al., 2011; Yang et al., 2015) although this method requires expensive specialized equipment. The 12S can also be detected using a lateral flow immunoassay (Yang et al., 2015). An

alternative ELISA method has been developed and was used in this study. This method uses llama single-domain antibody fragments (VHHs) specific for 12S capsid components in a double antibody sandwich (DAS) ELISA to specifically quantify the 12S capsid particles present in a sample (Harmsen et al., 2011). The VHHs, also called Nanobodies®, are single-domain functional antibody fragments that are fully capable of antigen binding without requiring domain pairing (Harmsen and De Haard, 2007). The word "double antibody" in DAS ELISA means that the same llama single-domain antibody (M3ggsVI-4Q6E) is used for both trapping and detection of 12S capsid particles. The advantages of VHHs DAS ELISA is that it is easier to perform, has higher sensitivity as well as higher sample throughput than SDG and HPLC (Harmsen et al., 2017).

During FMD virus replication, in addition to 146S, there are empty capsid particles that are naturally produced. These empty capsid particles are the complete form of the FMDV capsid without the viral RNA inside. These are often referred to as "natural" empties or 75S since they sediment at coefficient of 75 sucrose gradient. Although the 75S particles resemble the 146S in structure and antigenicity, they are inherently less stable (Basavappa et al., 1994). However, it has been demonstrated that 75S particles of FMD virus serotype A Cruzeiro are able to produce useful level of immunity but less effective than the intact capsid (146S) (Doel and Chong, 1982b). These different FMD virus capsid protein particles (146S, 75S and 12S) are present in FMD virus stocks and more so in FMD vaccine due to the production process (Rowlands et al., 1975). Moreover, the seven FMD virus serotypes are markedly different in their capsid stability. For instance, serotype A is reported to be relatively more stable than serotype O to heat and decreases in pH (Doel and Baccarini, 1981). However, since serotype A are reported to have high antigenic diversity (Knowles and Samuel, 2003); it is possible that there will be differences in the capsid stability between the serotype A virus lineages.

Although protective antibody responses usually target epitopes expressed on the FMD virus capsid, serological immune responses of infected hosts recognise epitopes that are present both on the intact and dissociated capsid components (Cartwright et al., 1980). However, the 12S capsid particle of FMDV do not stimulate significant levels of neutralizing antibody (Cartwright et al., 1980; Randrup, 1954). The hypotheses of this chapter are that dissociated virus particles (DPVs) are present in virus and stocks used for the VNT and LBPE; that the presence of these DPVs compete with the ability of FMDV antibodies present in hyper-immune serum to bind to intact capsids (146S particles); and this will in turn influence the results of the *in-vitro* vaccine matching methods as illustrated in Figure 4.1 and 4.2, causing a reduction in antibody titres measured by both VNT and LBPE methods. This chapter explores these hypotheses through a series of experiments and also investigates the related question of whether viral capsid integrity

is the same for the antigenically diverse serotype A viruses of FMDV, which would cause any effect detected to be different for different viruses.

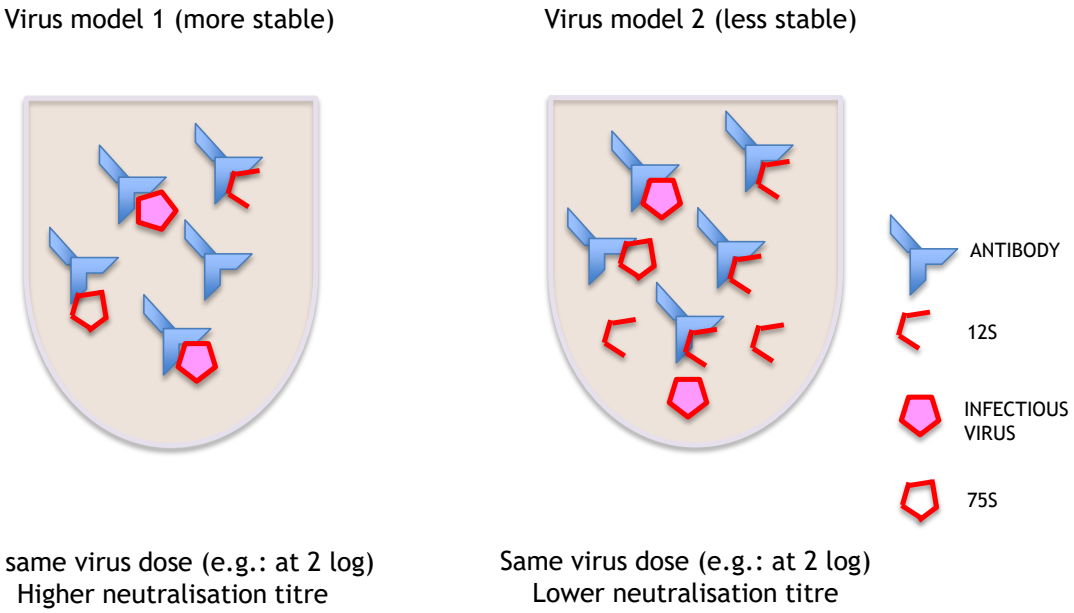


Figure 4.1: The hypothesised impact of dissociated FMD virus particles (12S) on VNT. In this model, the dissociated capsid particles interact with the FMDV-specific antibodies available resulting in more free-infectious virus that can infect cells and cause cytopathic effect. Consequently, the presence of FMD virus dissociated capsid particles reduce the neutralization titre.

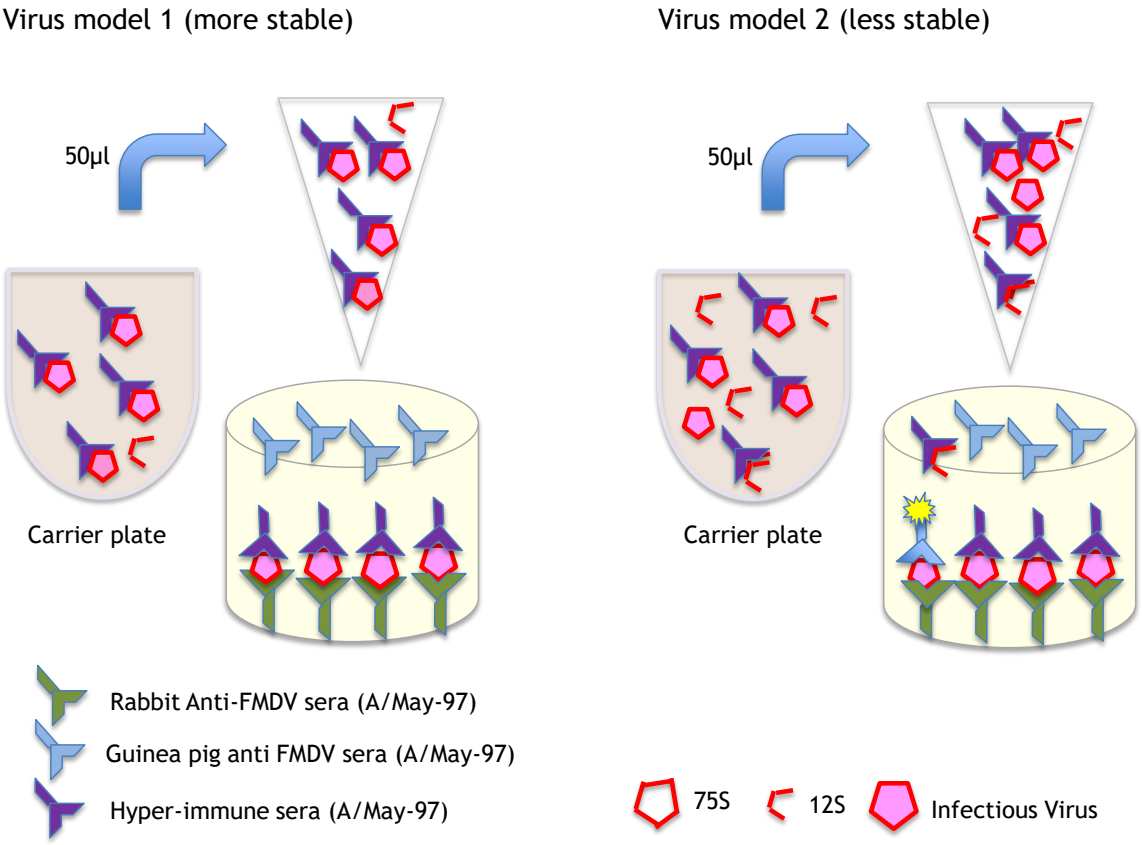


Figure 4.2: The hypothesised impact of dissociated FMD virus capsid particles (12S) on LPBE. In this model, the dissociated FMD virus capsid particles bind to the antibodies available in the sera of the liquid phase. Infectious free virus can then bind to the guinea pig anti-FMDV, which capture the chromogen-substrate. Consequently, the presence of dissociated capsid particles increases the colour density that correlates to a reduction in the total antibody titres.

4.3 Materials and methods

4.3.1 Study design

The study design of this chapter involved four main experiments as described in Table 4.1. In this study two different preparation of FMD virus that are non-glycerinated and glycerinated were used. Glycerol is routinely added as excipient to FMD virus stocks in order to increase their stability for future diagnostic serological assays such as VNT and LPBE. In Experiment 4.1, non-glycerinated A/May-97 (vaccine virus) and A/MAY/2/2011 (field isolate) were heat-treated at 45°C to assess the effect of mild heat treatment on the production of 12S capsid particles measured using VHHs DAS ELISA and the impact of these dissociated viral particles (DPVs) on FMD virus titres. Experiment 4.2 was performed with thermostability assay using the particle stability thermal release assay (PaSTRy) to

determine the capsid dissociation point of the vaccine virus (A/May-97) and two field isolates from the same region (A/MAY/2/2011 and A/VIT/13/2015). The findings from Experiment 4.1 and 4.2 were used to select the applicable temperature for Experiment 4.3. Experiment 4.3 then tested whether the VHHs DAS ELISA generated different results when glycerinated FMD viruses (vaccine virus: A/May-97 and field isolates: A/MAY/2/2011 and A/VIT/13/2015) were subjected to three temperatures (51°C, 56°C and 61°C). This experiment optimised the temperature and conditions required to fully dissociate the FMD viruses to produce Dissociated Virus Particles (DVPs) (described in detail in section 4.3.7). Next, the optimal conditions were used to investigate the impact of glycerinated DVPs on FMD virus titres, and antibody responses using VNT and LPBE methods. For Experiment 4.3 three different individual sera collected at 21st and 56th day post vaccination (dpv) (Animal IDs: 281, 283 and 284; described in Chapter 3 section 3.3.2) were tested. Finally, the fourth experiment (Experiment 4.4) was performed to assess whether there is variability in 12S particle produced by different FMD viruses of serotype A.

Table 4.1: The series of experiments carried out for chapter 4.

	Experiment 4.1	Experiment 4.2	Experiment 4.3	Experiment 4.4
Viruses: Vaccine virus Field virus	A/May-97 A/MAY/2/2011	A/May-97 A/MAY/2/2011 A/VIT/13/2015	A/May-97 A/MAY/2/2011 A/VIT/13/2015	FMDV isolates from 2 different lineages: A/ASIA/Sea-97 and A/ASIA/Iran-05
Temperature for heat-treatment	45°C	Range of temperatures: 25°C to 94°C*	51°C 56°C 61°C	51°C 56°C 61°C
Excipient**	No glycerol	No glycerol	50% glycerol	50% glycerol
Detection method used to determine FMDV capsid degradation and FMDV specific antibody responses	VHHs DAS ELISA Virus titration	PaSTRy	VHHs DAS ELISA Virus titration VNT LPBE	VHHs DAS ELISA

* N.B.: PaSTRy analyses were performed using a real-time PCR machine

** Glycerol can be added at the ratio 1:1 to stabilise FMD viruses prior to storage. Therefore, adding glycerol to the viruses mimics the current VNT and LPBE methods used within the WRLFMD at Pirbright (where glycerol is added to FMD virus stocks for long-term storage). In this study, no glycerol was added to the viruses in experiments 4.1 and 4.2, whereas the other two experiments 4.3 and 4.4 glycerol was added to the untreated and the heat-treated viruses (Table 4.1).

4.3.2 FMDV isolates

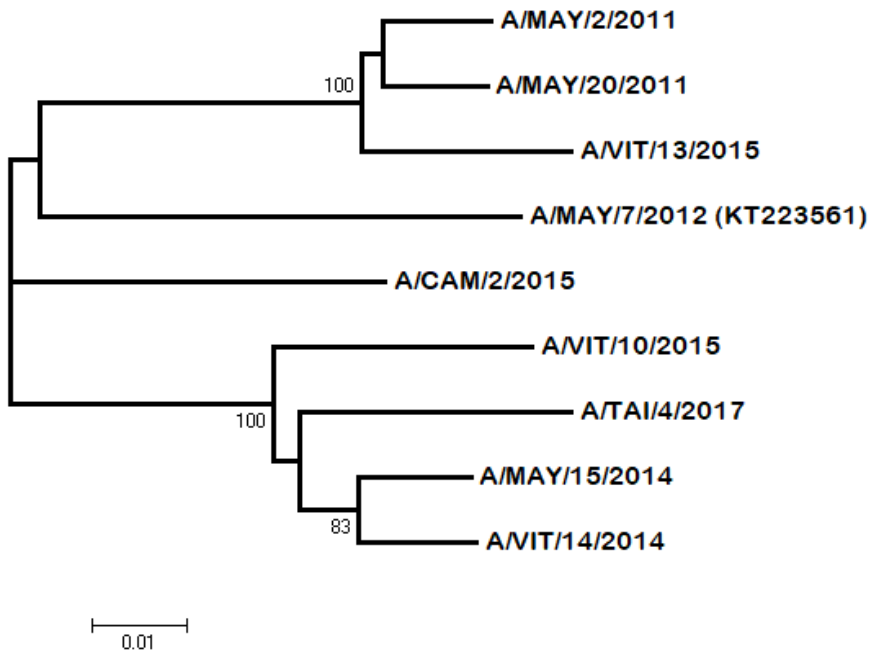
Two FMD vaccine viruses and sixteen field isolates representing two serotype A lineages (A/ASIA/Sea-97 and A/ASIA/Iran-05) were selected from the repository held at the FAO World Reference Laboratory for FMD (WRLFMD) at The Pirbright Institute (TPI). For detail of the viruses see Appendix ii). All field isolates were treated with stabilised di-ethyl ether to destroy any adventitious lipid-containing organisms before propagation. These viruses were previously characterized by phylogenetic analyses (Figure 4.3) based on VP1 coding sequences (Knowles NJ, 2016).

4.3.3 Virus propagation

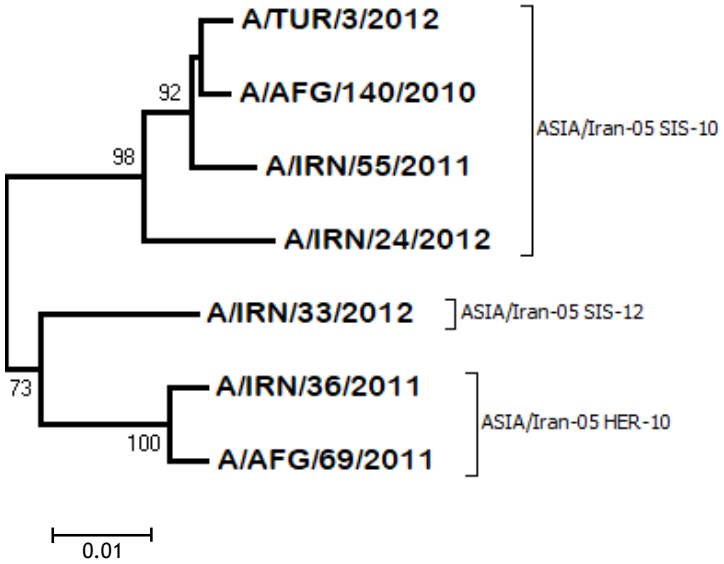
All viruses were propagated in 175ml flasks (Greiner Bio-One) on a 100% confluent monolayer of IB-RS-2 cells (De Castro, 1964). The media used for propagation of the viruses comprised HEPES Modified Eagles Medium supplemented with 0.2% field antibiotic containing mixture of penicillin (10MU), neomycin (25,000µg/ml), polymyxin B (100,000U/ml) and amphotericin B (field antibiotic from central services unit [CSU, TPI]) and 0.4% sodium hydroxide solution (NaOH from Sigma-Aldrich). Sterile glycerol (bidistilled analar from VWR International) was added to the FMDV at ratio of 1:1 after the viruses were harvested except for the viruses propagated in experiment 4.1. For this experiment A/May-97 (vaccine virus), and A/MAY/2/2011 (field isolate), were titrated and tested straight after harvesting; no glycerol was added (Table 4.1). For each experiment, all viruses were passaged to the same passage number.

For the preparation of material for PaSTRy analysis in experiment 4.2, three FMD viruses were selected: A/May-97 (vaccine virus), A/MAY/2/2012 and A/VIT/13/2015 (field isolates). These three viruses were propagated in 175ml flasks (Greiner Bio-One) on a 100% confluent monolayer of foetal goat epithelium cell lines (ZZR cells, (Brehm et al., 2009) and passaged twice to propagate high amounts of virus required for purification prior to testing. These viruses were not glycerinated before testing.

A/ASIA/Sea-97



A/ASIA/Iran-05



N.B.: All viruses were passaged in IB-RS-2 cells to the same passage no: 3 (RS 3) before use

Figure 4.3: Phylogenetic analyses of field virus isolates used in this study. Midpoint-rooted Neighbour-joining phylogenetic trees were constructed based on RNA sequences and visualised using MEGA 6.06. Bootstrap values above 70 are displayed next to branches. The phylogenetic tree was constructed by Dr Katarzyna Bachanek-Bankowska.

4.3.4 Viral capsid dissociation

Heat treatment, by placing the sample in a water bath for 30 minutes, was used to dissociate the FMDV capsid. For experiment 4.1, the temperature used was 45°C. For the other two subsequent experiments; experiment 4.3 and experiment 4.4, the viruses were heat-treated for 30 minutes in a water bath at 51°C, 56°C or 61°C (Table 4.1).

4.3.5 Llama single-domain antibody fragments, 12S double antibody sandwich ELISA

The VHHs DAS ELISA to detect the FMDV capsid particle (12S) was carried out following the method described by Harmsen et.al (Harmsen et al., 2011) (Figure 4.4). All samples were tested in duplicate. Briefly, ELISA plates were coated with 100µl of M3ggsVI-4Q6E antibody (kindly provided by Dr Eva Perez, TPI) at a concentration of 0.5 mg/l in carbonate-bicarbonate buffer to capture FMDV antigen. The plate was then incubated at 4°C overnight. After washing three times using phosphate buffer solution with Tween (0.05% Tween 20) (PBST):), 50µl of each sample was added in the designated wells. The plates were sealed and incubated at 37°C on a shaker for one hour. After washing three times, the ELISA plates were incubated again for one hour with 100µl per well of biotinylated version of the same M3ggsVI-4Q6E antibody (diluted to 0.1 ml/l in VHHs ELISA buffer) for detection of the captured 12S. The bound biotinylated M3ggsVI-4Q6E antibody was detected with 100 µl per well of streptavidin-HRP (SIGMA-ALDRICH) at a 1:1,000 dilution in VHHs ELISA buffer before adding 50µl of O-phenylenediamine dihydrochloride (OPD; Sigma, P-8412) mixed with 30% hydrogen peroxidase per well. The VHHs ELISA buffer contained 1% skimmed milk; 0.05% Tween; 0.5M NaCl; 2.7mM KCl; 2.8mM KH_2PO4; 8.1mM Na_2HPO_4 at pH4.4. Finally, after 50ul per well of 1.25M sulphuric acid stopping solution was added, absorbance measurements were read at 490nm. Unlike the LPBE, the higher the OD reading, the higher amount of 12S FMDV capsid particle was present in the sample.

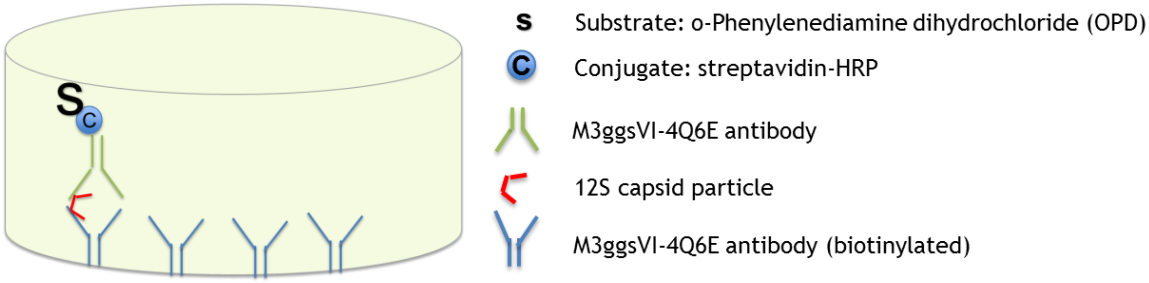


Figure 4.4: The principle of the VHHs 12S DAS ELISA. The M3ggsVI-4Q6E antibody are bound to the surface of an ELISA plate well to capture the 12S capsid particle (denoted as). The same M3ggsVI-4Q6E antibody (biotinylated) is then used to detect the captured 12S capsid particle. Peroxidase-conjugated streptavidin-HRP ( ) is added, which will bind to the M3ggsVI-4Q6E antibody. The presence of enzyme is detected by substrate reaction (S).

4.3.6 Virus titration

In order to assess the impact of 12S capsid particles on the FMDV titre, a titration series of the non-treated and heat-treated viruses were performed simultaneously using IB-RS-2 cells for experiments 4.1 and 4.3 (Table 4.1). Prior to the virus titration of experiment 4.3, the non-specific cytopathic effect (CPE) of glycerol (routinely added as excipient to virus stocks) was assessed. Glycerol was added to media at ratio of 1:1 with cell supernatants for each of the three viruses (A/May-97, A/MAY/2/2011 and A/VIT13/2015) and heat-treated at 61°C for 30 minutes, after which the viruses were tested in duplicate on the same plate titrated using a two-fold dilution series. The virus titration for experiment 4.1 and 4.3 were performed on the three viruses of the untreated and heat-treated at three different temperatures 51°C, 56°C or 61°C for 30 minutes. These experiments consisted of a four-fold titration, in duplicate carried out in flat-bottom 96 well micro-titration plates (Fisher Scientific) using a pre-diluted virus, which has an expected virus dose of 100$TCID_{50}$. The plates were sealed and incubated at 37°C for three days before being examined by microscope for CPE. The 50% endpoint titre was calculated following the Spearman-Kärber method (Kärber, 1931; Spearman, 1908).

4.3.7 Virus neutralisation test (VNT)

The method for the VNT was described in Chapter 3, material and method section 3.3.5. Both the vaccine and field viruses were tested simultaneously under the same condition with modification (as described in (i) and (ii) below) to capture the effect of 12S capsid particle on VNT. Two preparations ((i) and (ii)) were made for both the vaccine viruses and field viruses:

(i). Untreated - The viruses were prepared as standard with media (HMEM, field antibiotics and NaOH) as diluent to dilute the untreated and sera.

(ii). "Dissociated Virus Particles" (DVPs) - Heat-treated viruses (detail in section 4.3.4) were used as diluent (to replace the media) to dilute the untreated and sera tested.

For experiment 4.3, three virus doses were used. Dilutions were used so that a four-fold dilution fell on either side of the estimated 100$TCID_{50}$. The r1 values of both the untreated and DVPs were calculated from neutralization titres at a virus dose of 100$TCID_{50}$. Comparison of the neutralisation titres of the untreated and DVPs were carried out using a paired T test using Minitab version 17.

Note:

When one of the three virus doses showed no CPE (neutralization) in all designated wells the maximum, titre (3.15 log_{10}) was accepted to draw the linear regression line in order to estimate the neutralisation titre.

4.3.8 Liquid phase blocking ELISA (LPBE)

The liquid phase blocking ELISA (LPBE) was carried out for experiment 4.3 to assess the impact of 12S capsid particles on FMDV-specific antibody titres. Unlike the LPBE method used in Chapter 3, (described in material and method section 3.3.6), both the vaccine and field viruses were infectious (live viruses) used in the test. The untreated virus and DVPs preparations for LPBE were made the same way as VNT (detailed in 4.3.7). Both the untreated and DVPs of the vaccine virus and field virus were tested simultaneously as described in chapter 3, materials and methods section 3.3.6 (described by Hamblin et al. 1986 (Hamblin et al., 1986)). In these experiments, post-vaccination sera collected at 21 and 56dpv from three different cattle (Animal IDs 281, 283 and 284) were used. For the

field isolate, LPBE was performed using only the sera from one individual Animal ID 284 at 21 and 56dpv. For this chapter, only optical density (OD) values were recorded and the total antibody titres were not calculated.

4.3.9 FMDV purification and capsid integrity assessment of homologous and heterologous viruses using PaSTRy method

Three FMD viruses (vaccine virus: A/May-97, field isolates: A/MAY/2/2011 and A/VIT/13/2015) were tested using the PaSTRy method. Purification was carried out using a sucrose cushion method (Brown and Cartwright, 1963). Briefly, the cell lysates from infected monolayers of ZZR cells (in 175ml flasks) were clarified by centrifugation at 2,060 g at 4 °C for 20 min to remove debris and 7.5% (w:v) PEG 6,000 was used to precipitate the virus in the supernatant. Precipitated viruses were resuspended in PBS, clarified and then pelleted over a 30% sucrose cushion by centrifugation at 104,000 g at 12 °C for 2.5 h. Pellets were resuspended in PBS, overlaid onto a 15–30% sucrose gradient and then fractionated by centrifugation at 104,000 g at 12 °C for 3 h. The concentration of virus was determined by spectrophotometric quantification using the following formula: (OD260 × Total volume)/7.6) = mg of virus.

The PaSTRy method that measures viral RNA when the capsid is dissociated was performed in accordance to Kotecha et al (Kotecha et al., 2016) using an Agilent MX3005 PCR machine (Walter et al., 2012). A total of 0.4 µg of virus and SYBR green-II dye (Molecular Probes, Invitrogen; final dilution 1:1000) was used to quantify the RNA released. Reactions were set up in 96-well PCR plate. Temperature was ramped from 25°C to 94°C in 0.5°C increments with 10 seconds intervals between temperatures. Fluorescence was read with the excitation and emission spectra at wavelengths of 490nm and 516nm to detect the release of viral RNA. Virus purification and PaSTRy method was carried out in collaboration with Dr. Julian Seago, from TPI.

4.4 Results

4.4.1 Experiment 4.1

In this experiment, the VHHs DAS ELISA was conducted to assess the effect of mild heat treatment on non-stabilised (not glycerinated) FMD viruses for the production of 12S capsid particles. Using this assay, the OD value measured were an indicator of the presence of 12S capsid particles. The increase in the OD value after the mild heat treatment at 45°C of the A/May-97 (vaccine virus) was obvious (Figure 4.5a), but for A/MAY/2/2011 (field isolate) the increase in OD was very small (0.049, Figure 4.5b). Consistent with these results, the baseline OD values for these two viruses were also very different, with A/May-97 (vaccine virus) a higher OD (by a factor of 2.8 times) was measured which was indicative of more 12S capsid particles being present in the non-heat-treated samples. Although the amount of 12S was not directly quantified in the assay, these observations suggest that the capsid integrity of the field isolate was more stable compared to the vaccine virus under storage conditions and after mild heat treatment. The virus titration performed in this experiment showed that the vaccine virus which was heat-treated at 45°C had a lower virus titre (5.43 $\log_{10}$) compared to the untreated vaccine virus (5.84 $\log_{10}$). However, for the field isolate, the untreated virus has a virus titre of 6.63 $\log_{10}$ which was only slightly lower than the heat-treated field virus (6.78 $\log_{10}$). These findings indicted that mild heat-treatment at 45°C was not sufficient to completely dissociate both the FMD viruses. Therefore, higher temperatures were used for the subsequent experiments 4.2, 4.3 and 4.4.

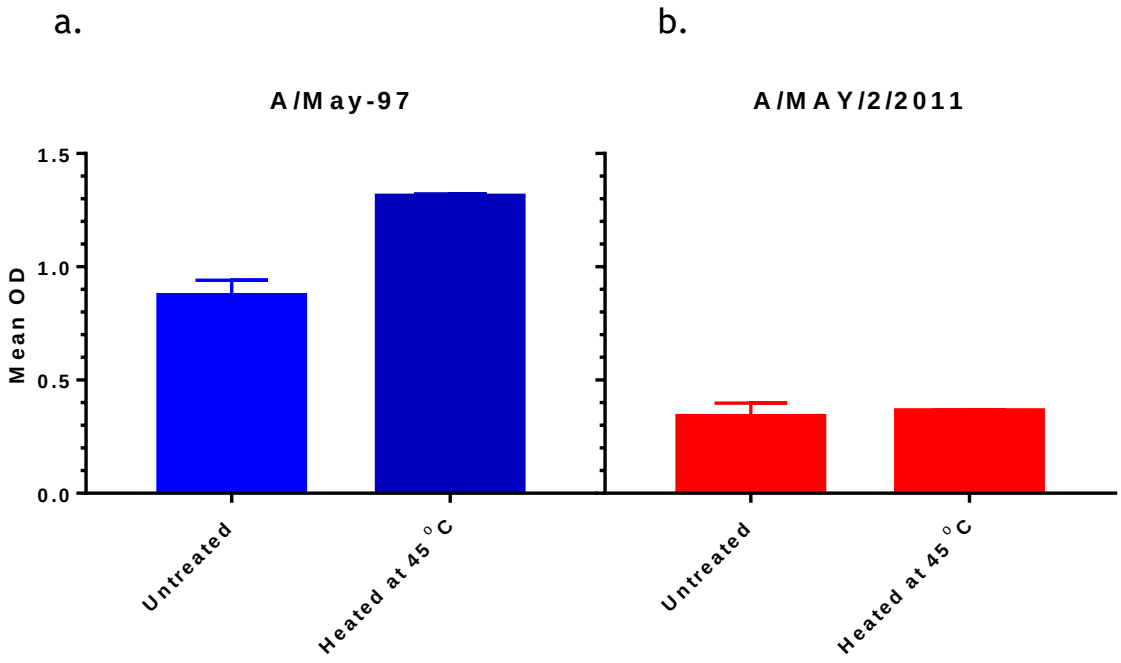


Figure 4.5: The amount of 12S capsid particle in FMDV measured using VHH DAS ELISA. Panel (a) A/May-97 (vaccine virus) and panel (b) A/MAY/2/2011 (field isolate). The amount of 12S capsid particles are measured by mean optical density (OD) on the y-axis. On the x-axis are the treatment given to the viruses. The higher the OD value the more 12S capsid particles are present.

4.4.2 Experiment 4.2

Viral capsid integrity assessment using the PaSTRy method

The relative stability of the FMDV vaccine virus (A/May-97) and two field isolates (A/MAY/2/2011 and A/VIT/13/2015) were determined using PaSTRy analysis. First negative derivative plots of the respective dissociation curves are shown in Figure 4.6. The results show that A/May-97 (vaccine virus) was the most unstable, exhibiting peak RNA release (T_r), monitored as an indicator of capsid dissociation, at 53.5˚C. In comparison, the field isolates exhibited T_r values of 56.5˚C (A/MAY/2/2011) and 56.0˚C (A/VIT/13/2015).

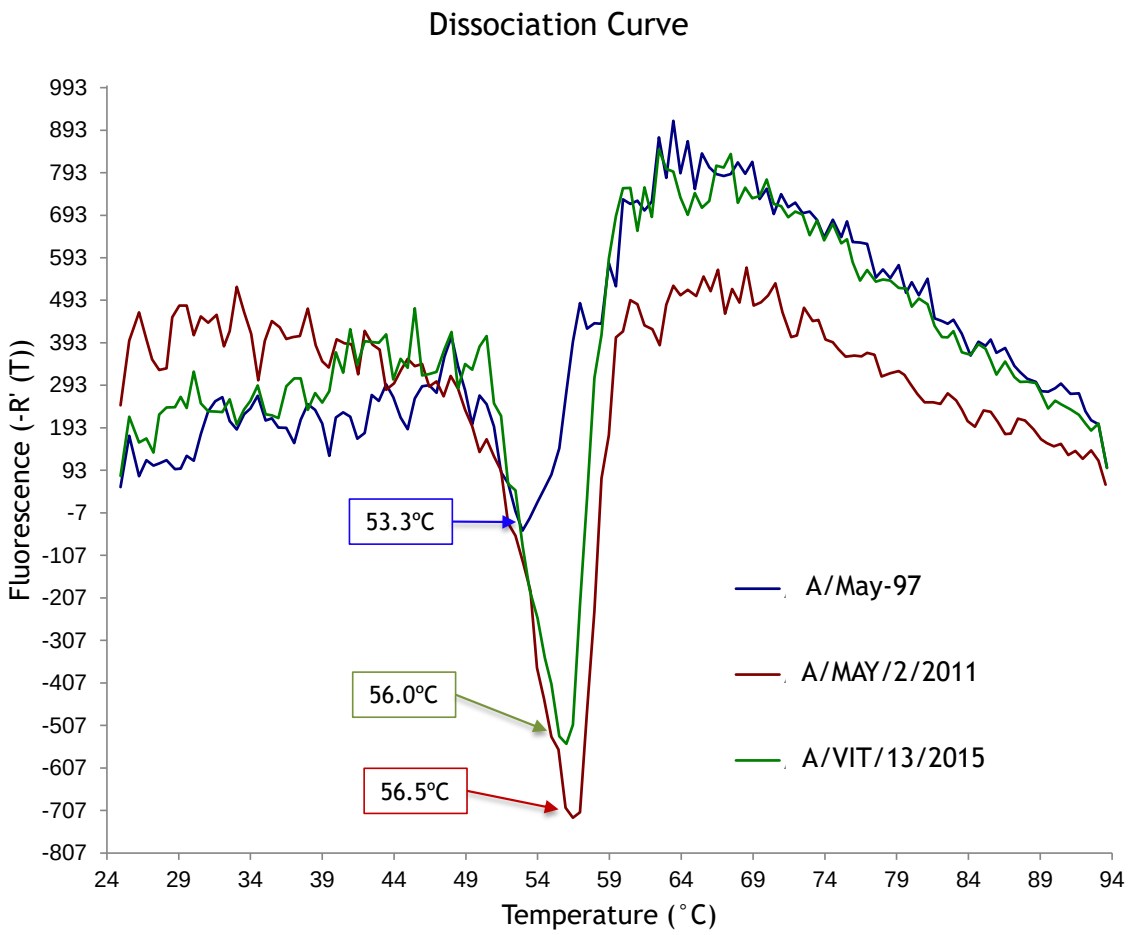


Figure 4.6: FMDV capsid integrity monitored by PaSTRy method which detects the release of the viral genome over a temperature gradient as an indicator of capsid dissociation. Dissociation curves (negative first-derivative plot) obtained for A/May-97 (blue), A/MAY/2/2011 (red) and A/VIT/13/2015 (green). Arrows indicate the temperature of viral RNA release (Tr) and hence capsid dissociation for each virus. These results are representative of three independent assays.

4.4.3 Experiment 4.3

This experiment assessed the effect of heat treatment on glycerinated (stabilised) FMD viruses and the impact of DPVs upon the results generated by VNT and LPBE methods.

4.4.3.1 VHHs DAS ELISA

The effect of heat treatment of glycerinated viruses on the production of 12S capsid particles was assessed in a vaccine virus (A/May-97) and two field viruses (A/MAY/2/2011 and A/VIT/13/2015). The three viruses were heat-treated at 51°C, 56°C and 61°C. All three glycerinated viruses tested showed different (in the range 0.1 to 0.2) initial OD values without any heat treatment, less variable and lower than the two non-glycerinated viruses from Experiment 4.1. With the heat treatment, the mean OD values for all three viruses increased with the increment of the temperatures (Figure 4.7a, b and c), and all three viruses showed the highest mean OD values after heat-treatment at 61°C (Figure 4.7a, b and c), but still did not achieve the OD values of the non-glycerinated viruses (from Experiment 4.1).

Preliminary experiments showed that when glycerol was added to media at a ratio of 1:1, a non-specific cell toxicity was generated similar in appearance to the viral cytopathic effect, and this response could also be detected when the glycerol was diluted further to 1/2, 1/4 and 1/8, although this non-specific effect disappeared when glycerol was diluted at 1/16. Virus titrations were carried out on the three different FMD viruses for both the untreated and heat-treated at the three different temperatures simultaneously using the same IB-RS-2 cell suspension to determine whether the viruses had completely dissociated (i.e. no “live” FMD virus that indicates that 146S were absent). All of the three undiluted virus stocks [A/May-97 (vaccine virus) and A/MAY/2/2011 and A/VIT/13/2015 (field isolates)] contained glycerol at 1:1 ratio but this effect was reduced through dilution in media from the left to the right in the plate.

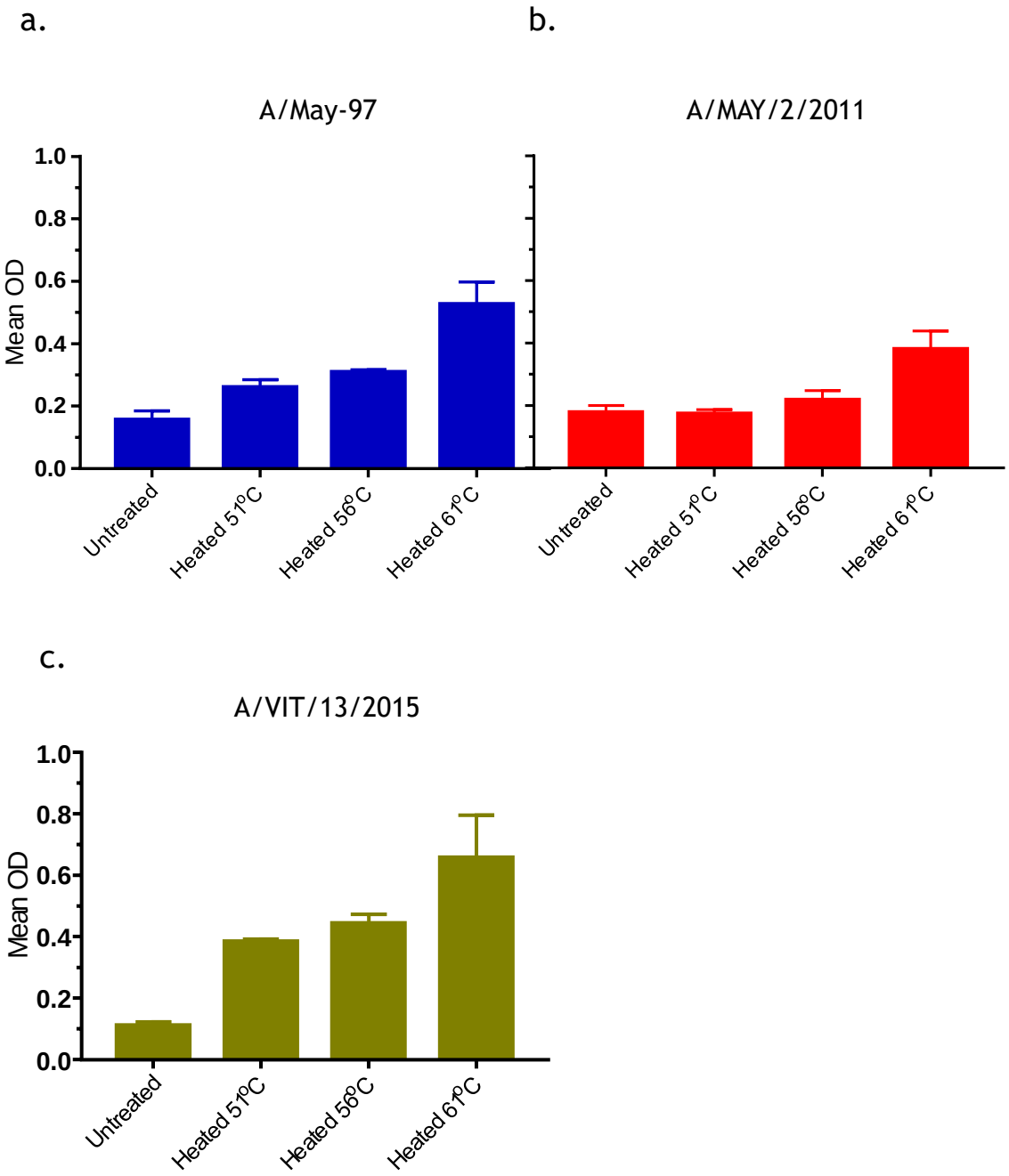


Figure 4.7: Effect of heating on the production of 12S capsid particles for the vaccine virus and field isolates of FMDV measured using the VHHs DAS ELISA. The three different colour panels represent (a) vaccine virus: A/May-97 (blue), (b) field isolate: A/MAY/2/2011 (red) and (c) field isolate: A/VIT/13/2015 (olive). The relative amount of 12S capsid particles are measured by mean optical density (OD) on the y-axis. On the x-axis are the treatment given to each of the viruses.

4.4.3.2 Virus titration

As expected, the virus titre of all the three viruses decreased with the heat treatment at 51°C and further decreased with heat treatment at 56°C. Finally, with heat treatment at 61°C, all three viruses failed to generate CPE in the cell cultures (no titre) (Figure 4.8). Results from this experiment indicated that heat-treated of glycerinated FMDV at 61°C for 30 minutes completely dissociated the viral capsid.

4.4.3.3 Virus neutralisation titre

The effect of dissociated capsid particles on VNT and LPBE were investigated using individual cattle sera collected at 21 and 56dpv. The neutralisation titres determined using the A/May-97 (vaccine virus) and A/MAY/2/2011 (field isolate) of FMDV containing the DVPs material were consistently lower than the neutralisation titres generated with the control viruses (Figure 4.9a to 4.9f and 4.10a to 4.10f) for all three virus doses. However, variation was observed for sera of animal ID 284 at 21dpv and sera of animal ID 283 at 56dpv against the A/May/2/2011 (field isolate) at virus dose 1.43 $\log_{10}$ (Figure 4.8f and 4.9d). Subsequently, when adjusted to 100$TCID_{50}$ the neutralization titres of both viruses containing DPVs were always lower than the untreated controls viruses for all individual sera of animal ID at 21 and 56dpv (Table 4.2). However, all neutralisation titres of all sera tested were above the $\log_{10}$ 1.4 cut-off. The paired T-test indicated that the neutralisation titres of the untreated virus were significantly higher compared to the neutralization titres of DVPs (p<0.05). The r1 values for the neutralization titres showed variable, values above and below the suggested vaccine-match cut-off (0.3) as in Table 4.3.

Virus dilution	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6.0	6.6
A/May-97 (Untreated)	+	+	+	+	+	+	-	-	-	-
	+	+	+	+	+	+	-	-	-	-
A/May-97 (Heated at 51°C)	+	+	+	+	+	-	-	-	-	-
	+	+	+	+	-	-	-	-	-	-
A/May-97 (Heated at 56°C)	+	+	+	+	-	-	-	-	-	-
	+	+	+	+	-	-	-	-	-	-
A/May-97 (Heated at 61°C)	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-

Virus dilution	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6.0	6.6
A/MAY/2/2011 (Untreated)	+	+	+	+	+	+	+	-	-	-
	+	+	+	+	+	+	+	-	-	-
A/MAY/2/2011 (Heated at 51°C)	+	+	+	+	+	+	-	-	-	-
	+	+	+	+	+	+	-	-	-	-
A/MAY/2/2011 (Heated at 56°C)	+	+	+	+	+	-	-	-	-	-
	+	+	+	+	-	-	-	-	-	-
A/MAY/2/2011 (Heated at 61°C)	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-

Virus dilution	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6.0	6.6
A/VIT/13/2015 (Untreated)	+	+	+	+	+	+	+	+	+	-
	+	+	+	+	+	+	+	+	-	-
A/VIT/13/2015 (Heated at 51°C)	+	+	+	+	+	+	-	-	-	-
	+	+	+	+	+	-	-	-	-	-
A/VIT/13/2015 (Heated at 56°C)	+	+	+	+	+	-	-	-	-	-
	+	+	+	+	-	-	-	-	-	-
A/VIT/13/2015 (Heated at 61°C)	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-

Figure 4.8: Virus titres of three untreated FMD viruses compared to the heat-treatment at 51°C, 56°C and 61°C. Individual CPE data (indicated by a +) for vaccine virus A/May-97 (blue), field viruses A/MAY/2/2011 (red) and A/VIT/13/2015 (olive) is shown where the top row shows the dilution of the virus from 1.2 log10 to 6.6 log10 with a 0.6 log10 (4x) interval (in duplicate).

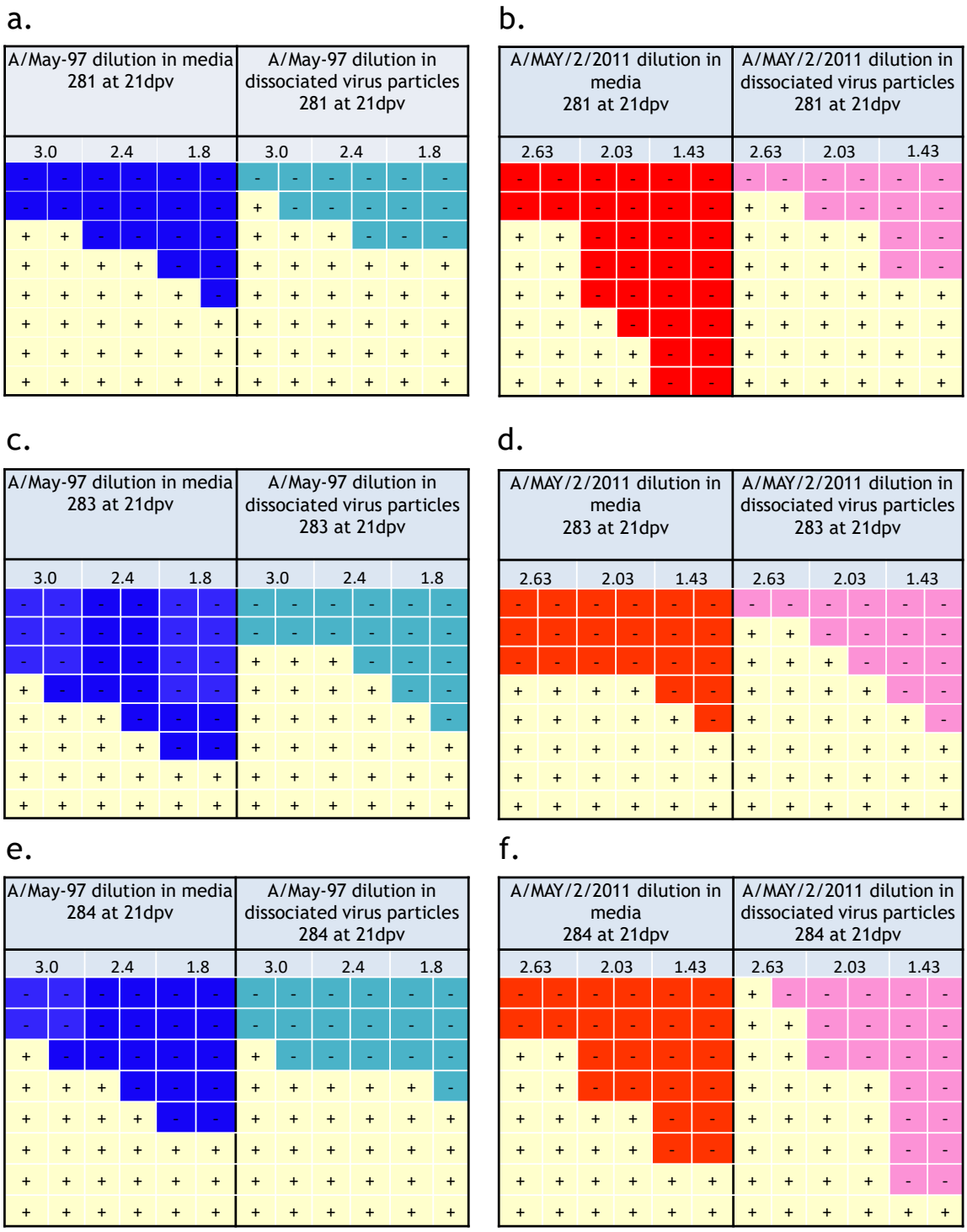


Figure 4.9: Neutralisation titres of individual sera for animal ID 281, 283 and 284 collected at 21dpv. The three different panels where (+) and (-) represents wells with and without CPE, respectively are (a) and (b) for animal ID 281 against vaccine virus and field isolate, (c) and (d) for animal ID 283 against vaccine virus and field isolate and (e) and (f) for animal ID 284 against vaccine virus and field isolate. Boxes with the shades of navy blue and light blue represent the neutralization titres against vaccine virus at three virus doses (3.0, 2.4 and 1.8 log10). Boxes with shades of red and pink represent the neutralization titres against field isolate at three virus doses (2.63, 2.03 and 1.43 log10). The maximum titre (3.15 log10) was accepted for DVPs of the heterologous virus for individual sera of animal ID 281 collected at 21dpv (b).

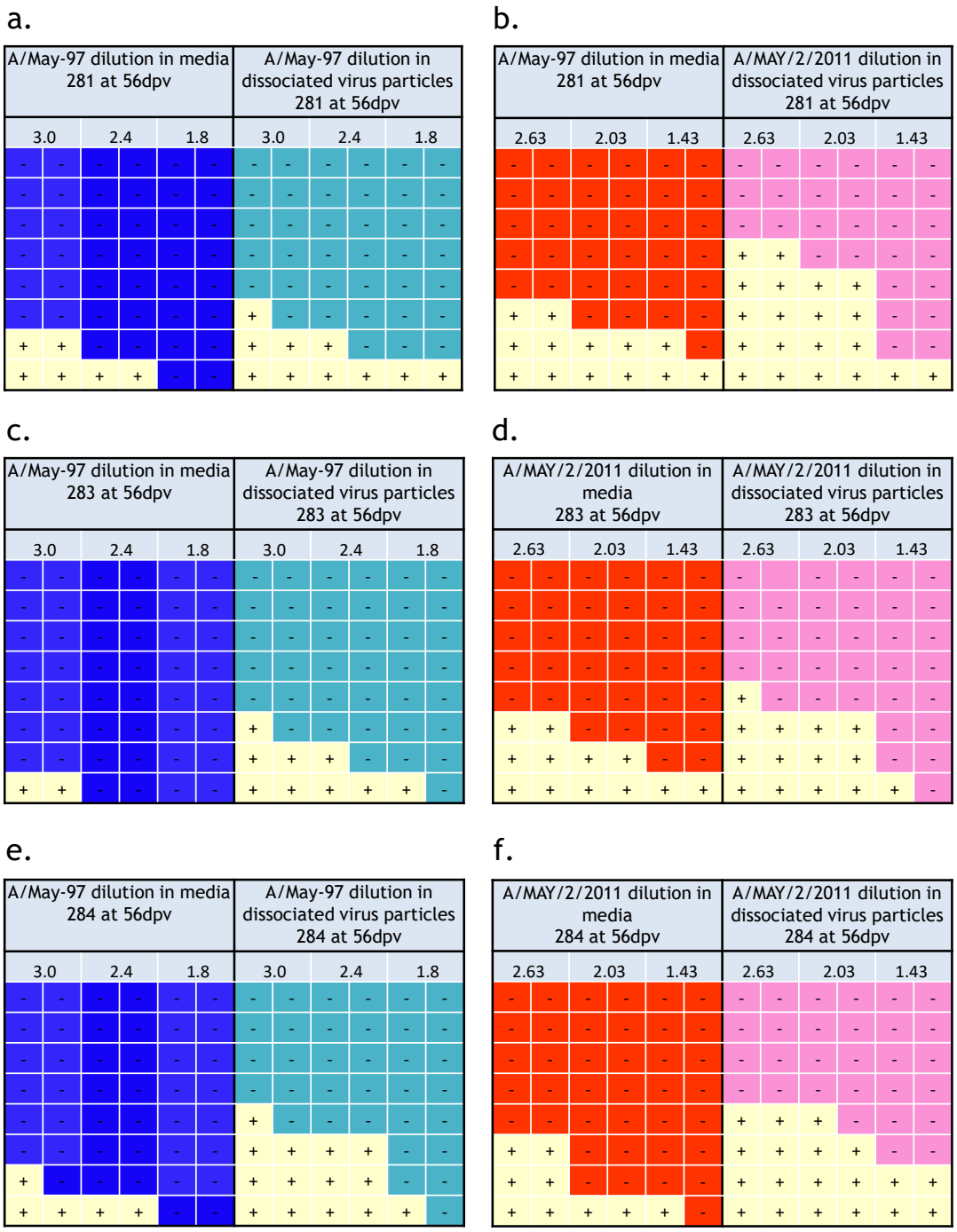


Figure 4.10: Neutralisation titres of individual sera for animal ID 281, 283 and 284 collected at 56dpv. The three different panels where (+) and (-) represents wells with and without CPE, respectively are (a) and (b) for animal ID 281 against vaccine virus and field isolate, (c) and (d) for animal ID 283 against vaccine and field isolate and (e) and (f) for animal ID 284 against vaccine virus and field isolate. Boxes with the shades of navy blue and light blue represent the neutralization titres against vaccine virus at three virus doses (3.0, 2.4 and 1.8 log10). Boxes with shades of red and pink represent the neutralization titres against field isolate at three virus doses (2.63, 2.03 and 1.43 log10). The maximum titre (3.15 log10) was accepted for DVPs of the field isolate for individual sera of animal ID 281, 283 and 284 (a, c and e respectively) of sera at collected 56dpv against the untreated vaccine virus.

Table 4.2: The neutralisation titres at 100TCID$_{50}$ for A/May-97 (vaccine virus) and A/MAY/2/2011 (field isolate) including DVP compared to the untreated controls. Three sera of different individual animals were used for these studies was collected at 21 and 56dpv. Neutralisation titre of >log$_{10}$1.4 is indicative of protective cut-off.

Animal Id	Sampling time	Viruses	Neutralisation titres (Untreated)	Neutralisation titres (DVP)
281	21dpv	Vaccine virus	1.96	1.60
283	21dpv	Vaccine virus	2.40	1.85
284	21dpv	Vaccine virus	2.10	1.76
281	56dpv	Vaccine virus	3.21	2.80
283	56dpv	Vaccine virus	3.46	2.91
284	56dpv	Vaccine virus	3.26	2.76
281	21dpv	Field isolate	2.46	1.47
283	21dpv	Field isolate	1.82	1.58
284	21dpv	Field isolate	1.98	1.85
281	56dpv	Field isolate	2.52	2.18
283	56dpv	Field isolate	2.57	2.48
284	56dpv	Field isolate	2.72	2.22

Table 4.3: The corresponding r1 values of the untreated and DVPs of the homologous and heterologous viruses for three different sera at 21 and 56dpv.

Animal Id	Sampling time	r1 value (Untreated)	r1 values (DVP)
281	21dpv	3.16	0.73
283	21dpv	0.26	0.53
284	21dpv	0.75	1.25
281	56dpv	0.20	0.24
283	56dpv	0.13	0.37
284	56dpv	0.29	0.29

N.B.: In red are r1 values below the antigenic-match value (0.3) recommended by the OIE

4.4.3.4 Liquid phase blocking ELISA (LPBE)

The effect of the DVPs on FMDV-specific antibody titres (for three individual sera, collected at 21dpv and 56dpv) measured with LPBE were assessed. The total antibody titres against the A/May-97 (vaccine virus) for all three sera with animal IDs 281,283 and 284 at 21dpv and 56dpv showed that the mean optical density (OD) of the DVPs were consistently higher than the OD of the untreated virus (Figure 4.11a to 4.11d and 4.12a and 4.12b). In LPBE, higher ODs related to lower total antibodies titres. The LPBE was extended to determine the total antibodies titre of sera with animal ID 284 at 21 and 56dpv against the A/MAY/2/2011 (field isolate) to determine the impact of 12S capsid particle. Similarly, the total antibodies titres against the field isolate using the same sera of animal ID at 21dpv and 56dpv showed higher mean optical density (OD) of the DVPs compared to the untreated field isolate of FMDV (4.12c and 4.12d).

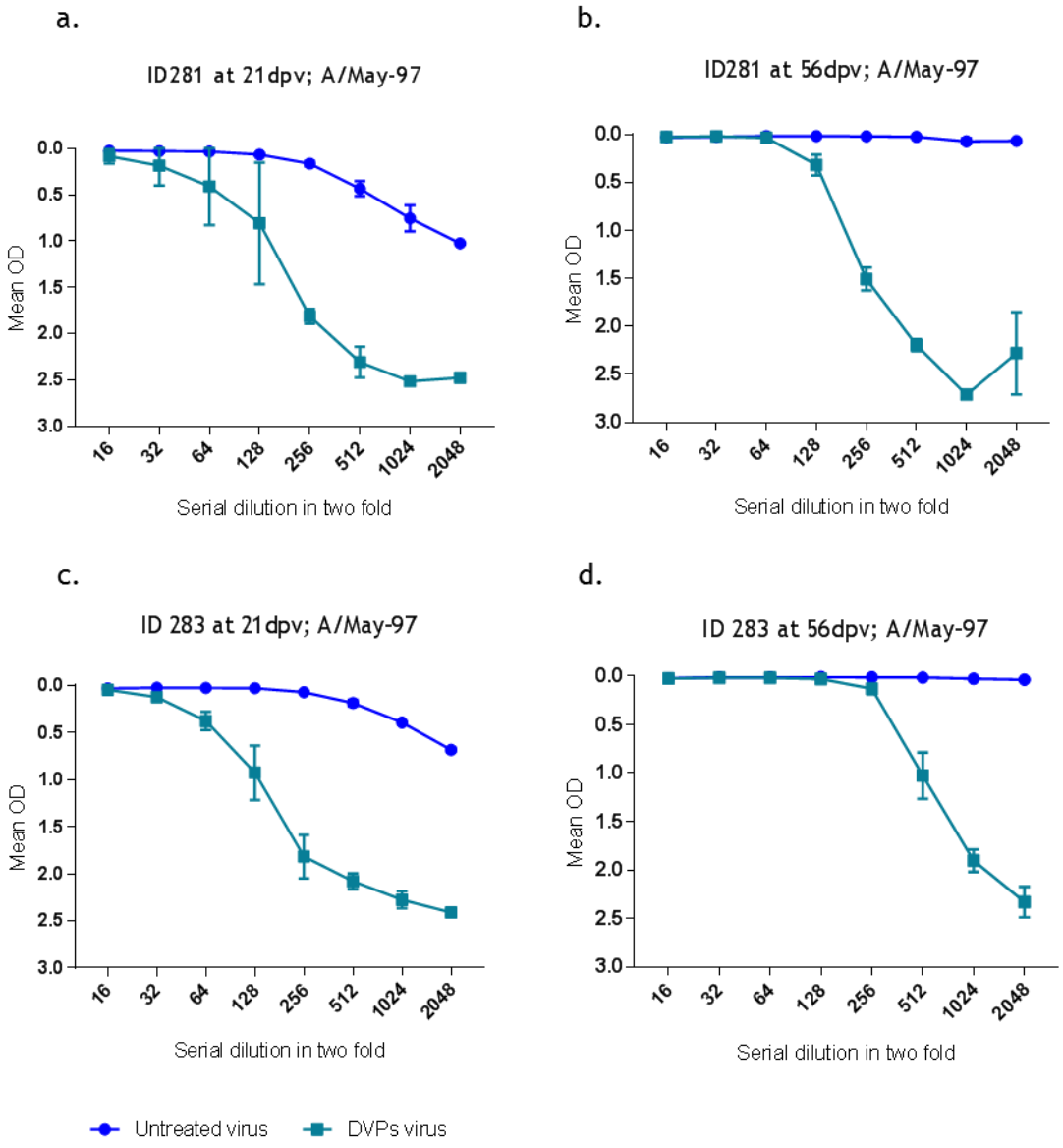


Figure 4.11: Mean OD values generated with LPBE for two individual sera of animal ID 281 and 283 against A/May-97 (vaccine virus). The four panels are (a) animal ID 281 at 21dpv, (b) animal ID 281 at 56dpv, (c) animal ID 283 at 21dpv and (d) 2 animal ID 283 at 56dpv. On the x-axis are the 2-fold serial dilutions of the sera. Line and markers with blue and green represent the mean OD values for the homologous virus.

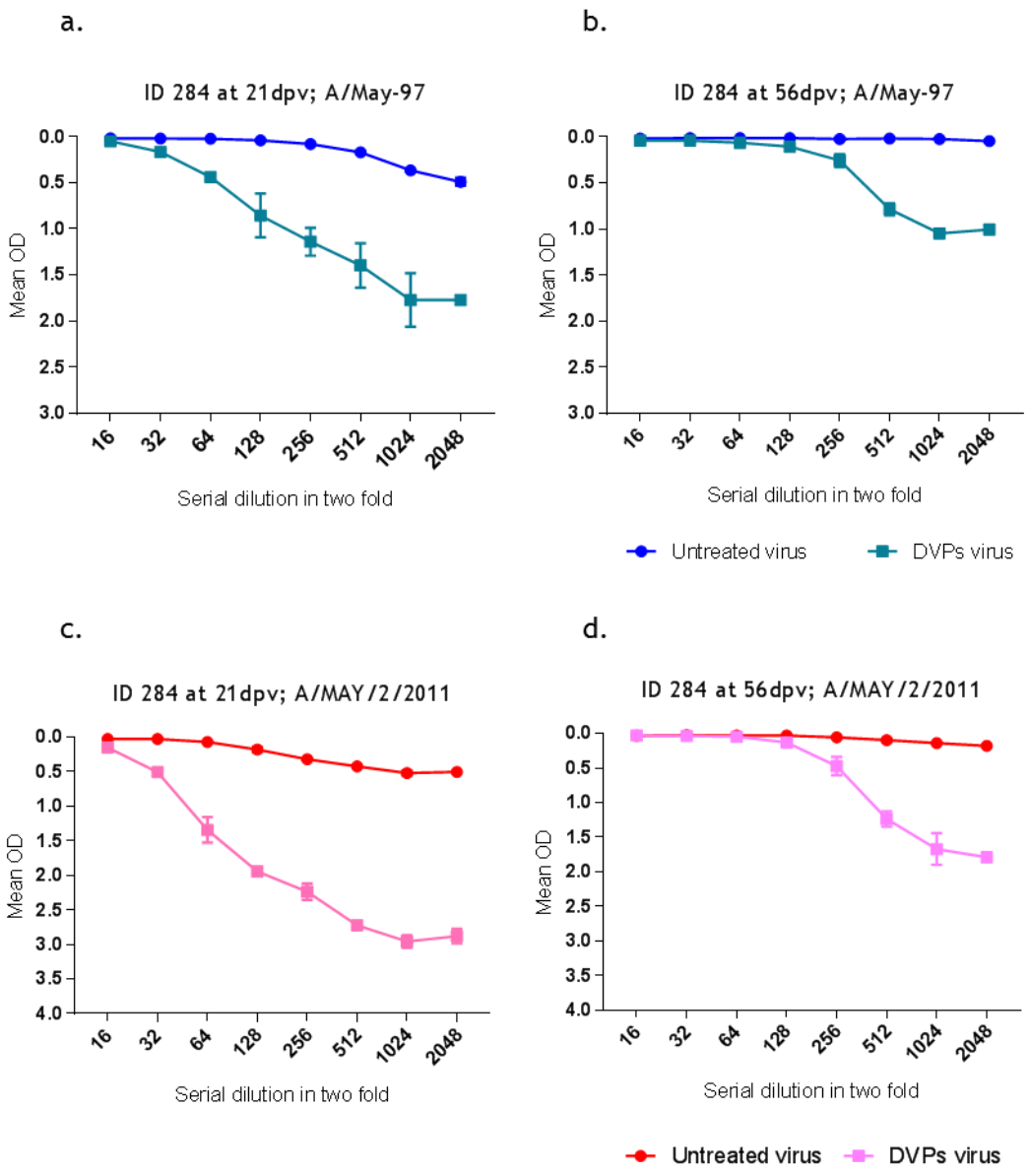


Figure 4.12: Mean OD values generated with LPBE for individual sera of animal ID 284 against A/May-97 (vaccine virus: blue and green plots) and A/MAY/2/2011 (field isolate: red and pink plots). The four panels are (a) animal ID 284 at 21dpv against vaccine virus, (b) animal ID 284 at 56dpv against vaccine virus, (c) animal ID 284 at 21dpv against field isolate and (d) animal ID 284 at 56dpv against field isolate. On the x-axis are the 2-fold serial dilutions of the sera.

4.4.4 Experiment 4.4

Impact of heat treatment on capsid integrity; comparison of FMDV field isolates from two different serotype A lineages (A/ASIA/Sea 97 and A/ASIA/Iran 05)

The impact of heat treatment on the capsid integrity by the production of 12S capsid particles for representative FMD viruses within two different lineages of FMDV serotype A were compared using the VHHs DAS ELISA. Viruses from both lineages showed a similar pattern with an increase in 12S capsid particles generated by heat-treatment as indicated by an increase in the OD values (Figure 4.13a and b). Without any heat treatment, all FMD viruses from both lineages contained 12S capsid particles corresponding within range of OD values between 0.175 to 0.277 for lineage A/ASIA/Sea-97 and 0.188 to 0.336 for lineage A/ASIA/Iran-05, respectively. Analysis of variance on the OD values of viruses of the same lineage after heat treatments indicated that there was a significant difference between the heat treatment groups of the lineages for both A/ASIA/Sea-97 ($P<0.05$) and A/ASIA/Iran-05 ($p<0.05$). However, comparison of the mean OD values between the two lineages receiving the same heat treatments showed no significant different between the two lineages $p>0.05$.

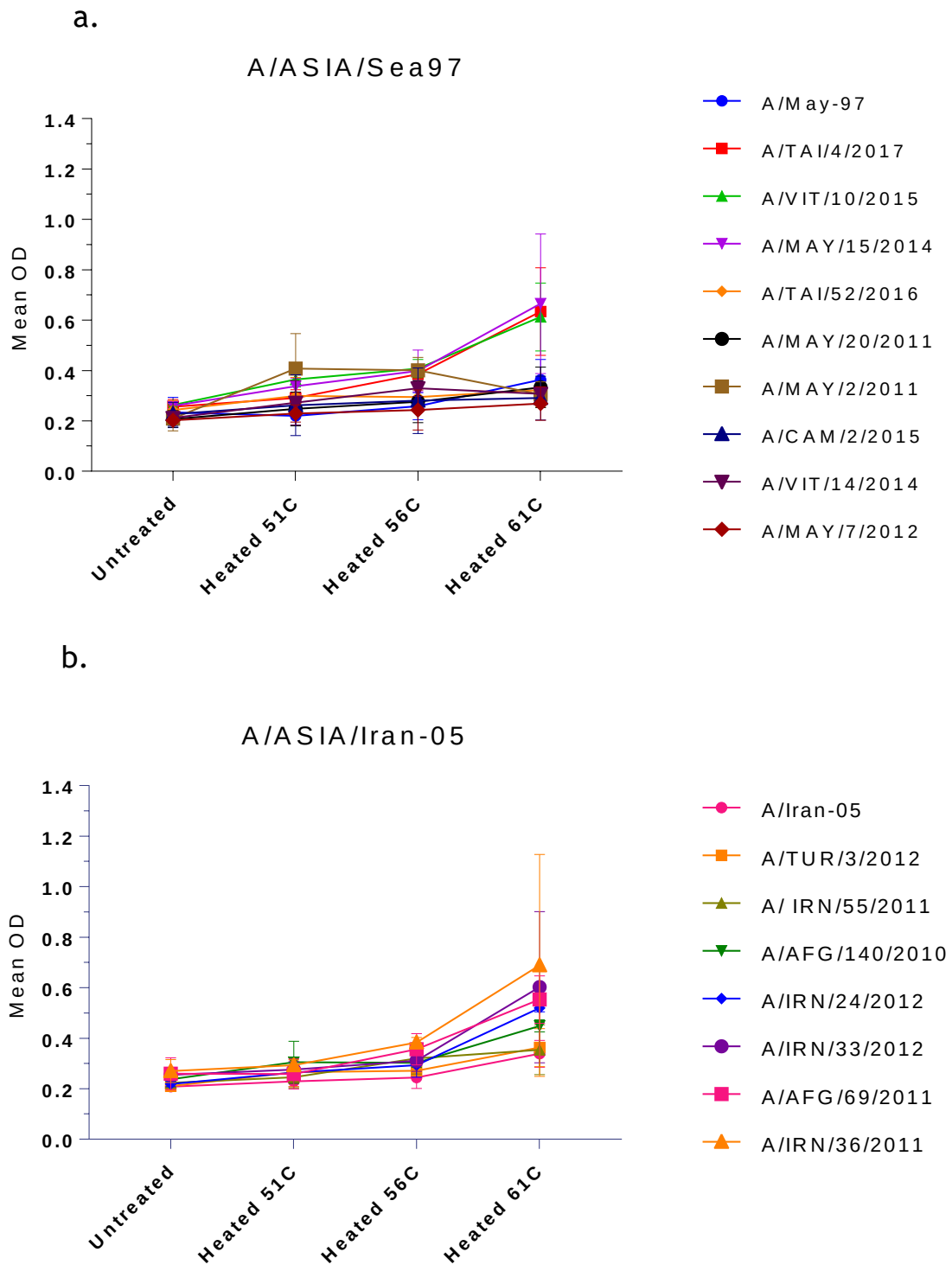


Figure 4.13: The effect of heating on the 12S capsid particle production of two different lineages of FMDV serotype A viruses measured using VHH DAS ELISA. Panel (a) A/Sea-97 and (b) A/Iran-05. The relative amount of 12S capsid particles are measured by mean optical density (OD) on the y-axis. On the x-axis are the treatment given to the viruses.

4.5 Discussion

The work in this chapter investigated the impact of viral capsid integrity of FMDV serotype A in order to understand whether dissociated viral particles (DVPs) can influence the results of *in-vitro* vaccine matching methods.

Heat-treatment was used to mimic long-term storage of FMDV; to break down FMDV 146S capsid into component fragments such as 12S which were measured using a specific ELISA. Different temperatures were used to assess the relative stability of two different serotype A field viruses to heat treatment, where a greater increase in ELISA signal was observed for A/VIT/13/2015 at lower temperatures as compared to A/MAY/2/2011. These results provide a first indication that the production rate of the 12S capsid particles are not the same for each virus; findings that were further supported by the results from the PaSTRy method, where A/MAY/2/2011 had the most stable capsid followed by the A/VIT/13/2015 and finally A/May-97 (vaccine virus). Unexpectedly the vaccine virus capsid integrity was found to be more sensitive to heat treatment as compared to the capsid integrity of the field isolates. The reason for vaccine virus capsid being less stable than the capsid of the field isolates is not known. However, it may be because the vaccine virus has been adapted in cell culture systems over many passages.

When added back into the FMDV test samples, the 12S capsid particles appeared to reduce the FMDV titres that were measured by CPE on the IB-RS-2 cells. For post-vaccination sera, the serum neutralization titres were consistently (and significantly) higher in the untreated sera than the paired samples containing DVPs. These differences between the measured neutralization titres were usually greater than a two-fold dilution (0.3 $\log_{10}$). Similar observations were made when the same individual sera were tested using LPBE, where the mean ODs for the FMD viruses containing DVPs were consistently higher than the OD of the untreated viruses. In other words, the added DVPs influenced the LPBE to lower the FMDV-specific antibodies that were detected. These findings are in agreement with the other reports that indicate 12S capsid particles can reduce the virus neutralization titres of guinea pigs and mice (Cartwright et al., 1980; Meloen et al., 1979; Rowlands et al., 1975).

The results from this study demonstrate that DVPs can influence the serum antibody titres measures by VNT and LPBE. When considering vaccine-matching studies, these effects may be particularly important in cases where the ratio between 146S (live FMDV) and the DVPs varies between the vaccine virus and field virus. There is evidence from this study that different FMD virus preparations have different levels of DVPs. For instance, while A/VIT/13/2015 (field isolate) virus had the highest virus titre (measured by VNT)

compared to A/May-97 (vaccine virus) and A/MAY/2/2011 (field isolate), the untreated sample for this virus generated the lowest OD values in the VHHs DAS ELISA. This may indicate that the A/VIT/13/2015 virus had proportionally more intact virus compared to the other two viruses. To further compare the differences in capsid stability between viruses, a pilot study (Experiment 4) was performed to examine the effect of heat-treatment on different field viruses, and although the changes were not significant across two FMDV lineages, small differences between isolates were observed (particularly at 51°C and 56°C); findings which further support the idea that the impact of heat on different viruses is not necessarily the same.

In addition to heat-treatment, it is also reported that long-term storage of inactivated FMD virus at -70ºC does not prevent 146S degradation, and storing FMD virus for long-term in liquid nitrogen is recommended since these conditions do not significantly affect the immunogenic properties of the virus (Ferris et al., 1984). Consideration of storage conditions is especially important since the *in-vitro* vaccine matching methods employed in FMD Reference Laboratories frequently use long-term stored FMD virus stocks. The act of freezing and thawing the viruses may also increase the dissociation of FMDV capsid particles present in a sample. Therefore, it is suggested to store FMD viruses in aliquot for single use in order to prevent the formation of additional dissociated capsid particles. The current *in-vitro* vaccine matching methods use glycerol as excipient to stabilise the viruses used in the tests. Other study suggests that glycerol stabilises the virus and hinders the production of capsid intermediates (Yang et al., 2017). Therefore, adding glycerol may reduce variability of *in-vitro* vaccine matching. Furthermore, inactivated virus using BEI has also been shown to reduce the dissociation of 146S particles. Moreover, the use of inactivated virus is much easier and safer to use for the LPBE that does not require live virus.

Previous studies using other methods such as SDG and HPLC only reported 12S capsid particles in heated or acid treated FMD viruses (Spitteler et al., 2011; Yang et al., 2013). Data from this study suggests that the VHHs DAS ELISA is more sensitive compared to the other methods, and may provide a simple method to assess FMDV capsid stability. In summary, the results presented in this chapter suggest that the hypothesis can be accepted: i.e., that 12S capsid particles can mop up antibodies that would otherwise be available for detection by *in-vitro* vaccine matching methods. This results in an artificial decrease in neutralisation titres (measured by VNT) and FMDV-specific antibody titres (measured by LPBE), and may play a central role as a factor that influences the variability of vaccine-matching tests.

Chapter 5:

Foot-and-mouth disease virus-specific serological immune responses of cattle in Peninsular Malaysia following vaccination

Acknowledgements:

Jamaliah Senawi acknowledges assistance from Ginette Wilsden and Clare Browning to perform virus neutralization testing of the serotype O viruses. Jamaliah Senawi generated all of the remaining laboratory data presented in this chapter.

5.1 Abstract

FMD vaccination together with other control measures has contributed to the success of eradication campaigns in countries such as those in Western Europe and most parts of South America. However, at present there are still many countries in Asia and African regions that are endemic for FMD despite the use of prophylactic and responsive vaccination. These FMD endemic countries are facing detrimental losses to livestock production and cost to control FMD, in addition to losing opportunities to access lucrative markets for livestock trade. In this chapter, a field experiment was carried out in a dairy cattle farm located in area with lower risk of FMD in Peninsular Malaysia to assess the post-vaccination antibody responses generated in after primary and booster vaccination in calves and adult cows. In this farm, serum samples were collected from young calves at different ages, with different levels of maternally derived antibody after primary and booster vaccination, while for cow serum samples were only collected after booster vaccination. Additionally, dairy cattle farms located in areas with lower and higher risk of FMD that received multiple FMD vaccinations were assessed for antibody against the non-structural protein of FMD virus. No NSP positive detected from all 48 cows and 51 calves at all sampling points of the selected dairy farm located in lower FMD risk area. In contrast, 32% of cattle were NSP positive in the high risk FMD area. For dairy farm located in lower risk area, all cows showed neutralisation antibody titre against the vaccine strains higher than the suggested protective cut-off points of both serotypes A (2.84 $\log_{10}$ $\pm$ 0.27 $\log_{10}$) and O (2.54 $\log_{10}$ $\pm$ 0.49 $\log_{10}$). Moreover, >90% of cows that received multiple FMD vaccinations showed neutralisation antibody titre against the field strains of serotype A (A/MAY/2/2011) and serotype O (O/MAY/10/2016) above the suggestive protective cut-off point. The calves showed that maternally derived antibody titre is higher in younger calves and reduces with age. Maternally derived antibody also interfered negatively with primary neutralisation titre but does not affect neutralisation antibody titre after booster vaccination. These findings indicate that the vaccine tested was suitable for use against the serotype A field viruses tested, despite that fact that r1-values generated from neutralisation titres in all groups (either after primary, booster or multiple booster vaccination) had a high degree of variability.

5.2 Introduction

Countries with FMD endemic status face annual losses due to impacts on livestock production and vaccination that alone is estimated at a value of US$ 6.5 to 21 billion (Knight-Jones and Rushton, 2013; Knight-Jones et al., 2017). Vaccination is an important tool that has contributed to the successful control and eradication of FMD in Western Europe and parts of South America (Clavijo et al., 2017; Leforban and Gerbier, 2002; Saraiva, 2004). In addition, "vaccination-to-kill" policies have been adopted in the Netherlands during 2001 FMD outbreak in Europe (Pluimers et al., 2002) and in Japan during the 2010 FMD outbreak in Miyazaki (Muroga et al., 2012). These policies shown to have contained the FMD outbreaks rapidly in FMD free countries and have helped to lift international trade restrictions. However, currently there are still many countries in Asia and African region that are endemic to FMD and are therefore without an official OIE status (O.I.E, 2018).

The country of Malaysia comprises Peninsular (P) Malaysia and the States of Sabah and Sarawak on the Island of Borneo (see Figure 5.1). P. Malaysia is connected to mainland Southeast Asia through Isthmus of Kra in the north. Malaysia has a multiracial population with unique cultural, social and livestock husbandry practices. The livestock industry only makes a small contribution to the country's economy mostly through export of poultry and processed livestock products (D.V.S., 2018). Pig production sustains the domestic consumption with exports of product to Asian countries. On the other hand, most (57%) of the ruminant livestock production is from a traditional husbandry system, while cattle that are reared integrated into oil palm plantations account for a smaller proportion (23%) of the industry. In both systems, livestock are free to feed in an open area and chances for animals to co-mingle with other herds are high particularly in the traditional system where risks for disease transmission are very high. These systems currently produce far less ruminant meat and milk than is required by the population in the country. As a result, Malaysia is dependent on imports of live ruminants, meat, milk and other dairy products from neighbouring countries, to cater for the need of the people (D.V.S., 2018). These factors emphasise the importance of regional programmes with strong cooperation between neighbouring countries and effective control at borders to successfully control FMD in Malaysia (Adullah, 2014).

In Malaysia, FMD has never been reported in Malaysian Borneo (Sabah and Sarawak) (Figure 5.1). In contrast, there has been a long history of FMD in P. Malaysia. According to Wallace, (Wallace, 1936), the first reports of a disease that resembled FMD in P. Malaysia was recorded in 1860 followed by another episode that occurred in 1909. In 1936,

a clinically similar disease was reported in the states of Perak and Selangor, Malaysia affecting 551 animals (Wallace, 1939). The first confirmed case of FMD in P. Malaysia was in 1973, when FMD virus subtype A_{22} was detected by the WRLFMD (Pirbright, UK) in samples sent to the UK (Chong, 1979). In December 1982, a disease control policy to vaccinate all susceptible livestock on P. Malaysia was implemented after an FMD eradication policy was strongly objected by the people (Babjee, 1994; Chong, 1979; Department of Veterinary Services Malaysia, 1995; Thuraisingham, 1977). However, the effectiveness of this vaccination policy was questioned in view of FMD outbreaks that occurred; initially in Pahang in 2001 (Senawi, 2012), which led to FMD outbreaks not only in the northern states of P. Malaysia, but also in all other states in P. Malaysia (Ramanoon et al., 2013). Despite continuous efforts to control FMD in P. Malaysia, the disease has expanded and there have been more FMD outbreaks recorded with more FMDV serotypes and strains over time. The range of different FMDV serotypes (and lineages) shown to be present in the country include FMDV A/ASIA/Sea-97, O/SEA/Mya-98, O/SEA/Cam-94 (2001-2003), O/ME-SA/PanAsia (2000-2001), O/ME-SA/PanAsia-2 (2003-2009), O/CATHAY (2005) and Asia 1 (Abdul-Hamid et al., 2011) as well as the FMDV O/ME-SA/Ind-2001e sub lineage that has also been recently detected (WRLFMD, 2018).

The use of prophylactic vaccination as part of FMD control measures in an endemic area has to be continuously assessed to ensure the effectiveness of the vaccine that is used (Ferrari et al., 2016a). FMDV-specific antibody responses in vaccinated animals can be measured using structural protein-specific tests such as VNT or SP-ELISAs (Anna Ludi, 2017). However, interpretation of post-vaccination serological results is not always straight-forward due to the lack of clearly defined protective cut-offs for these tests (especially for heterologous protective responses). Furthermore, post-vaccination serological patterns in endemic countries are often complicated by the presence of FMDV-specific antibodies resulting from natural infection. In addition, maternally derived neutralisation titres have been documented to have negative influence in eliciting protective immune response induced by vaccination in young calves (Nicholls et al., 1984a). In cases where it is important to distinguish vaccinated animals from those that are naturally infected, an NSP-ELISA that detects antibodies against the FMDV non-structural protein can be used (Sorensen et al., 1998). This approach works as long as the vaccine used has been purified to remove non-structural proteins. However, antibodies against non-structural proteins take a minimum of about 8 days post infection to be detected (Sorensen et al., 1998) compared to the antibodies against the structural proteins which are generated much more quickly.

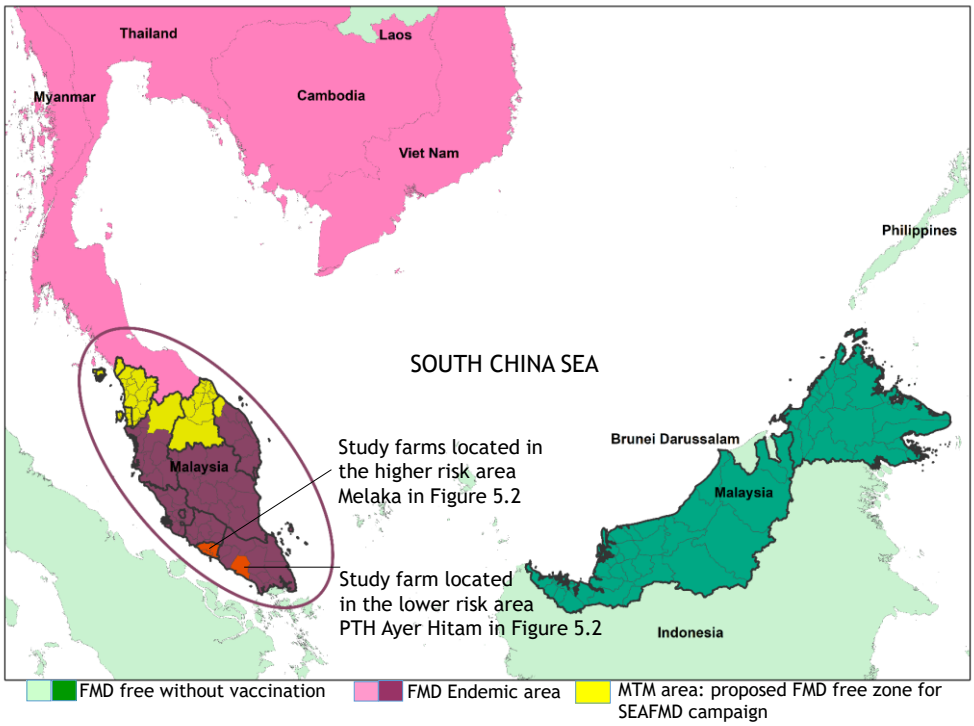
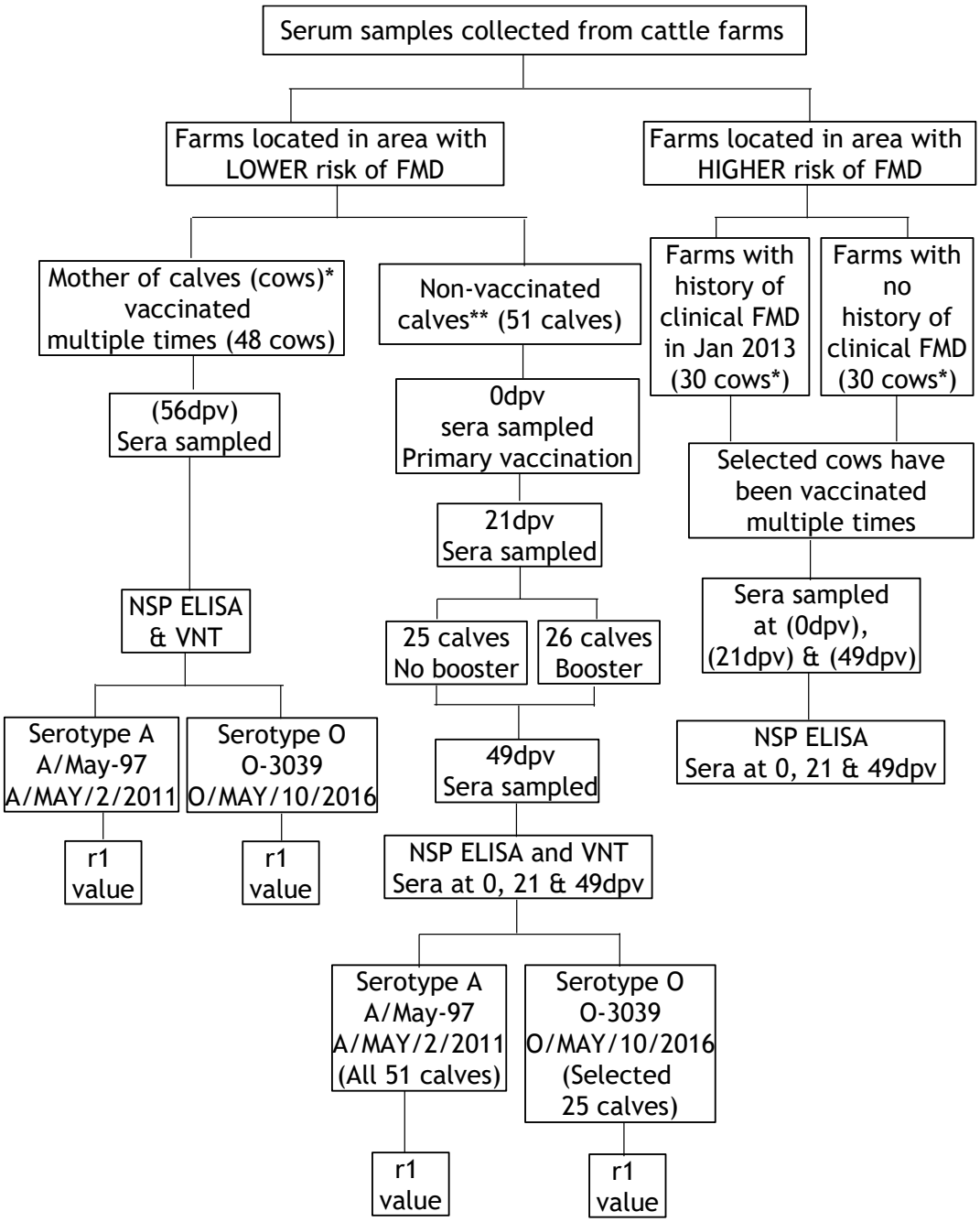


Figure 5.1: Map of Malaysia; in relation to countries in mainland Southeast Asia where FMD is endemic (pink). FMDV free areas are indicated in green. The proposed FMD free zone for Malaysia as part of the OIE SEAFMD campaign is indicated with yellow, and the states where the farms are located for this study are identified.

The previous chapters in this thesis have tested the antigenic relationship between a commonly used FMD vaccine (A/May-97) and a representative field virus from the A/ASIA/Sea-97 lineage. The results from these studies highlight (i) the variability of the *in-vitro* vaccine-matching tests and (ii) the uncertainty that can sometimes arise when using these tests to define heterologous protection. The objectives of this chapter were (i) to investigate whether the *in-vitro* vaccine matching using VNT method may provide similar picture to the field situation in a herd of cattle in Malaysia, (ii) to describe and evaluate the neutralisation titre following primary and booster vaccination in a herd of cattle in Malaysia, (iii) to assess the impact of maternally derived neutralisation antibody on the calves' neutralisation titre in a herd of cattle in Malaysia.

5.3 Materials and methods



N.B:
* Selected cows were vaccinated multiple times.
** Each calf was paired with the mother for testing. Two mothers were not available for testing.
(0dpv), (21dpv), (49dpv) and (56dpv): Not the actual 0dpv, 21dpv, 49dpv and 56dpv because the cows were vaccinated for multiple times

Figure 5.2: Schematic diagram showing the outline of serum samples collected from farms in P. Malaysia and the tests carried out.

5.3.1 Background information about the study farms and sera collection

For this study, dairy cattle herds located in two areas (states) in P. Malaysia were selected based on the risk of FMDV infection in the areas. One area was considered a lower-risk for FMD while the second area was a higher-risk area for FMD (Figure 5.2). The lower-risk farm was selected for its location, good biosecurity and zoo-sanitary control measures and husbandry practices. The farm, Pusat Ternakan Haiwan (PTH) Ayer Hitam, Kluang, Johor was located in the southern P. Malaysia (Figure 5.1) where FMD occurrence was lower than other states. This cow and calf unit is government owned and has Mafriwal dairy cattle (about 400 heads) and Friesian Shahiwal (about 100 heads). All cattle on this farm are vaccinated for FMD twice a year starting at six months of age and there have never been any clinical cases of FMD reported. All eligible cattle receive FMD vaccination during the first week of January and June every year. However, no regular post-vaccination monitoring program has been carried out on this farm to assess the performance of the FMD vaccine. For this study, a group of 51 calves, aged two to seven months were selected which had not previously received an FMD vaccine. Forty-eight out of 51 mothers (cows) of the selected calves were also included in the study. Sera were collected from all 48 cows at 56dpv and from all 51 calves at 0dpv. At 21dpv, serum samples were collected from all 51 calves after which the calves were divided into two groups. One group of 26 calves were given a booster vaccination at 21dpv, and the other group that contained 25 calves were not boosted. Finally, serum samples were collected from all calves at 49dpv.

For the second area that has a higher risk of FMD, serum samples from dairy cattle were collected to assess whether there was evidence for subclinical infection in an FMD vaccinated population. This part of the study measured NSP-specific antibody responses to recognise FMDV-infected animals in the vaccinated herds. The state of Melaka was chosen for this purpose because it has dairy cattle herds that are well monitored by the State Department of Veterinary Services (DVS). Melaka is a small state divided into three districts located in the mid-west P. Malaysia with total area of 1615.92 square kilometres. Melaka has total dairy cattle population of 2256 heads (D.V.S., 2018). No cases of clinical FMD have been recorded in dairy cattle farms in Melaka since early 2013 to November 2016. A total of 60 cows from nine private dairy cattle farms located in three different districts of Melaka were selected. Thirty of these cows were selected from herds with no history of clinical FMD, whereas the other 30 cows were selected from herds where the last clinical cases due to FMD were recorded in January 2013. Cows were aged between 2 to 4 years at the start of the study (0dpv) and were examined for FMD lesions (both old and new) during sera collection at 0, 21 and 49dpv (Figure 5.2).

5.3.2 Vaccine and Vaccination

All FMD vaccines used in P. Malaysia are produced by MERIAL/Boehringer Ingelheim and therefore this vaccine was tested on all the study farms recruited for this study. This is an aqueous vaccine with a potency of at least $6PD_{50}$ purchased by the Government of Malaysia for farmers that keep ruminants in P. Malaysia. The adult cows in the herds had received FMD vaccination on multiple occasions ranging from seven times in three years to 22 times in 10 years with the last FMD vaccination on 7th June 2016. The calves were vaccinated on 2nd August 2016 which was designated as 0 days post vaccination (dpv). At 0dpv, 2 ml of inactivated polyvalent (quadrivalent) FMD vaccine containing A/May-97, O_1 Manisa, O-3039 and Asia 1 Shamir (Aftovaxpur® Merial Animal Health Ltd) was administrated subcutaneously in the front of the shoulder of the vaccinated calves. Booster vaccination was carried out using the same dose and route at 21dpv to 26 selected calves (as described above). On the days of vaccination and sampling, every cow and calf selected for the study were physically examined to ensure no clinical signs of FMD were present.

The same vaccine and route of administration was used for the cows selected from the higher risk area; where cattle received multiple vaccination following the manufacturer recommendation starting from January 2013. These cows received booster vaccination at the start of the study ((0dpv): not first vaccination). (Figure 5.2).

5.3.3 Collection and processing of blood samples.

Sera were collected from coccygeal vein of each cow and calf using 10ml red top vacutainers without any anticoagulant (BD Franklin Lakes USA). After collection, the tubes were transported in a cool box to a regional veterinary laboratory in P. Malaysia (Makmal Veterinar Kawasan Salak Tinggi) and placed in a refrigerator at 4°C overnight. The tubes were then centrifuged at 2000g for 5 minutes using a stand-alone centrifuge (Kubota 5800). Collected sera were placed in water-bath at 56°C for 30 minutes to inactivate any viruses and complement factors that might have been present (according to Pirbright Institute biosecurity regulations) before being aliquoted into 1.8 ml cryo-vials. Aliquots of sera were stored at -20°C until transported to The Pirbright Institute for testing.

5.3.4 Non-structural protein ELISA

Sera collected at all the sampling points were tested using a commercially available kit (PrioCHECK® FMDV-NS) produced by Prionics Lelystad B.V. (Catalogue no 7610440). NSP ELISA results were used for two purposes: firstly, to ensure that all cows and calves selected for this study in PTH Ayer Hitam (lower-risk herd) were not infected with FMDV prior to, or during the study, and secondly, to test all 60 sera from the cows from Melaka (higher-risk herds) to assess whether the animals might have been infected with FMDV. Briefly, on the first day of the test, 80µl of prepared ELISA buffer was dispensed into all wells. A total of 20µl of the negative control, the weak positive control, positive control and test serum samples were dispensed into the designated wells in duplicates. The plates were sealed and left at room temperature (+19°C to +25°C) overnight. The plates were washed with washing buffer (provided in the kit) six times. The plates were dried by tapping them onto a lint-free absorbent towel. A total of 100µl of prepared conjugate was dispensed into all wells before the plates were sealed and incubated at room temperature for an hour (+19C to +25C). The plates were emptied and washed again. After drying the wells, 100µl of chromogen/substrate solution (provided in the kit) was added. The plates were then sealed and incubated at room temperature (+19C to +25C) for 20 minutes. A total of 100µl stop solution (provided in the kit) was dispense to all wells and the side of the plates were tapped to ensure even mixing before the plates were read ELISA reader machine (V-MAX model) at 450nm filter to measure the optical density (OD). The mean OD values were calculated for percentage of inhibition (PI) using the formula:

$$PI = 100 - \frac{\text{(OD of test or control samples)}}{\text{OD max}} \times 100$$

According to kit guidelines, samples ≥ 50% PI were considered as positive for FMDV NSP-specific antibodies.

5.3.5 Virus neutralisation test

The method for the VNT used the protocol described in Chapter 2 (Material and methods section; neutralisation titres). All serum collected from PTH Ayer Hitam (a total of 210 sera: 48 cow's and 51 calves sera at 0, 21 and 49dpv) were tested for antibodies against A/May-97 (homologous virus) and A/MAY/2/2011 (heterologous virus), representing the FMD vaccine virus and a representative A/ASIA/Sea-97 field isolate, respectively.

A subset of sera collected from cows and calves from PTH Ayer Hitam were also tested with VNT for FMDV serotype O-specific antibody responses. For these analyses, a total of 25 sera from mother cows and sera from paired 25 calves were selected. For the 25 sera from calves, 17 calves received the booster vaccination in comparison to 8 calves that received only a single dose of vaccine. A total of 100 sera: 25 cows and 25 calves' sera at 0, 21 and 49dpv) were tested for O-3039 (vaccine virus) and O/MAY/10/2016 (field isolate).

For both serotype A and O viruses, selected cows' sera at 56dpv and selected calves' sera at 0, 21 and 49dpv were tested with VNT. All sera were tested in batches where the same individual sera tested against the homologous/vaccine virus and heterologous/field isolate were performed simultaneously at the same time with the same cell (CSFV positive IB-RS-2) suspension.

5.3.6 Determination of the r1 value

In order to measure the antigenic cross-reactivity between the vaccine viruses and field isolates, r1 values were calculated from the neutralisation titres generated from the VNT of individual sera as described in Chapter 2 (Determination of r1 value section). The r1 values were calculated for individual sera of the cows and sera of the individual calves aged between six and seven months of age (for both 21dpv and 49dpv) against both FMDV serotype A and O. The cut-off values at 0.3 used were according to those recommended by the OIE (OIE, 2017).

5.3.7 Statistical analysis

Analysis of variance using a general linear model were carried out on the data gathered for FMDV serotype A and serotype O. The linear model was built independently for serotype A and serotype O to assess the mean difference and interaction between factors. The factors were (i) viruses: homologous/vaccine virus and heterologous/field isolate, (ii) vaccination: primary and booster, (iii) day post vaccination (dpv): 0, 21 and 49dpv. Tukey's pairwise comparison tests were also performed independently for serotype A and serotype O to identify interaction between factors. All statistical analyses were performed using Minitab (version 18). One-way ANOVA was also performed on the net antibody produced (neutralisation antibody titre) after the primary vaccination of the homologous virus for all age groups as well as for the net antibody produced after booster vaccination for all age groups.

5.4 Results

5.4.1 Detection of antibodies against FMDV non-structural proteins

In order to detect FMDV infection in the lower-risk area study herd, NSP ELISAs were performed for all 48 cow sera at 56dpv as well as for all 51 calf sera at 0dpv, 21dpv and 49dpv. All these serum samples generated negative results (PI < 50%) using the NSP ELISA (at all sampling times).

To investigate the occurrence of NSP-specific antibodies within the higher-risk area of FMD, sera collected from 60 animals at three different sampling time points ((0dpv), (21dpv) and (49dpv)) were tested with NSP ELISA. As a result, NPS-specific antibodies were detected in nine out of the 60 cows (15%) (≥ 50% PI for NSP ELISA) at all three sampling points. In addition, four animals (6.7%) were found to be NSP positive at two sampling points ((0dpv) and (21dpv)) and six animals (10%) generated positive NSP results at just one sampling point, either at (0dpv) (four animals) or at (49dpv) (two animals).

The detection of NPS-specific antibodies depended on the history of clinical FMD in the herds held within the higher-risk area of FMD. Thus, in herds with no clinical history of FMD, four out of 30 animals (13%) involving three out of four (75%) herds were positive for NSP-specific antibodies at any time point of the experiment (Figure 5.3a), while NSP-specific antibodies were detected in 15 out of 30 animals (50%) involving all five (100%) in herds with history of clinical FMD in January 2013 (Figure 5.3b).

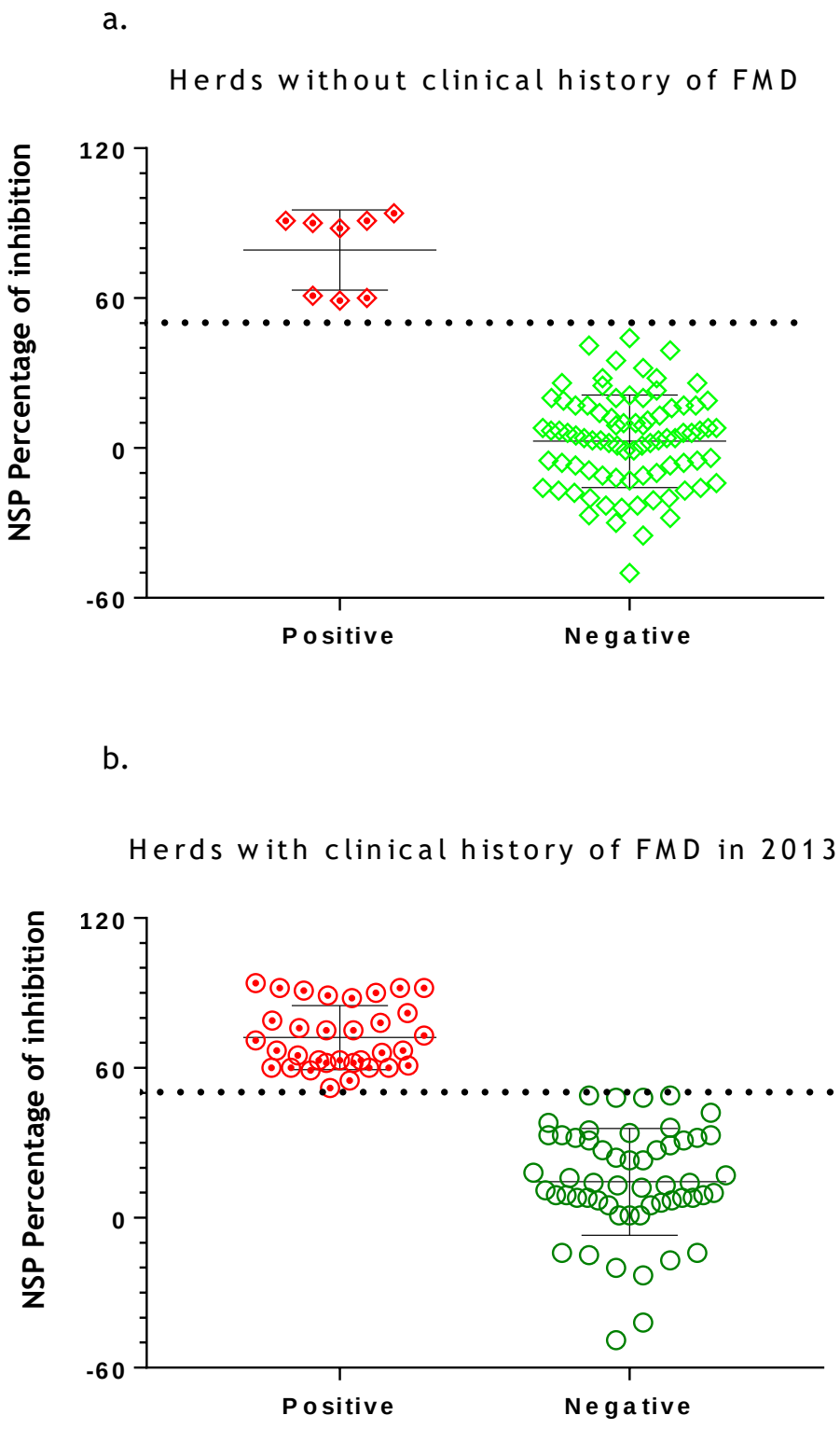


Figure 5.3: NSP positive and negative sera from cattle herds located in an area with higher-risk of FMD. Panel (a) herds without any clinical history of FMD and (b) herds with history of clinical FMD in January 2013. The black error bars represent the standard deviation from the mean NSP percentage of inhibition (PI). The black dotted lines represent the test cut-off value for NSP positive samples (PI ≥ 50% was considered positive NSP).

5.4.2 FMDV-specific neutralising antibody responses in cows

All VNT experiments described in this chapter were carried out using sera collected from cows held in the farm located in an area with lower-risk of FMD.

5.4.2.1 FMDV-serotype A-specific responses measured in adult cow sera

As can be seen in Figure 5.4, all 48 adult cows had homologous neutralisation titres against the A/May-97 vaccine virus, a component of the Aftovaxpur® used in Malaysia (see 5.3.2 section), that were found to be above the suggested protective cut-off of 1.4 $\log_{10}$ for FMDV serotype A (Barnett et al., 2003). For these samples, the neutralisation range observed was 2.17 $\log_{10}$ to 3.53 $\log_{10}$, with a mean neutralisation titre $\pm$ standard deviation of 2.84 $\log_{10} \pm$ 0.27 $\log_{10}$. When these sera were tested using the heterologous virus (A/MAY/2/2011; field isolate), the overall level of heterologous neutralisation antibody titres was lower compared to homologous titres (Figure 5.4). Only three out of the 48 adult cow sera had neutralisation titres that were below the suggestive protective cut-off level (Barnett et al., 2003) (Figure 5.4) suggesting a heterologous percentage protection of 93.8%. These three sera were from the cows that showed the lowest neutralisation titre for the homologous virus. These heterologous $\log_{10}$ neutralisation antibody titres ranged from 0.82 to 2.70, with a mean $\log_{10}$ neutralisation titre $\pm$ standard deviation of 2.11 $\pm$ 0.43.

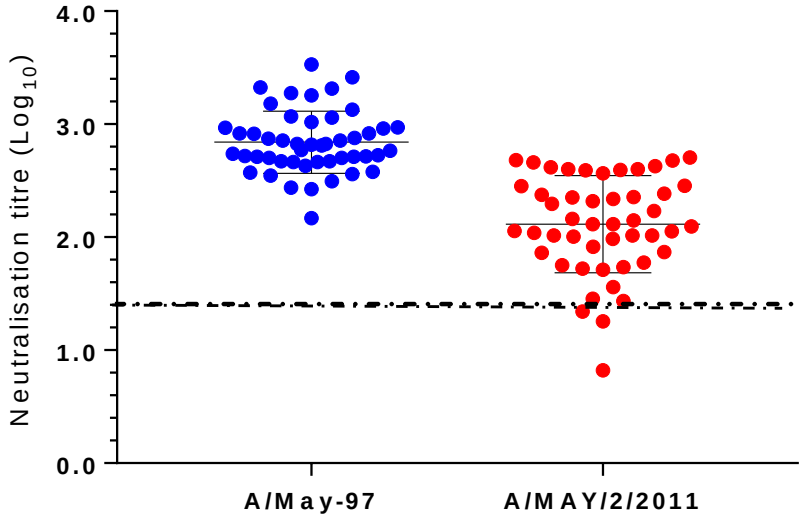


Figure 5.4: Homologous and heterologous FMDV-serotype A-specific neutralisation antibody titres. Neutralisation antibody titres (log10) of individual adult cows is projected against homologous (A/May-97) and heterologous (A/MAY/2/2011) viruses. The sera were collected after multiple vaccination with Aftovaxpur®, a quadrivalent vaccine containing FMDV A/ASIA/Sea-97 (A/May-97), O1 Manisa, O-3039, and Asia 1 Shamir. The suggestive protective cut-off point for serotype A is indicated by the black dashed line at 1.4 log10 (Barnett, 2003). The black error bar indicates the standard deviation from the mean neutralisation titre.

5.4.2.2 FMDV-serotype O-specific responses measured in adult cow sera

For serotype O, VNT was only performed on a reduced subset of the antisera and for only one (O-3039) of the two (O-3039 & O_1 Manisa) vaccine virus components that were present in the of the Aftovaxpur® quadrivalent vaccine (see section 5.3.2). All 25 (100%) adult cow sera generated neutralization titres against this vaccine virus that were above the suggestive protective cut-off point for FMDV serotype O of 1.6 $\log_{10}$ (Barnett et al., 2003) as shown in Figure 5.5. The highest neutralization antibody titre against this vaccine virus was 3.3 $\log_{10}$, whereas the lowest neutralization titre against the vaccine strain was 1.67 $\log_{10}$. The mean neutralisation antibody tires against the vaccine virus was 2.54 $\pm$ 0.49 $\log_{10}$.

The neutralisation antibody titres against the heterologous virus (O/MAY/10/2016: field isolate) had a range of 2.88 $\log_{10}$ to 1.21 $\log_{10}$. The mean neutralisation antibody titre was 1.94 $\log_{10}$. Four out of 25 (estimated 16%) individual sera tested showed neutralization

antibody titres against the field isolate below the suggestive protective cut-off 1.6 log_{10} (Figure 5.5).

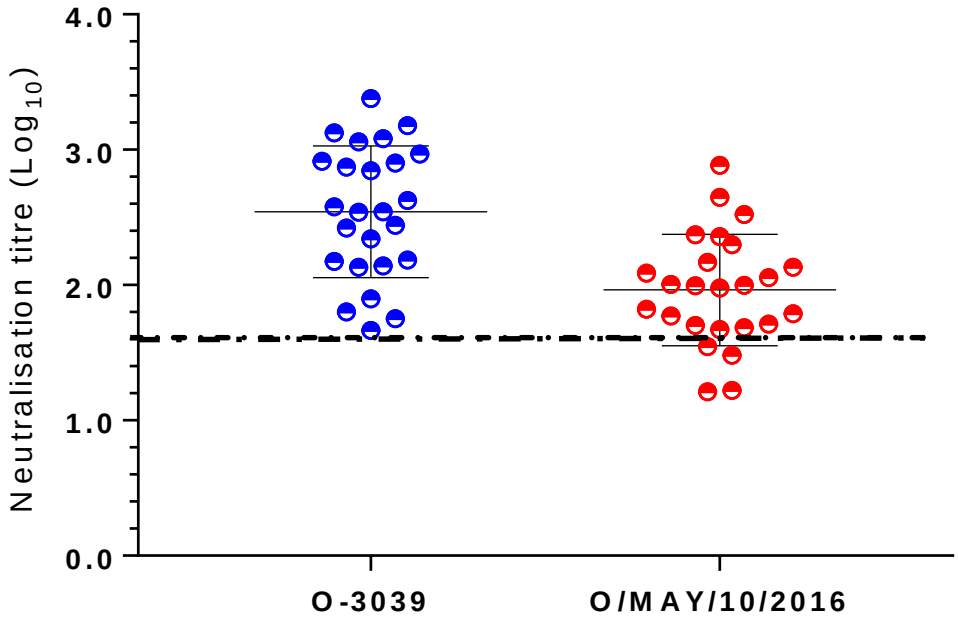


Figure 5.5: Homologous and heterologous FMDV-serotype O-specific neutralisation antibody titres. Neutralisation antibody titres (log10) of individual adult cows is projected against vaccine strain (O-3039) and field strain (O/MAY/10/2016) viruses. The sera were collected after multiple vaccination with Aftovaxpur®, a quadrivalent vaccine containing FMDV A/ASIA/Sea-97 (A/May-97), O1 Manisa, O-3039, and Asia 1 Shamir antigens. The suggestive protective cut-off point for serotype O is indicated by the black dashed line at 1.6 log10 (Barnett, 2003) and the black error bars indicate the standard deviation from the mean neutralisation titre.

5.4.3 Neutralising antibody response in calves

FMDV-specific immune responses after primary and booster vaccination were investigated using sera collected from calves, aged two to seven months, held in farms located in an area of lower-frisk of FMD.

5.4.3.1 Maternally derived neutralisation titres at 0dpv for serotype A and O

For the first component of this study, maternally-derived neutralisation antibody titres against serotype A homologous (A/May-97) and heterologous (A/MAY/2/2011) viruses were measured in 51 unvaccinated calves (at 0dpv). Maternally-derived neutralisation titres against the homologous virus were highest in the calves aged two, and four-months

(which were above the suggested protective cut-off), but at three months old, only four out of nine individual sera had measurable maternally-derived neutralisation titres (Figure 5.6). At the age of 5 months, the maternally derived neutralisation titres were lower and all individual sera of calves in age groups six and seven months generated negative maternally-derived neutralisation titres. Similar patterns were observed between homologous and heterologous antibody responses of the individual titres were measured using the heterologous VNT assay. However, overall, the heterologous maternally-derived neutralisation titres were lower than the homologous, and below the suggested protective cut-off point (Figure 5.7).

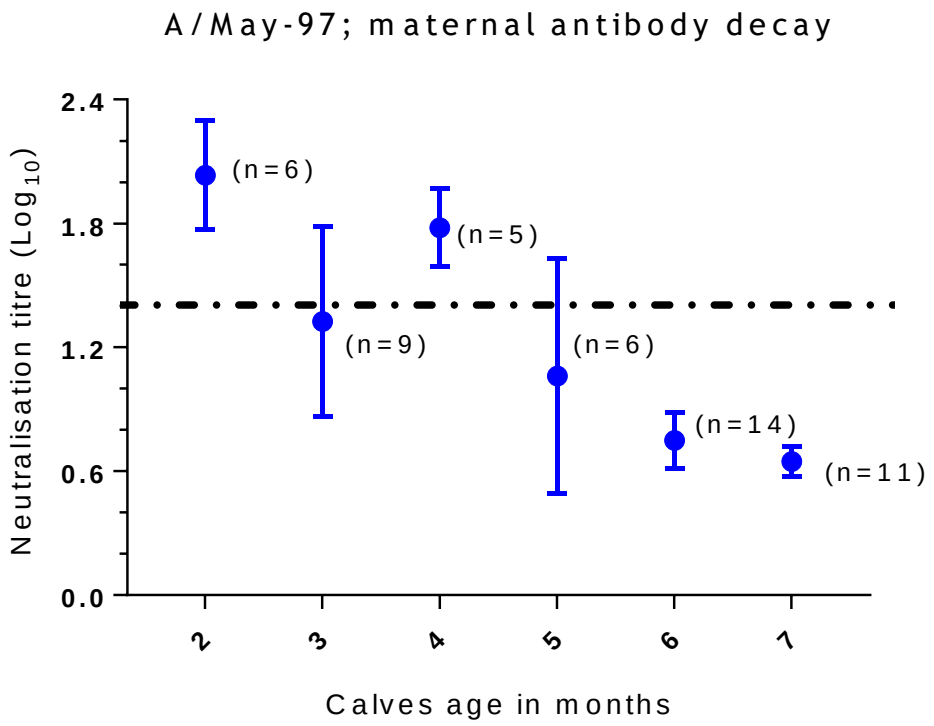


Figure 5.6: Maternally-derived neutralisation titres against the A/May-97 (homologous) vaccine virus in unvaccinated calves. The suggestive protective cut-off point for serotype A is represented by the black dashed line at 1.4 log10 (Barnett, 2003). The error bars indicate standard deviation from the mean; number of calves in an age group is indicated by n.

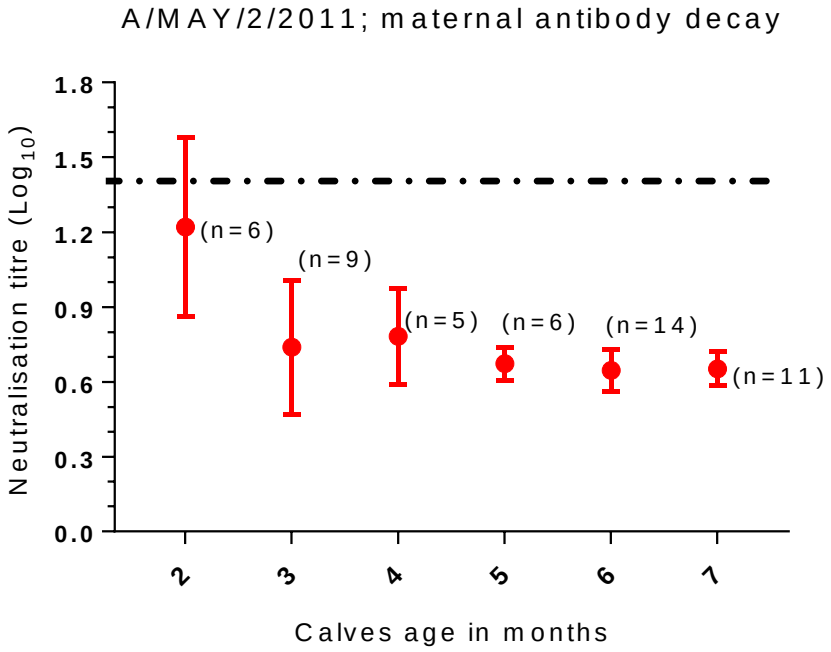


Figure 5.7: Maternally-derived neutralisation titres against the A/MAY/2/2011 (heterologous) field virus in unvaccinated calves. The suggestive protective cut-off point for serotype A is represented by the black dashed line at 1.4 log10 (Barnett, 2003). The error bars indicate standard deviation from the mean; number of calves in an age group is indicated by n.

As for FMDV serotype O, maternally-derived neutralisation titres were measured in 25 unvaccinated calves (at 0dpv). Maternally-derived neutralisation titres against the O-3039 vaccine virus were measurable in five out of eight calves aged between two to five months at 0dpv (Figure 5.9a) All individual sera of the calves aged five to seven months showed comparably lower maternally-derived neutralisation titres to the younger age groups. The same sera were also tested for heterologous maternally-derived neutralisation titres (against the O/MAY/10/2016 field isolate) with the highest maternally-derived neutralisation titre being 1.43 $\log_{10}$. (Figure 5.8b).

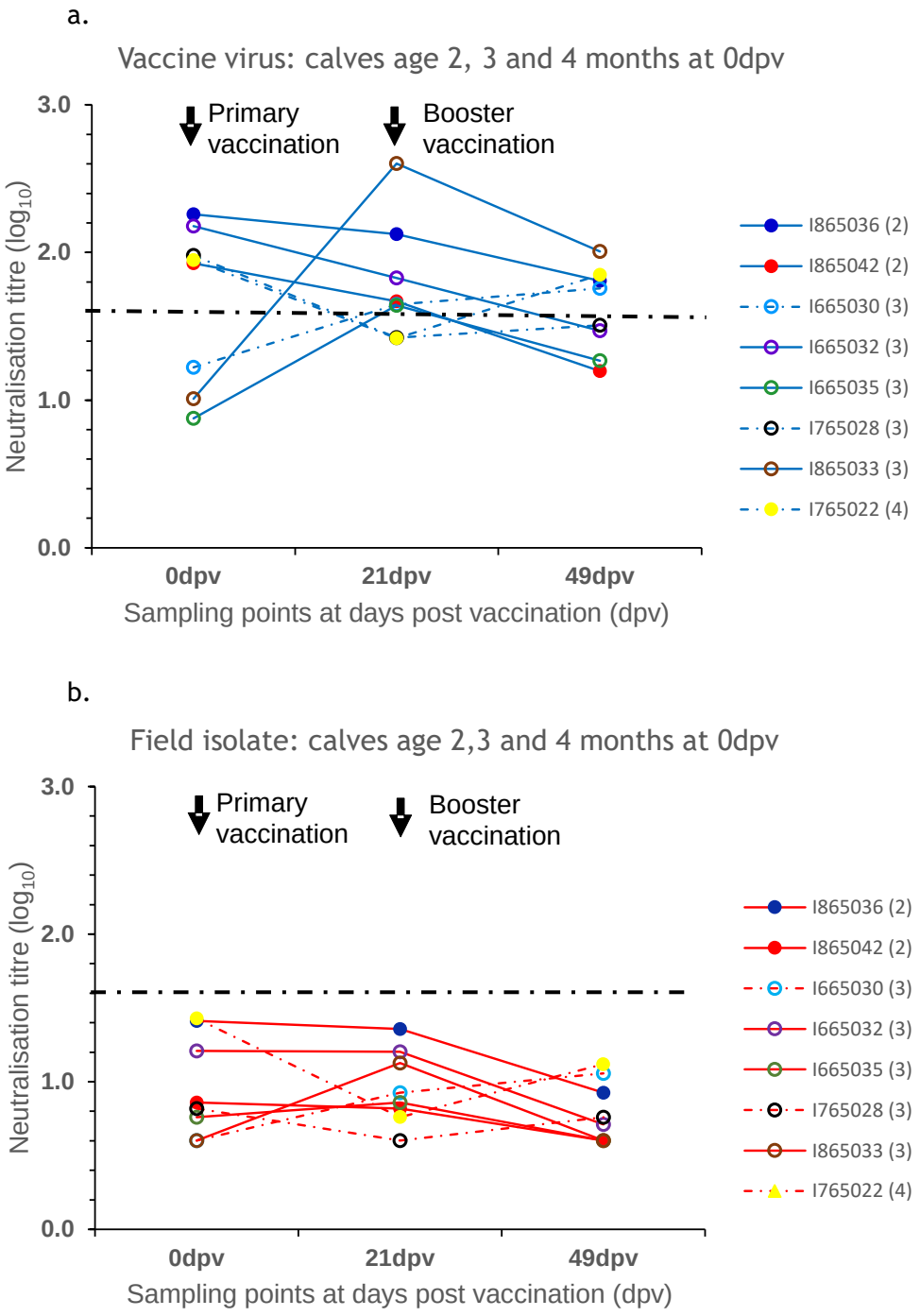


Figure 5.8: Serotype O FMDV-specific antibody responses after vaccination. Maternally-derived neutralisation titres (0dpv), neutralisation antibody titres after primary (21dpv) and booster vaccination (49dpv) for homologous (panel a) and heterologous (panel b) viruses are shown in calves aged 2, 3 and 4 months at the start of the study. Solid lines represent antibody responses in calves that did not receive booster vaccination, while dashed lines represent antibody responses in calves that received booster vaccination at 21dpv. Blue lines trace neutralisation titres of homologous virus while red of heterologous virus. Open and closed circles with different colours indicate individual calves, while age groups are noted in brackets following the calves' identification number. The suggestive protective cut-off point is represented by the black dashed line at 1.6 log10 (Barnett, 2003).

5.4.4 Effect of primary vaccination on FMDV-specific antibody responses for serotypes A and O

The individual neutralisation antibody titres against vaccine viruses (A/May-97 and O-3039) and field isolates (A/MAY/2/2011 and O//MAY/10/2016) were measured at 21dpv in order to access the impact of primary vaccination upon the FMDV-specific immune response of calves with varying levels of maternally-derived neutralisation titre.

For serotype A, the primary vaccination neutralisation titre against the vaccine virus (A/May-97) of all individual calves' sera were negatively correlated with the maternally derived neutralisation titre (p<0.0001) (Figure 5.9).

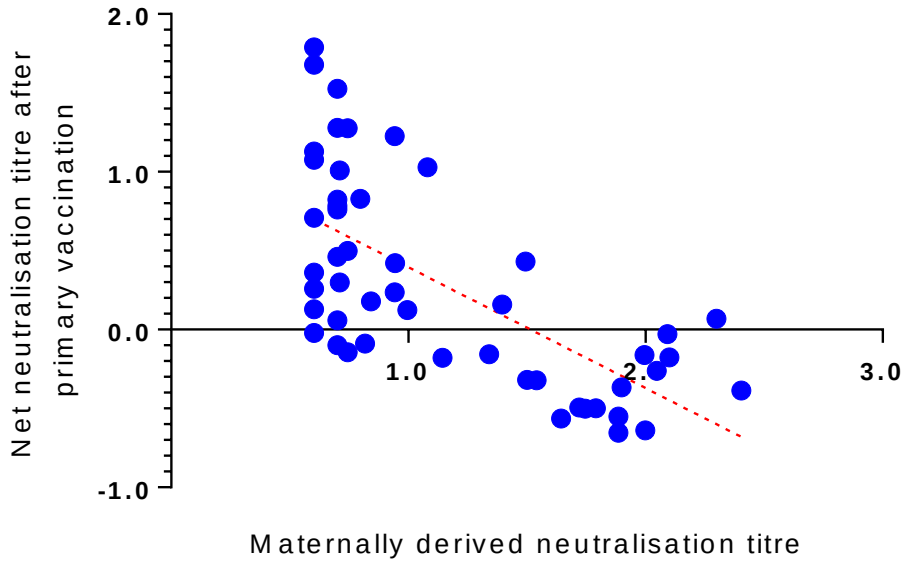


Figure 5.9: Negative effect of maternally derived neutralisation titre on primary neutralisation titre for serotype A (vaccine virus). The red-dotted line indicates the regression trend.

More specifically, animals with the highest (above the 1.4 $\log_{10}$ protective cut-off point) maternally-derived neutralisation titres produced lower FMDV-specific neutralisation antibody titres after primary vaccination as compared to calves with maternally-derived neutralisation titres measured below the protective cut-off point (Figures 5.10 a, c, e and 5.11 a, c, e). For instance, calves aged two and four months showed lower mean $\log_{10}$ neutralisation titre against A/May-97 after primary vaccination dropping from 2.03 to 1.65, and 1.78 to 1.32, respectively. Analysis of variance indicated there were significant

differences (p<0.05) in the net antibody titres produced after primary vaccination between animals with the highest maternally derived neutralisation titres in groups age two- and four-months as compared to calves' sera in the older age groups (at six and seven months of age). In all four calves with low maternally-derived neutralisation titres against the homologues A/May-97 virus in the three-month old group, an increase in the neutralisation antibody titre after the primary vaccination was observed (Figure 5.10c). A similar trend of an increase of maternally-derived neutralisation titres after primary vaccination response were observed for the individual sera of calves in the older age groups of five, six and seven months (Figure 5.11a, c, e).

At 95% confidence interval the mean net antibody titres against vaccine virus produced after the primary vaccination for two-month age group was -0.34 (95% CI -0.74, 0.06), for the three-month age group was 0.11 (95% CI -0.22, 0.44), for the four-month age group was -0.46 (0.90, -0.24), for the five-month age group was $\log_{10}$ 0.03 (95% CI -0.37, 0.43), for the six-month age group $\log_{10}$ 0.67 (95% CI 0.41, 0.94) and finally for age group of seven-month was 0.74 (95% CI 0.44, 1.04). No difference between the mean neutralisation titres produced after booster vaccination for calves in all age groups was observed. The same sera were also tested against serotype A heterologous virus (A/MAY/2/2016: field isolate) showed in principle similar pattern in response to primary vaccination compared to response against the homologous virus (A/May-97: vaccine virus) as in Figure 5.10b, d, f and 5.11b, d, f. However only two out of the 51 calves had maternally-derived neutralisation titre above the suggested protective cut-off of 1.4 $\log_{10}$ at 0dpv. Both calves were two months old (Figure 5.10b, d, f and 5.11b, d, f).

For serotype O, a similar pattern of neutralisation antibody titres was observed when measured against the vaccine virus (O-3039) and the field isolate (O/MAY/10/2016) after the primary vaccination at 21dpv. All 5 calves that initially had maternally-derived neutralisation titres against vaccine virus above the suggested protective cut-off, produced lower neutralisation antibody titres after primary vaccination (Figure 5.8a). Only two calves that belonged to age groups of five, six and seven months, produced neutralisation antibody titres above the suggested protective cut-off after the primary vaccination against the vaccine virus (O-3039) (Figure 5.12c). In contrast, calves that had maternally-derived neutralisation antibody titres against the field isolate (O/MAY/10/2016) showed reduction or no difference after the primary vaccination was given (Figure 5.8b). No sera in any age group showed neutralisation antibody titres against the field isolate (O/MAY/10/2016) above the suggested protective cut-off point after the primary vaccination (Figure 5.12b, d, f).

5.4.5 Effect of booster vaccination on calves at 49dpv on FMDV-specific serotype A and O antibody responses

The effect of booster vaccination on calves with different levels of maternally-derived neutralisation titres against vaccine viruses (A/May-97 and O-3039) and field viruses (A/MAY/2/2011 and O//MAY/10/2016) were measured in sera collected at 49dpv.

For serotype A, all individual sera from calves that received booster vaccination at 21dpv showed an increase in neutralisation antibody titres against the vaccine virus (A/May-97) virus at 49dpv, irrespective of their age when they were first vaccinated (Figure 5.10 and Figure 5.11). Only one individual serum had a neutralisation antibody titre against the A/May-97 virus below the suggested protected cut-off point despite an increase after the booster vaccination (Figure 5.11a). On the other hand, the majority (76%) of individual calves that were not given booster vaccination at 21dpv demonstrated a reduction in their serum neutralisation antibody titres against vaccine virus (A/May-97) at 49dpv. However, there were two individual calves that did not show a reduction in their serum neutralisation antibody titres against the A/May-97 virus which remained above the suggested protective cut-off despite no booster vaccination being given (Figure 5.10 c and Figure 5.11 c). The effect of booster vaccination on the neutralisation antibody titre against the field isolate (A/MAY/2/2011) differed in some individuals, in the young calves age two, three and four months old, to the effect observed for vaccine virus (A/May-97). There were individual sera that showed a reduction in their neutralisation antibody titres against field isolate (A/MAY/2/2011) despite of the booster vaccination given at 21dpv (Figure 5.10 d, b and e). There were also individual sera that showed an increase in their neutralisation antibody titre against field isolate (A/MAY/2/2011) measured at 49dpv even without booster vaccination (Figure 5.10 d, b and e). For serotype O, the booster vaccination resulted in an increase in neutralisation antibody titres against vaccine virus (O-3039) above the suggested protective cut-off point in most animal tested except for 5 individual calves aged two, six and seven months (Figure 5.8a, 5.12c and 5.12e). All calves that did not receive booster vaccination showed a reduction in specific neutralisation antibody titres against vaccine virus (O-3039). Booster vaccination also increased the neutralisation antibody titres against field isolate (O/MAY/10/2016), except in four individual calves that belonged to the six and seven-month age groups (Figure 5.12d and f). However, booster vaccination was not able to increase the neutralisation antibody titre against field isolate above the suggested protective cut-off point except for all calves aged two, three and four-months old (Figure 12b) and five individual sera in the five, six, and seven-month old groups (Figure 5.12b and 5.12d).

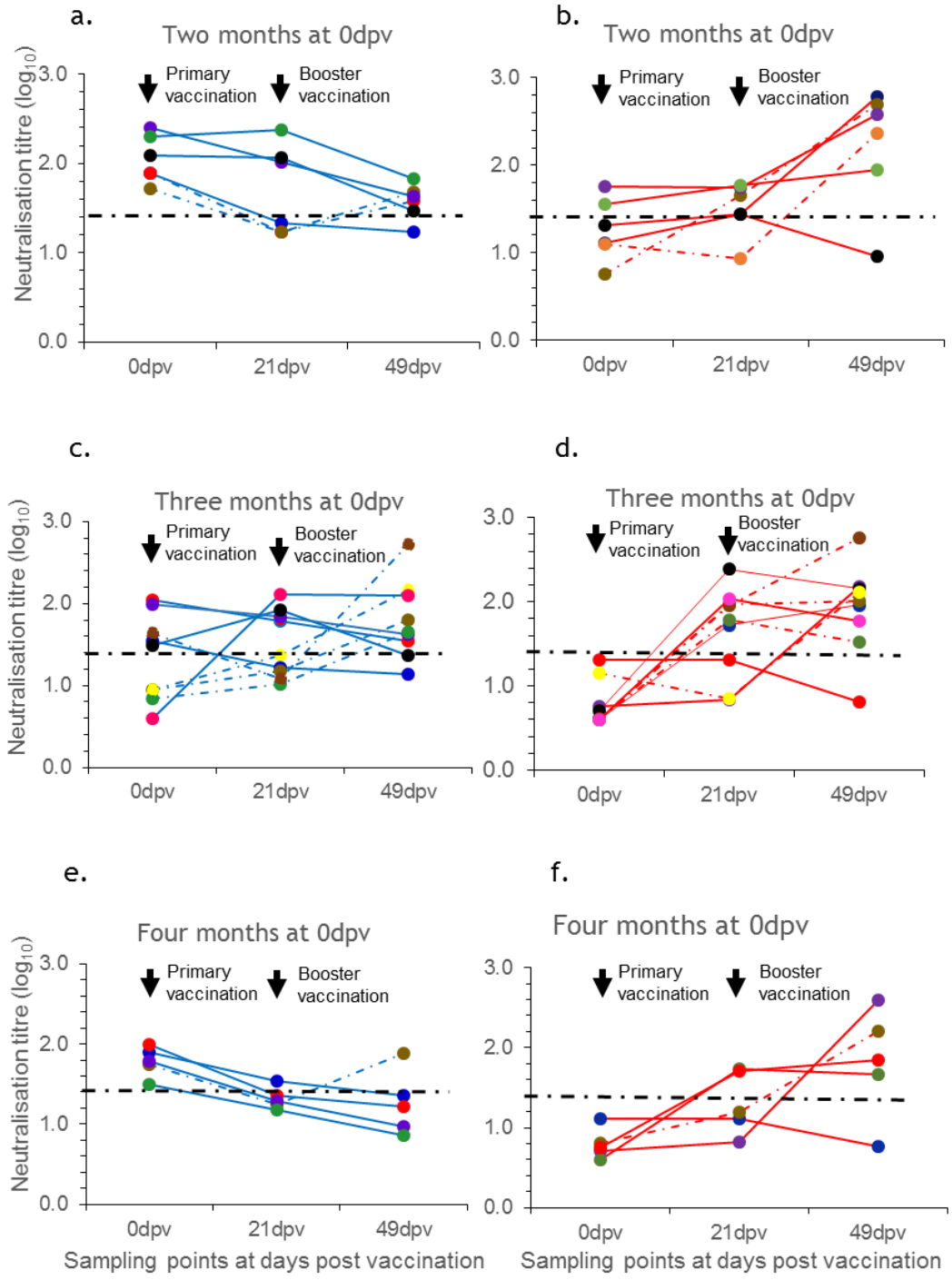


Figure 5.10: Generation of serotype A FMDV-specific antibody responses after vaccination in calves aged two, three, and four months at the start of the study. Data presented shows maternally-derived neutralisation titres (0dpv), primary vaccination (21dpv) and booster vaccination (49dpv) neutralisation antibody titres against vaccine virus (5.9a, c and e) and against field isolate (5.9b, d and f) of the serotype A. Solid lines represent calves that did not receive booster vaccination (in blue for vaccine virus, in red for field isolate), while dashed lines represent calves that received booster vaccination at 21dpv. The suggestive protective cut-off point is represented by the black dashed line at 1.4 log10 (Barnett et al., 2003).

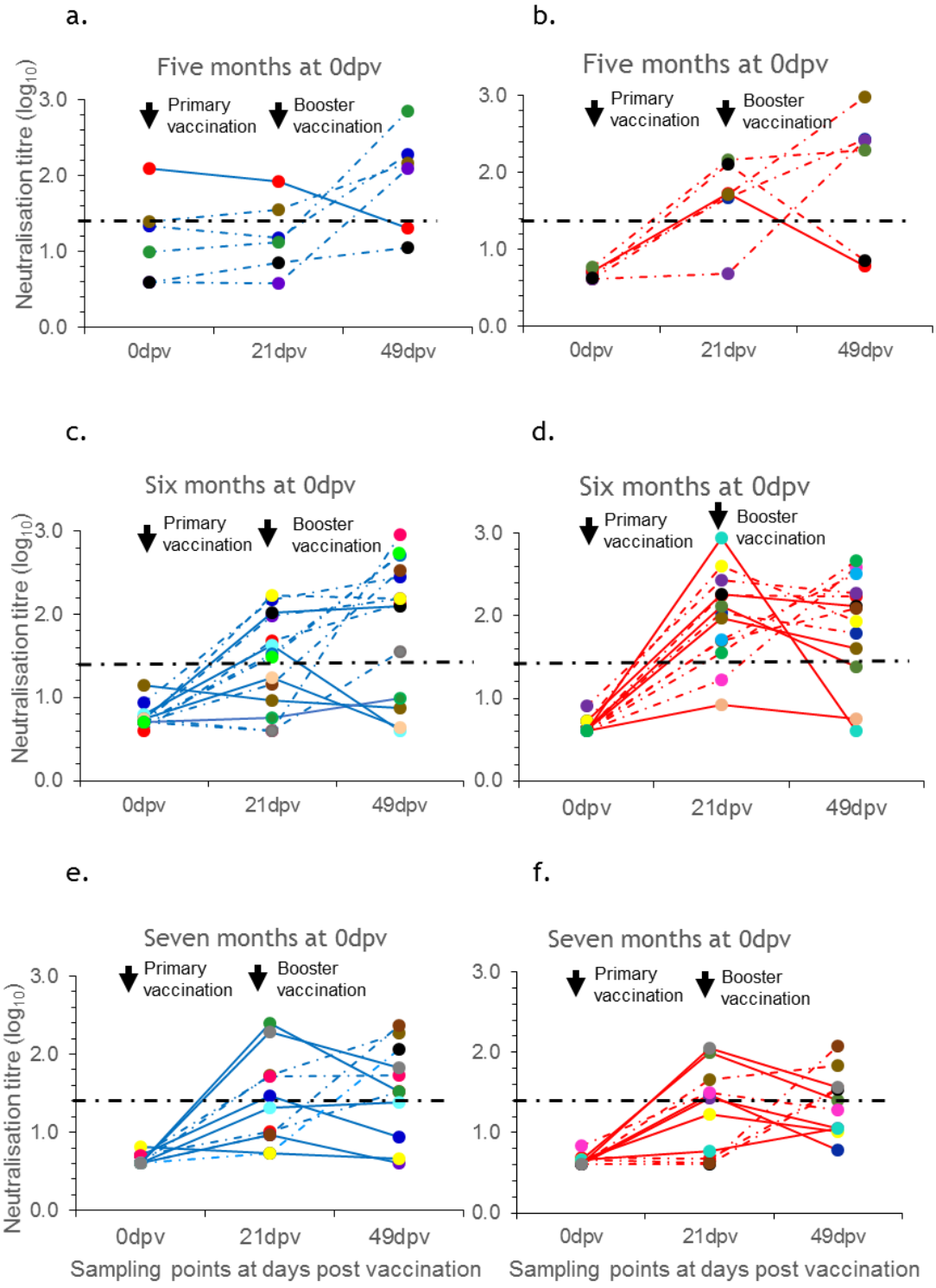


Figure 5.11: Generation of serotype A FMDV-specific antibody responses after vaccination in calves aged five, six, and seven months at the start of the study. Data presented shows maternally-derived neutralisation titres (0dpv), primary vaccination (21dpv) and booster vaccination (49dpv) neutralisation antibody titres against vaccine virus (a, c and e) and against field isolate (b, d and f) of the serotype A. Solid lines represent calves that did not receive booster vaccination (in blue for vaccine virus, in red for field isolate), while dashed lines represent calves that received booster vaccination at 21dpv. The suggestive protective cut-off point is represented by the black dashed line at 1.4 log10 (Barnett et al., 2003).

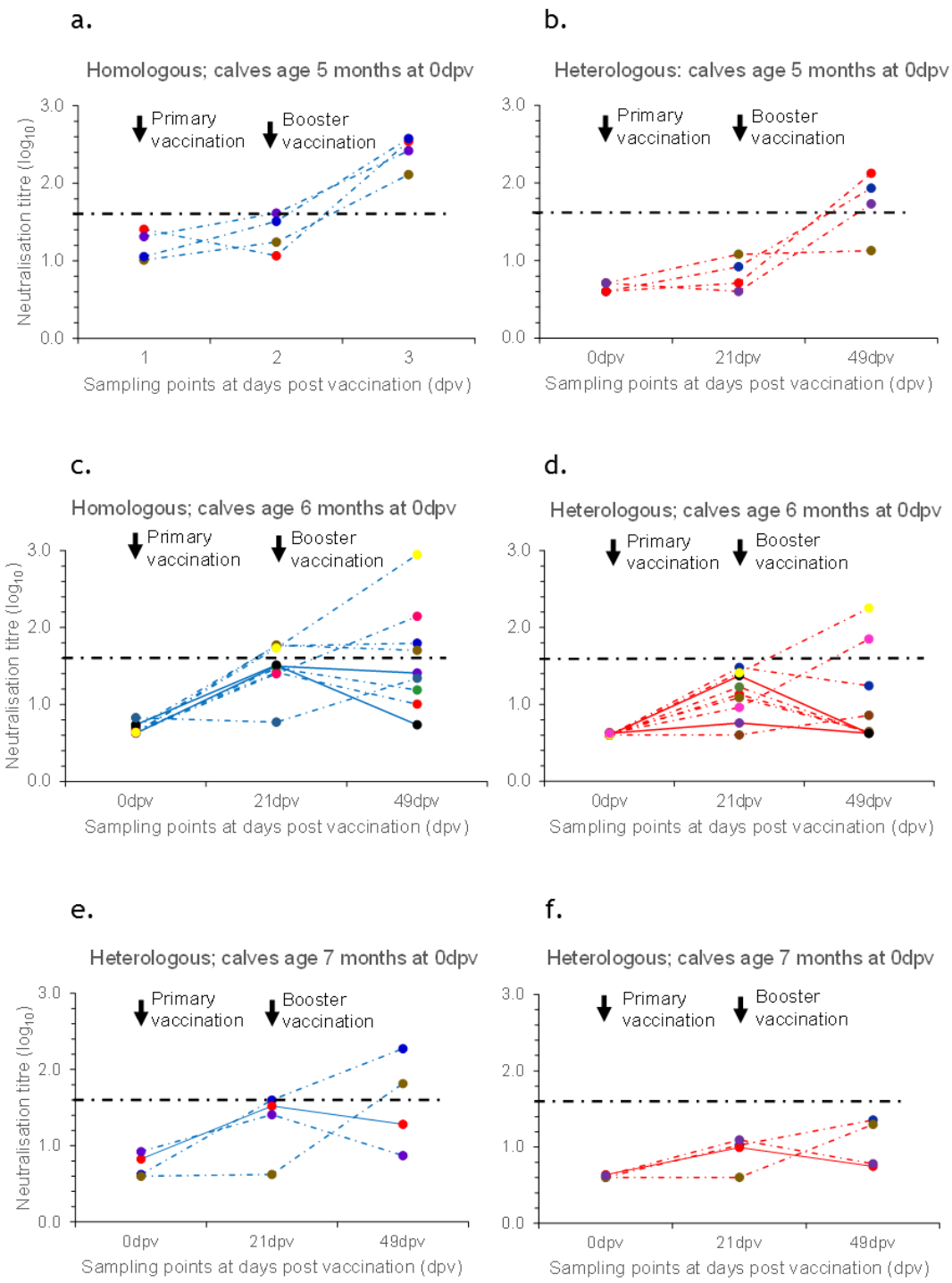


Figure 5.12: Generation of serotype O FMDV-specific antibody responses after vaccination in calves aged five, six, and seven months at the start of the study. Data presented shows maternally-derived neutralisation titres (0dpv), primary vaccination (21dpv) and booster vaccination (49dpv) neutralisation antibody titres against vaccine virus (a, c and e) and against field isolate (b, d and f) of the serotype O. Solid lines represent calves that did not receive booster vaccination (in blue for vaccine virus, in red for field isolate), while dashed lines represent calves that received booster vaccination at 21dpv. The suggestive protective cut-off point is represented by the black dashed line at 1.6 log10 (Barnett et al., 2003).

5.4.6 Calculated r1 values

The antigenic relationship (r1 values) between the vaccine viruses and field viruses were calculated for all individual 48 cows, 51 calves after primary vaccination (serum collected at 21dpv) and 26 calves that were boosted (serum collected at 49dpv).

The antigenic relationship between the A/May-97 vaccine virus (homologous virus) and the A/MAY/2/2011 field isolate (heterologous virus) for the individual sera of the cows ranged from 0.01 to 0.59 (with the mean r1 value of 0.24). The majority (31 of 48 sera) generated an r1 value less than the suggested vaccine-match cut-off value of 0.3 (OIE, 2017) (Figure 5.13a). Interestingly, two sera with the lowest neutralisation titres generated r1 values above the suggested vaccine-match cut-off (sera ID 17Y4481: r1 value 0.37 and sera ID 1724626: r1 value 0.38).

For sera of the calves' sera after primary vaccination at 21dpv against the serotype A the r1 value generated from VNT titres showed wider range from 0.10 to 22.71. Majority of the calves (57%) showed r1 value more than 1.0, 35% calves showed r1 value above the suggested vaccine-match cut-off (0.3) and below 1.0 and only 9% showed r1 value below the suggested vaccine-match cut-off (Figure 5.13b). Calves that received booster vaccination showed different distribution of r1 value compared to calves after the primary vaccination. There were 46% of the calves that received booster vaccination show r1 value above the suggested vaccine-match cut-off (0.3) and below the 1.0. Only about 7.7% of the calves that received booster vaccination showed r1 value less than the suggested vaccine-match cut-off, whereas the rest of the calves that received booster vaccination (42%) showed r1 value more than 1.0 (Figure 5.13c).

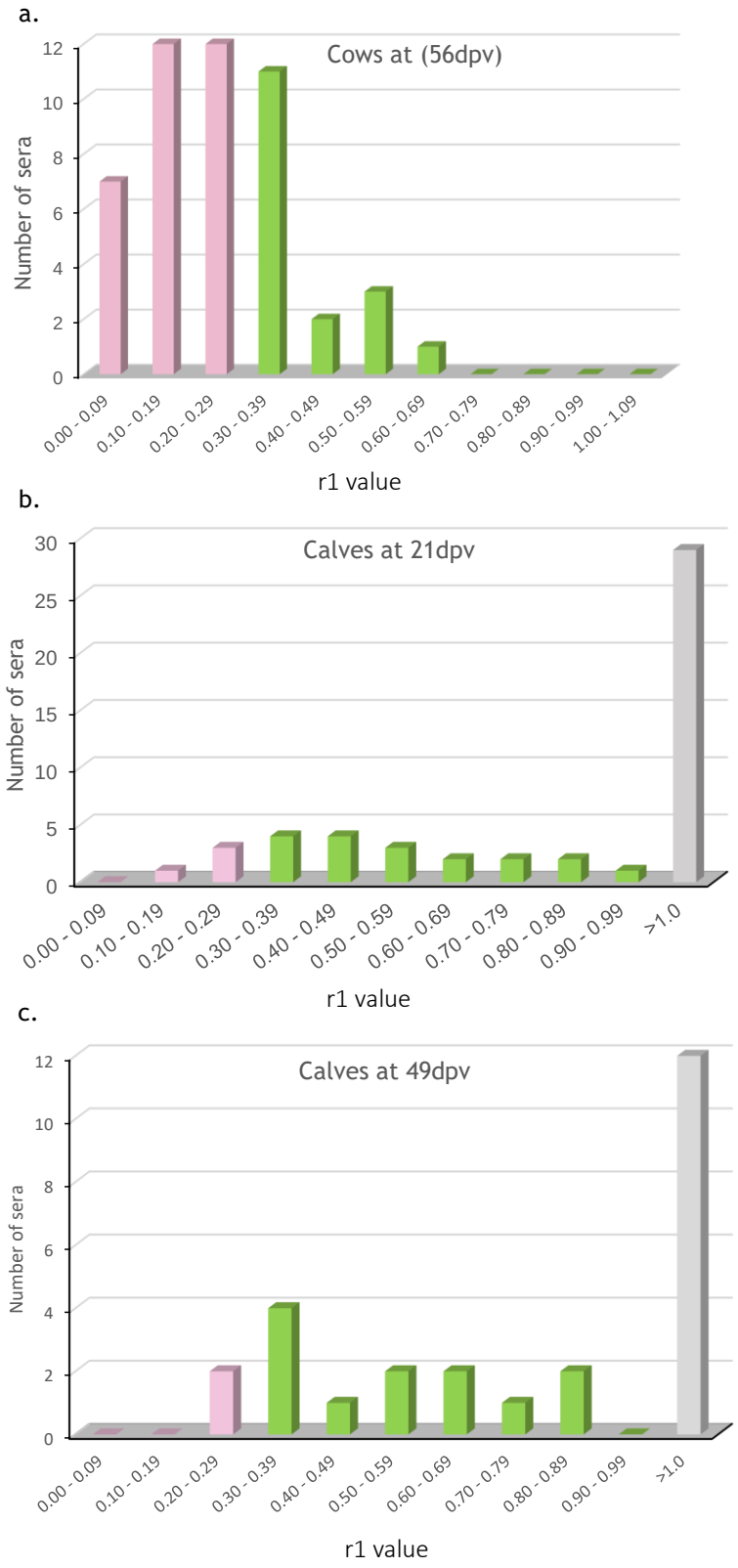


Figure 5.13: The r1 values generated from neutralisation titres of adult cows and calves against the vaccine virus and field isolate of A/ASIA/Sea-97. Panel (a) cows at 56dpv after multiple vaccination, (b) of calves after primary vaccination and (c) of calves after booster vaccination. Bars in pink represent sera that have r1 value below the protective cut-off and bars in green represent sera with r1 value above the suggested protected cut-off (0.3) (OIE 2018). The grey bars represent sera with r1 values that were more than 1.0.

5.4.7 Statistical analysis for FMDV serotype A and O specific antibody responses

Analysis using a general linear model built on the antibody responses indicated that there was significant difference between the mean neutralisation antibody titres of the homologous and the heterologous virus of serotype A (p=0.004). There was also a significant difference (p=0.0001) in the mean neutralisation antibody titres at different sampling points (0dpv, 21 and 49dpv). Tukey pairwise comparison indicated significant increase in the mean neutralisation titres between 21dpv and 49dpv in sera that was boosted, but for the non-boosted sera there was no significant difference in the mean neutralisation antibody titre for the same time points. Tukey pairwise comparison also indicated significant higher maternally derived neutralisation titre against the homologous virus than heterologous viruses. Analysis of variance for the FMDV serotype O: O-3039 and O/SEA/Mya-98 showed there was a significantly higher in the mean neutralisation titre against the vaccine virus and the field isolate (p=0.001). The difference in the mean neutralisation titre against the vaccine virus and the field isolate was also significant at 0, 21 and 49dpv with the (p=0.002).

5.5 Discussion

Post-vaccination monitoring (PVM) is very important in FMD endemic areas that use vaccination as part of their FMD control program (Ferrari et al., 2016a). PVM ensures that the vaccination carried out is beneficial and that the control program is working effectively. However, in endemic areas it can be challenging particularly in countries where resources and access to data are limited. Therefore, the PVM guide describes the general key elements in how to perform post vaccination evaluation to meet the country's specific objectives which most of the time are related to the stage of PCP that the country has achieved. There are four main elements in the PVM guide. The first is the selection and purchase of a suitable vaccine that includes the use of r1 values (for all the vaccine components) as an indication of adequate antigenic matching. This element also includes ensuring that the vaccine used is of suitable quality (dependent upon potency and purity of the vaccine). The second part of the PVM guide covers the importance of defining the objectives of the vaccination program that are specific to the country's needs. The third and fourth elements describe how to measure and evaluate antibody response in the vaccinated herds and how to undertake vaccine effectiveness studies. In addition, the PVM guide highlights the importance of ensuring an intact cold chain during the distribution and delivery of the vaccine.

The OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE, 2017) provides guidelines on production of good quality vaccines in a chapter describing the principles of veterinary vaccine production. Good quality vaccines contain FMDV strains that match field isolates causing FMDV infection in a given area. It is essential to ensure the antibodies elicited by the vaccine virus are most likely to confer protection. At the same time, it also crucial to ensure that the vaccine is safe for use and free from a possible contamination with other organisms. Furthermore, good quality vaccine should be purified from FMD virus NSPs so that naturally infected animals can be discriminated from vaccinated animals. Vaccine potency also is very important. It is usually determined by establishing the percentage of protection either in *in-vivo* challenge or *in-vitro* conditions and serological antibody response of the vaccinated population are measured either by VNT or ELISA. Studies shown that high potency FMD vaccines (of at least $6PD_{50}$) were more likely to confer protection even when r1 values generated were low (Brehm et al., 2008).

In this study, all sera collected from cows and calves, located in an area with lower risk of FMD, were negative for NSPs. Adult cows that had been vaccinated multiple times over the years showed similar neutralisation antibody titre levels for homologous and heterologous viruses against the A/ASIA/Sea-97 lineage as well as against the O-3039 (a serotype O component in the vaccine) vaccine virus and O/MAY/10/2016 field virus. It has been suggested that for herd immunity at least 80% vaccination coverage in the herds is needed (Leforban and Gerbier, 2002; Lombard and Schermbrucker, 1994; Lubroth et al., 2007). In this study, all sera of the cows showed this level of neutralisation antibody titres against the homologous virus and only small proportion (6.25%) did not achieve the suggested protective cut-off point (1.4 $\log_{10}$) against the heterologous virus for the A/ASIA/Sea-97 virus (Barnett et al., 2003). As for the FMDV O-3039 vaccine virus, all 25 sera of the cows tested showed neutralisation antibody titre above the suggested protective cut-off point (1.6 $\log_{10}$) (Barnett et al., 2003). A total of 21 out of 25 (84%) sera of the cows showed neutralisation antibody titre against the heterologous virus above the suggested protective cut-off (1.6 $\log_{10}$). In this study, the protective cut-offs point was based on challenge experiments by Barnett, et al (Barnett et al., 2003). Ideally, the protective cut-off point should be made to match the disease and population dynamic of the herds studied (Ferrari et al., 2016a). Other researchers have also suggested to determine more suitable field-based correlates for vaccine assessment (Lyons et al., 2017). Based on the neutralisation antibody titres against homologous (vaccine virus) and heterologous (field isolate) of the adults as well as the NSP result, for this field study the suggested protective cut-off point suggested by Barnett 2003 can probably be considered appropriate. Furthermore, the suggested protected cut-off by Barnett were based on the

same FMDV linage (A/May-97). Although there are other researchers that have suggested different protected cut-off to be used based on different FMDV lineages (Goris et al., 2008; Maradei et al., 2008). Maternally derived antibodies are of IgG isotype transferred from cow to their new-born calf through colostrum that is absorbed by the gut (intestines) within the first 24 hours after birth. Therefore, IgG will be present in the bloodstream of calves and naturally declines over time. The results from this study measured higher and longer maternally-derived neutralisation titres against the homologous (vaccine) virus as compared to the heterologous virus (field isolate). This study also showed that maternally derived neutralisation titres against the homologous virus (vaccine: A/May-97) as well as the heterologous (A/MAY/2/2011: field isolate) decreased with calves' age (R^2 = 0.5 and P<0.0001). This study is in agreement with other reports (Cokcaliskan et al., 2017; Nicholls et al., 1984b) showing that maternally derived neutralisation titres in young calves can reach levels above the protective cut-off point, and for this study 16/20 of the individual sera tested at age two to four months reached this threshold for the homologous virus.

The negative effect of the maternally derived antibody on the generation of neutralising antibodies after primary vaccination in calves is in agreement with published reports (Elnekave et al., 2016, Bucafusco et al., 2014). However, in this study maternally derived antibodies did not directly impact upon booster vaccination responses; calves with maternally derived neutralisation titre against the homologous (vaccine) virus increased their neutralisation antibody titre after booster vaccination. Therefore, it can be concluded that booster vaccination can overcome the negative effect of maternally derived antibodies observed after primary vaccination. This finding is also in agreement with another report showing that booster vaccination increased FMDV-specific antibody responses (Cokcaliskan et al., 2017). It has been previously shown that B cells still differentiate and produce memory cells even when maternally-derived antibodies are present (Foote et al., 2007). This could explain the booster vaccination responses regardless of the maternally derived neutralisation titre at primary vaccination. In addition to the results for serotype A, similar data was generated for FMDV serotype O although in this case, the vaccine contains two components (O-3039 and O_1 Manisa) which were heterologous (from different lineage to the field isolates in P. Malaysia). The results presented here are different findings to a report which indicated that prophylactic vaccination using a different lineage than the challenge virus can overcome the problem of the negative influence of maternally derived antibodies (Dekker et al., 2014). Based on the negative effects of maternally derived antibodies on the neutralisation titre induced after primary vaccination against the serotype A virus, the result from this study reinforce the importance of vaccinating calves later than the recommendation at 2.5

months of age or 2 months in severe epidemiological circumstances (Doel, 2003), particularly when considering heterologous titres.

The r1 values generated for individual animals in this field study were very variable and similar to the r1 values calculated in the *in-vitro* vaccine matching in the other chapters in this thesis (particularly Chapter 3). Issues with the r1 value were also emphasised here in this chapter when the heterologous neutralisation titre were higher than the homologous virus to produce an r1 values greater than 1. Furthermore, some of the heterologous neutralisation antibody titres that were below the protective cut-off, produced r1 values above the suggested vaccine-match cut-off (0.3). As indicated in Chapter 3, pooling sera should be used for post vaccination monitoring to reduce variability of the antibody neutralisation titre of a herd. However, vaccine effectiveness evaluation studies in an endemic area should focus on the neutralisation antibody titre after vaccination being above the suggested protective cut-off point before r1 values are calculated, for example above 1.4 $\log_{10}$ for A/May-97 and above 1.6 $\log_{10}$ for the O-3039 vaccine components. Another important way to assess vaccine effectiveness in endemic counties is by monitoring FMDV infections in young adult animals in vaccinated herds (between 1 to two years of age) using NSP ELISA. This method can also be used as an indicator whether the vaccine used is effective or not. The fact that P. Malaysia is endemic for FMD makes the NSP ELISA test crucial to ensure that the neutralisation titre measured in this study were due to the vaccination and not natural FMDV infection. It can be concluded that FMD vaccination coupled with good zoo sanitary and husbandry practiced at PTH Ayer Hitam dairy cattle herd has shown to be effective to control FMD in the lower risk area. NSP ELISA testing was also performed on all serum samples collected from dairy cows that were reared in an area of higher-risk for FMD. This area experienced FMD outbreaks in January 2013 and sera collected from affected farms showed a higher proportion (50%) of NSP positive cows than sera collected from the same risk area but without any clinical history of FMD (13%). It may not possible to discriminate whether the NSP detected in sera collected from herds with history of clinical FMD in January 2013 were due the clinical FMD episode in January 2013 or more recent infection (or persistent infection). This is considering the fact that NSPs antibodies may be detectable for a longer period than antibodies against structural proteins (SPs), and can be detected after 6 months post infection (Sorensen et al., 1998). Furthermore, the NSP ELISA cannot discriminate subclinical infection from the persistently infected animals (Paton et al., 2006). However, the fact that 13% NSP positive serum samples were collected from cows with no history of clinical FMD indicated that there is a potential for subclinical or persistent infection in these vaccinated animals. These herds were vaccinated regularly following the manufacturer recommendation to vaccinate animals

at 2.5 months of age and achieving the minimum 80% coverage of protective immune response in the herd so FMDV infection and spread should be prevented. However, sub-clinical (and unapparent) circulation of FMDV in previously vaccinated herds has been recently recognised to be problem (Farooq et al., 2018; Hayer et al., 2018; Lyons et al., 2017; Ranjan et al., 2018; Stenfeldt et al., 2016). Further studies are required to understand this problem, work that could be supported by probang sampling and testing from NSP positive vaccinated animals in an endemic area. Currently, NSP ELISA is the only serological tool available to discriminate FMDV infection and a response against vaccination. There are reports which indicate that repeated vaccination over several years can produce NSP positive results (Mackay et al., 1998) even when a purified vaccine has been used, although other studies have reported that multiple vaccination does not induce NSP antibodies (Niedbalski and Haas, 2003). More recent reports suggested that regularly vaccinated, NSP positive, young animals (between 1 to 2 years of age) with no clinical FMD may be used as indicators for subclinical disease (Lyons et al., 2017). Taken together with the results from the lower-risk herd (where all the samples were NSP negative), this study provides a framework for implementing FMD surveillance (and an assessment of vaccine performance) in P. Malaysia. In these future studies, it will be important that only young animals, aged between six to twelve months, are selected for surveillance purpose in order to increase confidence in the surveillance data (Ferrari et al., 2016b). This is because calves six to twelve month of age are known to be free of NSP, unless they are infected and their maternally derived antibodies have waned.

This study focused on the impact of maternally derived neutralisation titre on primary and booster vaccination and the effect of booster vaccination on the host's immune responses in an endemic area. It is important to consider the negative effect of maternally derived neutralisation titre when considering vaccination schedules, to identify the optimal age of vaccine delivery in calves. The results of this study also highlight the importance of using booster vaccination to protect the most susceptible, young animals against clinical FMDV infection. In this situation, the vaccine used elicited the immune response suggestive of protection despite inconclusive and variable r1 values. In general, this study emphasises the importance and priority that should be given to PVM in FMD endemic areas to ensure the vaccination programme to control FMD is effective. In conclusion, this study indicated that the current vaccine strains of A/May-97 used in Malaysia is suitable and most likely to confer protection against the contemporary field virus belonging to serotype A. Moreover, this serotype A vaccine strain will also be suitable for use in mainland SEA as FMD viruses in this endemic region share genetic and antigenic properties.

Chapter 6:

General discussion, conclusions and suggestions

This discussion chapter focuses on three main areas (i) review of the conclusions from the experimental studies, (ii) discussion of the main findings of the thesis in context with recent data from the Malaysian national surveillance programme for FMD, and (iii) a discussion of options for FMD control programme in Peninsular Malaysia.

6.1 Background and context

Vaccination plays major role as part of the control measures used in countries where FMD is endemic, as well as in "vaccinate–to-kill" or "vaccinate-to-live" policies used in previously FMD free countries in response to disease incursions to regain free status. In both situations, the vaccine is used to reduce the impact of FMD clinical disease and suppress the potential spread of FMD virus to susceptible animals. Therefore, it is vital that a suitable vaccine is selected, and that the performance of the vaccine is evaluated in order to achieve the purpose of vaccination. This thesis focuses on laboratory-based serological methods that are widely used for FMD vaccine matching with the main objective to understand the factors that influence test variability and limitations of these tests. The project focussed on viruses classified within the A/ASIA/Sea-97 lineage, as a model system representing an important FMD virus lineage that is currently circulating in the field in Peninsular (P.) Malaysia and other countries in Southeast Asia (SEA), and poses an on-going threat to Malaysia via land borders with Thailand and Myanmar.

It has been previously documented that the *in-vitro* vaccine matching tests used to generate r1 values using both VNT and LPBE methods, suffers from a high degree of test-to-test variability (Tekleghiorghis et al., 2014). This thesis investigated different factors that underpin the variability of these tests. As shown in Chapter 2, the replication cycle of cells used to propagate FMDV for VNT has a significant impact on variability of virus and neutralisation titres, leading to variability of vaccine matching test results. This suggests that laboratories should adopt a protocol in which they always use the cells at the same point of the cell replication cycle. In this context, it is easier to standardise the use of cells at 100% confluence. In Chapter 3, r1 values generated by VNT and LPBE using bovine vaccinal sera (BVS) produced after primary and booster vaccination highlighted the variability of these tests, especially with respect to the suggested antigenic-match cut-off, with some results above, and others below the suggested antigenic-match cut-off point for the same samples. Comparison between these two methods showed that less variation was observed in r1 values generated from the LPBE titres compared to r1 values generated by VNT. Subsequently, Chapter 4 emphasised the importance of the irreversible dissociation of FMD virus capsid proteins in *in-vitro* vaccine matching methods. This work showed that different FMD viruses have different dissociation temperature points and contained different amount of dissociated FMD virus capsid particles (12S), that leads to further variability of the *in-vitro* vaccine matching results.

The high degree of variability seen in these studies, helps us to understand the different factors that may underlie the range of r1 values that have been observed for closely related field samples. Retrospective data for field isolates from the A/ASIA/Sea-97

lineage against the A/May-97 vaccine is highly variable (data for samples tested during 2006 to 2017 is presented in Chapter 1; section 1.4.1), although there is no evidence from the field that the antigenic nature of these viruses has changed. Much of the difficulties that arise from the use of a strict and universal cut-off (at 0.3 for VNT and 0.2 - 0.4 for LPBE) to define whether a vaccine is antigenically related to a field virus. The use of such a cut-off is unrealistic since these *in-vitro* methods suffer inherent variability as evidenced in this thesis. Furthermore, the r1 values do not necessarily provide any indication about "protection" that might be afforded by the vaccines since the values are not usually connected to the results from challenge studies. The inherent limitations of the vaccine-matching tests are important since these assays are widely used to define antigenic relationships and make important decisions about the selection of FMDV vaccines for control programmes. For instance, the widespread use of A_{22} Iraq as a serotype A component in FMD vaccine in SEA (Horsington et al., 2018), even when A_{22} Iraq is not closely genetically related to A/ASIA/Sea-97.

Results in this thesis lead to the conclusion that relationship coefficient (r1 values) that are currently recommended by the OIE need attention and improvement. These data are often vital for international agencies that oversee FMD control (such as the OIE and FAO) as well as for endemic countries. However, these assays can only be performed by specialised laboratories equipped with high-containment facilities and access to the vaccine strains (that are sometimes subject to the property rights of the vaccine manufacturer). A simple suggestion to improve the current vaccine matching reports provided by reference laboratories is to include the neutralisation titres against the homologous and heterologous viruses and to link these titres to heterologous protective values (described later in this discussion).

6.2 Use of serological assays to define protection

This study showed LPBE is more repeatable than VNT as described in Chapter 3. However, LPBE method uses polyclonal antibodies and therefore measures the binding of all antibodies to FMDV, while VNT measures the neutralising antibody titre which are reported to correlate better to protection (Robiolo et al., 2010). In contrast to *in-vivo* challenge studies which are also criticised as they have issues of low precision unless large number of animals are used (Goris et al.), the current *in-vitro* methods (using VNT and LPBE) are simple and affordable. However, there are a few points that need consideration when using these *in-vitro* methods. At present, defined cut-offs for different serotypes and lineages have not been systematically approached outside of viruses that circulate in

South America. Additionally, the current protective cut-off values available in references are mostly derived from experimental conditions which is not related to field situation (due to un-natural route and very high dose (10,000 BID) of virus challenge used in these studies). This results in the need for of the neutralisation or total antibody titres cut-off for other parts of FMD endemic world which have different FMD virus pools and host population dynamics. Longitudinal observation surveillance data such as clinical or serological prevalence of vaccinated herds in combination with systematic vaccine evaluation is therefore suggested. This effort is vital for FMD endemic countries as part of regular post vaccination monitoring (Ferrari et al., 2016). However, it is currently not carried out in many FMD endemic countries probably due to the challenges and resources required. Activities related to evaluation of vaccine performance in the field within FMD endemic area include detailed planning, suitability of the herds and animal selected, follow up, sample processing and testing as well as data organisation and analysis. Furthermore, these activities are directly related and therefore involve a chain of well-planned procedures usually undertaken by local veterinary services. Animals and herds selected for the purpose of vaccine evaluation have to be tested with a reliable and accurate test to ensure that there is no FMDV infection at any stage (both apparent and unapparent). With the potential for sub-clinically and persistently infected animals, NSP ELISA needs to be performed to discriminate herds with these FMD before selection can be made. It is important to note that the use of NSP ELISA is useful for evaluating the status of a herd but not so for individual animals (Clavijo et al., 2004) which leads to high number of farms to be tested in order to find suitable herds that can be selected for PVM. An example of this situation is shown in Chapter 5 where even in herds with no clinical history of FMD, NSP positives were detected in 13% of 30 serum samples involving three out of four (75%) herds while farms with FMD history (three years before sampling) showed all five (100%) herds sampled are not fit for PVM. Furthermore, repeated vaccination may also result in NSP positive cattle particularly due to impurities in the FMD vaccine use (Lee et al., 2006). Also, the selected animals have to be old enough to ensure that no maternally derived antibodies present for these may interfere with the immune response induced by vaccination. Considering factors that influence the NSP ELISA it is advised to include only calves aged between six months to one year for FMD vaccine evaluation. The use of serological assays to define protection in an endemic country is studied in Chapter 5.

6.3 What do r1 values mean in the context of FMD vaccination in P. Malaysia?

Chapter 5 emphasised the similarity in antigenic relationship of the *in-vitro* findings with the field situation in a herd of dairy cattle in Malaysia. This field experiment provided evidence that the current vaccine used in the field elicited neutralisation titres after primary vaccination and the number of neutralising antibodies increased after booster vaccination to achieve the suggested protective cut-off point. These findings indicate that the serotype A component in the vaccine used in Malaysia is appropriate for the field strains of FMDV that are circulating (even though the r1-values are variable).

This study also showed the negative impact of the maternally derived antibody on neutralisation titre after primary vaccination. It is therefore recommended that first vaccination is administered in calves after the level of maternally derived antibodies had waned to a level that that will not interfere with the primary vaccination and booster vaccination are given after the primary vaccination following the manufacturer recommendations.

6.4 Other factors define FMDV vaccine performance

We have seen that vaccine-matching is one of the key elements in the selection of an appropriate FMDV vaccine. However, it is crucial that an assessment of a vaccine performance includes other elements in addition to antigen matching. The major elements that influence FMD vaccine performance can be classified into three main groups comprising host, vaccine and human factors (Figure 6.1). Human factors include ensuring that the cold-chain integrity from the manufacturer to the farm, time to vaccinate and vaccine delivery to individual animals are done correctly. The elements under indirect host factors are more commonly encountered in farm animals' particularly the negative effects of maternally derived antibodies which are essential to determine the optimum time for the first FMD vaccination, in order to reduce the "immunological gap" between the waning of maternal protection and the first vaccination where animals may be infected by the virus. The second vaccine factor is subdivided into virus-related and manufacturer-related components which include matching as indicated by the antigenic relationship of the vaccine strain (virus in the vaccine preparation) and the field isolate (virus isolated from diseased animal in the field).

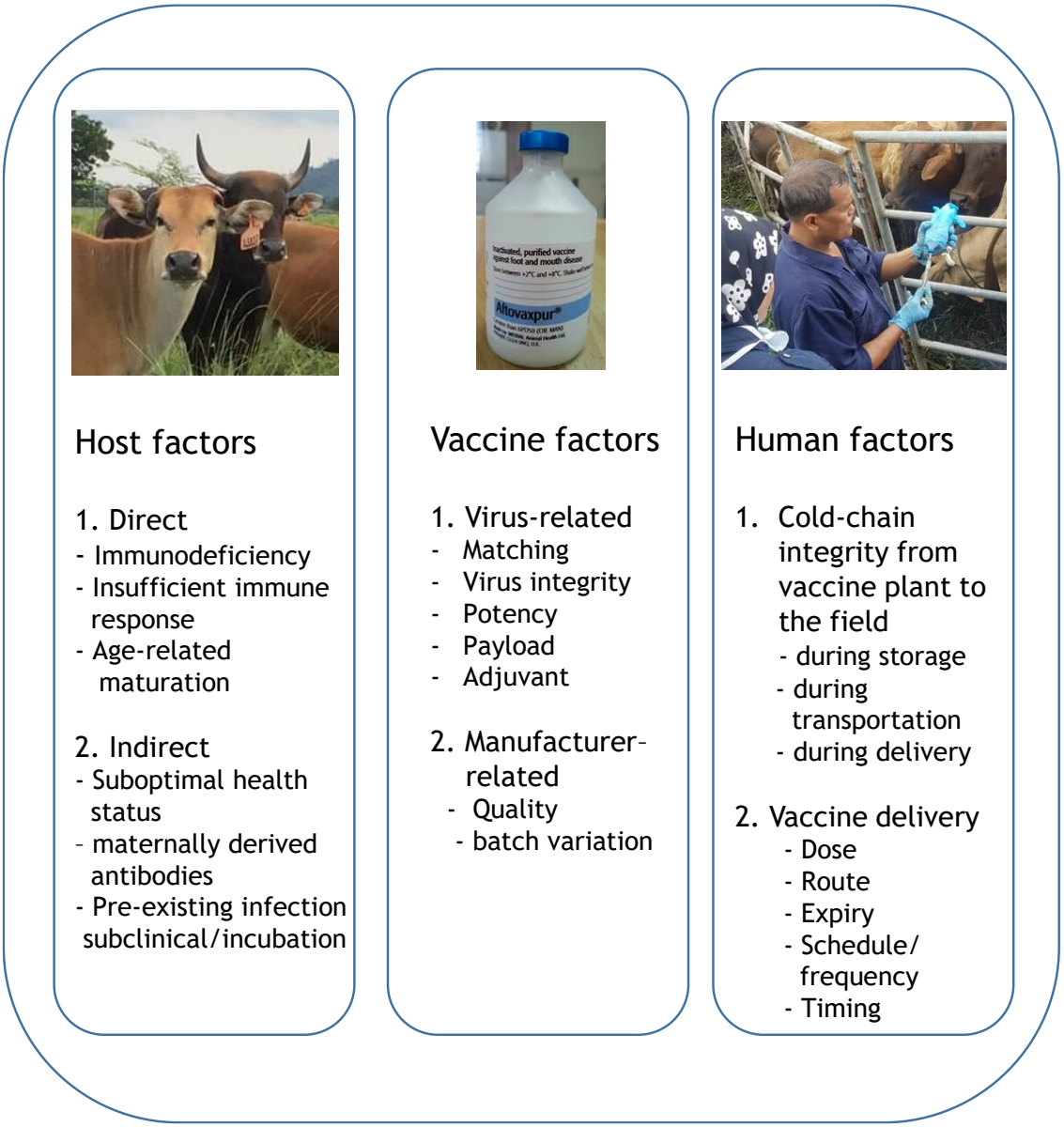


Figure 6.1: Schematic representation of the three main factors that influence the performance of FMD vaccines. Adapted from (Heininger et al., 2012; Lyons et al., 2016).

6.4.1 Future challenges for FMD control in Malaysia: continued threats posed by FMD incursions form neighbouring countries

The Malaysian national surveillance program for FMD for the year 2017 revealed that approximately 50% animals imported from Thailand and Myanmar and held at Quarantine stations in Malaysia, were found to have NSP antibodies against FMD virus.

Studies show that FMD cases in P. Malaysia are linked predominantly to the importation of live animals from neighbouring countries (Abila and Foreman, 2006; Adullah, 2014;

Gleeson, 2002; Maswati et al., 2000; Ramanoon, 2016). These findings were supported further by data from 2017 FMD national surveillance which showed that the estimated point prevalence for FMD (based on NSP ELISA tested in n = 9044 cattle sera) was highest in states bordering Thailand and Myanmar and progressively lower towards the south (Figure 6.2). More specifically, the overall point estimate for FMDV NSP positivity for cattle at individual level for the country was relatively low (32%) but significantly higher in the border states of Kelantan (53%; n = 816 serum samples), Perlis (49%; n = 728 serum samples) and Kedah (47%; n = 957 serum samples). Although the state of Perak is also neighbouring Thailand, the border terrain consists mainly of dense tropical jungle and mountainous terrain with the top of Mountain Inas as the highest point is difficult to penetrate. Therefore, importation of animals into this state is indirect, mostly via the states of Perlis, Kedah or the state of Kelantan. This shows that the existence of barriers (e.g. geographical) can reduce FMD incidence in the region.

The 2017 FMD national surveillance also revealed that estimated point prevalence for FMD in cattle at the farm level (71%) was higher than at the level of individual animals (32%) (Figure 6.3). This low level of NSP positive in multiple premises is most likely an effect of in-country livestock movement. Moreover, this observation is in agreement with a study on animal traceability in P. Malaysia which shows high movement with no specific pattern in cattle within and between districts/states in P. Malaysia (Bugis, 2018).

As evidenced following the 2017 FMD national surveillance program, water buffalo was shown to have the highest percentage of individual FMD NSP positive (43%), followed by cattle (32%), goat (19%), sheep (12%) and pigs (3%). However, the 3% serological prevalence in pigs is debatable since none of the 2647 pigs, from which sera samples were taken, showed any clinical sign of FMD. In P. Malaysia, pigs are reared in commercial pig farms with stringent rules after eradication of Nipah virus in 1999 (Mohd Nor et al., 2000). Clinical signs of FMD in commercial pig farms are relatively easy to identify since pigs are reared on concrete flooring, an environmental condition which often enhances severity of feet lesions in pigs. Possible explanation for the 3% NSP positive could be due to false positive since in reality there is no serology test that have 100% sensitivity and specificity at once even though pigs can also be sub-clinically infected with FMDV.

The 2017 FMD national surveillance program revealed 32% prevalence of NSP antibody positive cattle, however, without further specification into dairy and beef production. The distinction is important since, due to animal accessibility, vaccination is undertaken regularly only in dairy cattle. Interestingly, analysis of suspected FMD cases in cattle in Melaka (a state in the mid-west P. Malaysia) between 2016 and 2017 showed that, of 34 FMD suspected cases, 32 were beef cattle while only 2 dairy cattle. Further investigation on the two dairy farms revealed that the FMD clinical sign occurred in young animals and

no FMD incidence was noted in older animals which received booster vaccinations. On the other hand, only nine among the 32 suspected beef herds had history of FMD vaccination; six received FMD vaccine more than two years ago while in 17 herds last vaccination was administered more than one year ago. Higher serological positivity for FMD in water buffalo compared to cattle was also reported in Lao People's Democratic Republic and Vietnam (Blacksell et al., 2008; de Carvalho Ferreira et al., 2017).

The relatively low prevalence of the disease in pigs and dairy cattle provides the evidence that the current vaccine used to control FMD in P. Malaysia and the regime by which it is applied is effective. Currently, in Malaysia, vaccination against FMD in pigs and dairy cattle is compulsory and animals are vaccinated regularly following the manufacturer's recommendation.

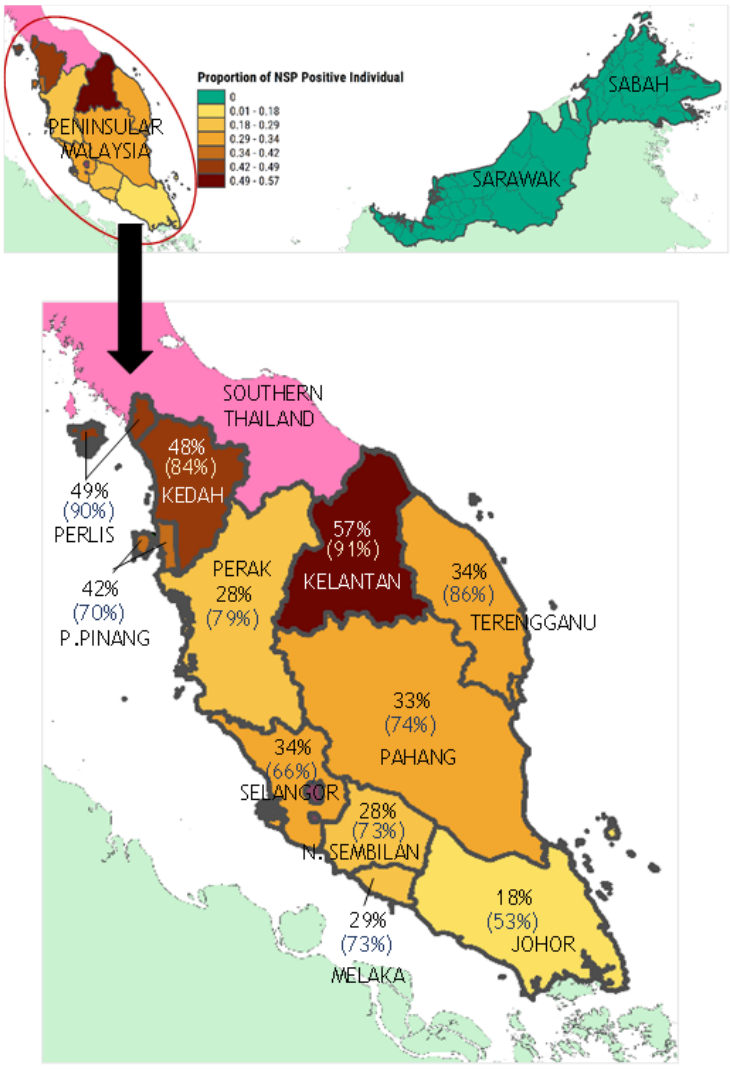


Figure 6.2: Results of the 2017 national FMD serological surveillance in P. Malaysia. Percentages point estimates of FMD prevalence in individual animals in each state as displayed; numbers in brackets () relate to percentages point estimates at the farm level in each state.

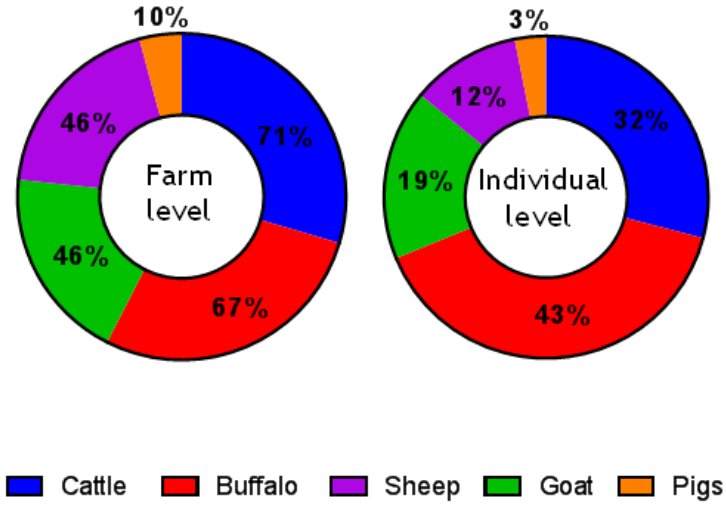


Figure 6.3: Percentages point estimates for FMD prevalence in livestock species in P. Malaysia in 2017. The percentages point estimates were established based on testing NSP positive animals.

6.4.2 Suggestion: control versus eradication of FMD

In addition to technical challenges, in endemic countries political decisions regarding FMD *control* vs *eradicate* need to be made and followed rigorously in form of implementation of polices. The extent to which these policies are adopted and implemented is dependent upon the economic status of the endemic country as well as local resources and priorities. The impact of FMD in relation to food security and livelihood of the nation play major role in the decision-making process.

As an example, the policy to control rather than eradicate FMD currently adopted in P. Malaysia was based on two main factors: (i) the high dependency on red meat and dairy products which need to be imported from other countries and (ii) the geographical location of P. Malaysia with common international borders with countries in SEA that are endemic for FMD. However, the policy used in P. Malaysia is different to that used in East Malaysia (Sabah and Sarawak) on the Island of Borneo, where FMD free zones (without vaccination) have been established (and recognised by the OIE). Maintenance of the FMD-free status in East Malaysia requires that stringent FMD control measures are applied such as strict controls on the movement of animals and animal products into this part of the country.

However, in P. Malaysia where FMD is endemic, FMD control policy demands compulsory vaccination but focusses mainly on pigs, dairy farms and beef cattle in defined FMD hotspots. The vaccine is provided free of charge to smallholders. Additionally, restrictions

on importation of live animals (pigs and dairy cattle breeds) into P. Malaysia from FMD free countries only are applied. However, the restrictions on importation do not apply to beef cattle and small ruminants. This is considered a major obstacle for successful disease control in the country. This is evident since nearly half (48%) of live cattle imported from Thailand and Myanmar were tested NSP positive at quarantine stations in the northern states of P. Malaysia in 2017. Therefore, it can be concluded that the FMD control policy in terms of importation of live animals into P. Malaysia needs improvements in order for the country to eradicate FMD.

Furthermore, most FMD incidences in P. Malaysia are recorded in beef cattle and small ruminants kept mainly in traditional and integration husbandry practice systems where vaccination is difficult. With the current FMD control measures in P. Malaysia, FMDV is likely to continue circulating within the small ruminant and beef cattle populations and pose a constant threat to the other susceptible livestock species. Therefore, to tighten the disease control a regular FMD vaccination program needs to be extended to include the small ruminant and beef cattle populations outside the areas considered as the disease hotspots.

Based on current knowledge, only when the above recommendations are fully implemented and practiced, eradication program for FMD that may include culling of clinically infected animals can be implemented in P. Malaysia. However, the biggest challenge that the country has to face in order to achieve FMD eradication is how to maintain the supply of live cattle and red meat with affordable prices in order to cater to the basic needs of the people. For instance, in 2017 about 24,517 live beef cattle were legally imported into P. Malaysia from Thailand for slaughter. There are also 20,556 live cattle imported into Malaysia from Australia for the same purpose. The value of ruminant meat and product imported into P. Malaysia is valued at US$1.7 billion in 2017.

6.5 Concluding remarks

i. The relationship coefficient (r1 values) that are currently recommended by the OIE need attention and improvement. Much of the difficulties that arise from the use of a strict and universal cut-off (especially the single cut-off of 0.3 for VNT, but also the lack of flexibility in the 0.2-0.4 cut-off for LPBE for different vaccines) to define whether a vaccine is antigenically related to a field virus. The use of such a cut-off is unrealistic since these *in-vitro* methods suffer inherent variability as seen in this PhD.

ii. Variability of *in-vitro* vaccine matching was in part due to the cellular cycle of the cells being used, the BVS and the stability of the antigen. By decreasing the variability of each of these three elements the *in-vitro* vaccine matching can produce more reliable and reproducible results.

iii. The host immune protective cut-off needs to be defined (using a range of available laboratory tests) for FMD viruses in different regions taking into consideration the epidemiological triad of FMD that has dynamic agent, host and environment factors.

iv. FMD is a transboundary animal disease. Therefore, it is recommended for field studies to be carried to determine suitability of a vaccine virus before decisions are made to change vaccine components (at the regional level).

v. Post Vaccination Monitoring for FMD has to be performed as part of surveillance program in FMD endemic countries that use vaccination as part of their FMD control measures to ensure their FMD control program is working (Ferrari et al., 2016).

vi. In general, field observations in P. Malaysia indicated that the vaccine used is beneficial and able to confer protection against the contemporary field virus. Since there is no significant antigenic variation in FMDV within Pool 1 it is predicted that same vaccine can be used in other countries in mainland SEA.

References

Abdul-Hamid, N.F., Hussein, N.M., Wadsworth, J., Radford, A.D., Knowles, N.J., King, D.P., 2011. Phylogeography of foot-and-mouth disease virus types O and A in Malaysia and surrounding countries. Infection Genetics and Evolution 11, 320-328.

Abila, R., Foreman, S. 2006. Control of foot and mouth disease in Southeast Asia. In Proceedings of the 11th Symposium of the International Society for Veterinary Epidemiology and Economics (Cairns, Queensland, Australia), 45-49.

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D., Brown, F., 1990. The structure of foot-and-mouth disease virus-implications for its physical and biological properties. Veterinary Microbiology 23, 21-34.

Adullah, U.N.B., 2014. Cattle importation in relation to occurance of foot-and-mouth disease in Peninsular Malaysia. University Putra Malaysia,

Aggarwal, N., Zhang, Z., Cox, S., Statham, R., Alexandersen, S., Kitching, R.P., Barnett, P.V., 2002. Experimental studies with foot-and-mouth disease virus, strain O, responsible for the 2001 epidemic in the United Kingdom. Vaccine 20, 2508-2515.

Alexandersen, S., Donaldson, A.I., 2002. Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. Epidemiology and Infection 128, 313-323.

Alexandersen, S., Kitching, R.P., Mansley, L.M., Donaldson, A.I., 2003. Clinical and laboratory investigations of five outbreaks of foot-and-mouth disease during the 2001 epidemic in the United Kingdom. Veterinary Record 152, 489-496.

Alexandersen, S., Mowat, N., 2005. Foot-and-mouth disease: host range and pathogenesis. Current Topics in Microbiology and Immunology 288, 9-42.

Alexandersen, S., Quan, M., Murphy, C., Knight, J., Zhang, Z., 2003. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. Journal of Comparative Pathology 129, 268-282.

Alexandersen, S., Zhang, Z., Donaldson, A.I., 2002. Aspects of the persistence of foot-and-mouth disease virus in animals--the carrier problem. Microbes Infect 4, 1099-1110.

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J.M., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. Journal of Comparative Pathology 129, 1-36.

Amass, S.F., Mason, P.W., Pacheco, J.M., Miller, C.A., Ramirez, A., Clark, L.K., Ragland, D., Schneider, J.L., Kenyon, S.J., 2004. Procedures for preventing transmission of foot-and-mouth disease virus (O/TAW/97) by people. Veterinary Microbiology 103, 143-149.

Amass, S.F., Pacheco, J.M., Mason, P.W., Schneider, J.L., Alvarez, R.M., Clark, L.K., Ragland, D., 2003. Procedures for preventing the transmission of foot-and-mouth disease virus to pigs and sheep by personnel in contact with infected pigs. Veterinary Record 153, 137-140.

Asfor, A.S., Upadhyaya, S., Knowles, N.J., King, D.P., Paton, D.J., Mahapatra, M., 2014. Novel antibody binding determinants on the capsid surface of serotype O foot-and-mouth disease virus. Journal of General Virology 95, 1104-1116.

Babjee, S.M., 1994. History, Development and Prospects of the Animal Industry and Veterinary Services in Malaysia. Department of Veterinary Services, Ministry of Agriculture Malaysia, Kuala Lumpur.

Bachanek-Bankowska, K., Di Nardo, A., Wadsworth, J., Henry, E.K.M., Parlak, U., Timina, A., Mischenko, A., Qasim, I.A., Abdollahi, D., Sultana, M., Hossain, M.A., King, D.P., Knowles, N.J., 2018. Foot-and-Mouth Disease in the Middle East Caused by an A/ASIA/G-VII Virus Lineage, 2015-2016. Emerging Infectious Diseases 24, 1073-1078.

Bachanek-Bankowska, K., Di Nardo, A., Wadsworth, J., Mioulet, V., Pezzoni, G., Grazioli, S., Brocchi, E., Kafle, S.C., Hettiarachchi, R., Kumarawadu, P.L., Eldaghayes, I.M., Dayhum, A.S., Meenowa, D., Sghaier, S., Madani, H., Abouchoaib, N., Hoang, B.H., Vu, P.P., Dukpa, K., Gurung, R.B., Tenzin, S., Wernery, U., Panthumart, A., Seeyo, K.B., Linchongsubongkoch, W., Relmy, A., Bakkali-Kassimi, L., Scherbakov, A., King, D.P., Knowles, N.J., 2018. Reconstructing the evolutionary history of pandemic foot-and-mouth disease viruses: the impact of recombination within the emerging O/ME-SA/Ind-2001 lineage. Scientific Reports 8, 14693.

Bachrach, H.L., 1968. Foot-and-mouth disease. Annual Review of Microbiology 22, 201-+.

Bachrach, H.L., Breese, S.S., Jr., Callis, J.J., Hess, W.R., Patty, R.E., 1957. Inactivation of foot-and-mouth disease virus by pH and temperature changes and by formaldehyde. Proceedings of the Society for Experimental Biology and Medicine 95, 147-152.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B., Palucka, K., 2000. Immunobiology of dendritic cells. Annual Review of Immunology 18, 767-811.

Barnett, P.V., Carabin, H., 2002. A review of emergency foot-and-mouth disease (FMD) vaccines. Vaccine 20, 1505-1514.

Barnett, P.V., Cox, S.J., 1999. The role of small ruminants in the epidemiology and transmission of foot-and-mouth disease. Veterinary Journal 158, 6-13.

Barnett, P.V., Statham, R.J., Vosloo, W., Haydon, D.T., 2003. Foot-and-mouth disease vaccine potency testing: determination and statistical validation of a model using a serological approach. Vaccine 21, 3240-3248.

Barteling, S.J., Meloen, R.H., 1974. A simple method for the quantification of 140S particles of foot-and-mouth disease virus (FMDV). Archiv für die gesamte Virusforschung 45, 362-364.

Bartley, L.M., Donnelly, C.A., Anderson, R.M., 2002. Review of foot-and-mouth disease virus survival in animal excretions and on fomites. Veterinary Record 151, 667-669.

Basavappa, R., Syed, R., Flore, O., Icenogle, J.P., Filman, D.P., Hogle, J.L., 1994. Role and mechanism of the maturation cleavage of VPO in poliovirus assembly: structure of the empty capsid assembly intermediate at 2.9 A resolution. Protein Science 3, 1651-1669.

Bastos, A.D., Boshoff, C.I., Keet, D.F., Bengis, R.G., Thomson, G.R., 2000. Natural transmission of foot-and-mouth disease virus between African buffalo (Syncerus caffer) and impala (Aepyceros melampus) in the Kruger National Park, South Africa. Epidemiology and Infection 124, 591-598.

Bates, D., Maechler, M., Bolker, B. & Walker, S., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67, 1-48.

Bautista, E.M., Ferman, G.S., Golde, W.T., 2003. Induction of lymphopenia and inhibition of T cell function during acute infection of swine with foot and mouth disease virus (FMDV). Veterinary Immunology and Immunopathology 92, 61-73.

Bautista, E.M., Ferman, G.S., Gregg, D., Brum, M.C., Grubman, M.J., Golde, W.T., 2005. Constitutive expression of alpha interferon by skin dendritic cells confers resistance to infection by foot-and-mouth disease virus. Journal of Virology 79, 4838-2847.

Baxt, B., Vakharia, V., Moore, D.M., Franke, A.J., Morgan, D.O., 1989. Analysia of neutralizing antigenic sites on the surface of Type-A2 Foot-and-mouth-disease virus. Journal of Virology 63, 2143-2151.

Bedson, S.P., Maitland, H.B., 1927. Further observations on foot and mouth disease. Section D. Experiments on the cultivation of the virus of foot and mouth disease. Journal of Comparative Pathology and Therapeutics 40, 79-93.

Belsham, G.J., 2005. Translation and replication of FMDV RNA. Current Topics in Microbiology and Immunology 288, 43-70.

Bielenberg, D.R., McCarty, M.F., Bucana, C.D., Yuspa, S.H., Morgan, D., Arbeit, J.M., Ellis, L.M., Cleary, K.R., Fidler, I.J., 1999. Expression of interferon-beta is associated with growth arrest of murine and human epidermal cells. Journal of Investigative Dermatology 112, 802-809.

Blacksell, S.D., Khounsy, S., Conlan, J.V., Gleeson, L.J., Colling, A., Westbury, H.A., 2008. Foot and mouth disease in the Lao People's Democratic Republic: II. Seroprevalence estimates, using structured surveillance and surveys of abattoirs. Revue Scientifique et Technique 27, 851-859.

Blackwell, J.H., Hyde, J.L., 1976. Effect of heat on foot-and-mouth disease virus (FMDV) in the components of milk from FMDV-infected cows. Journal of Hygiene 77, 77-83.

Blancou, J., 2002. History of the control of foot and mouth disease. Comparative Immunology, Microbiology and Infectious Diseases 25, 283-296.

Bo, L.L., Lwin, K.S., Ungvanijban, S., Knowles, N.J., Wadsworth, J., King, D.P., Abila, R., Qiu, Y., submitted. Foot-and-mouth disease outbreak due to an exotic serotype Asia 1 virus in Myanmar in 2017.

Bolwell, C., Clarke, B.E., Parry, N.R., Ouldridge, E.J., Brown, F., Rowlands, D.J., 1989. Epitope mapping of foot-and-mouth disease virus with neutralizing monoclonal-antibodies. Journal of General Virology 70, 59-68.

Booth, J.C., Rweyemamu, M.M., Pay, T.W.F., 1978. Dose-response relationships in a micro-neutralisation test for foot-and-mouth disease virus Journal of Hygiene 80, 31-42.

Brehm, K.E., Ferris, N.P., Lenk, M., Riebe, R., Haas, B., 2009. Highly sensitive foetal goat tongue cell line for detection and isolation of foot-and-mouth disease virus. Journal of Clinical Microbiology 47, 3156-3160.

Brehm, K.E., Kumar, N., Thulke, H.H., Haas, B., 2008. High potency vaccines induce protection against heterologous challenge with foot-and-mouth disease virus. Vaccine 26, 1681-1687.

Brito, B.P., Perez, A.M., Capozzo, A.V., 2014. Accuracy of traditional and novel serology tests for predicting cross-protection in foot-and-mouth disease vaccinated cattle. Vaccine 32, 433-436.

Brooksby, J.B., Rogers, J. 1957. Methods used in typing the virus of foot-and-mouth disease at Pirbright, 1950-55. In Methods of typing and cultivation of foot-and-mouth disease virus, European Productivity Agency of the Organisation for European Economic Co-operation, Project No.208 (Paris, France, European Productivity Agency of the Organisation for European Economic Co-operation (OEEC)), 31-34.

Brown, F., 2003. The history of research in foot-and-mouth disease. Virus Research 91, 3-7.

Brown, F. 2004. Stepping stones in foot-and-mouth research: a personal view, In: Sobrino, F., Domingo, E. (Eds.) Foot and Mouth Disease: Current Perspectives. 1-17.

Brown, F., Cartwright, B., 1961. Dissociation of foot-and-mouth disease virus into its nucleic acid and protein components. Nature 192, 1163-1164.

Brown, F., Cartwright, B., 1963. Purification of radioactive foot-and-mouth disease virus. Nature 199, 1168-1170.

Brown, F., Crick, J., 1959. Application of agar-gel diffusion analysis to a study of the antigenic structure of inactivated vaccines prepared from the virus of foot-and-mouth disease. Journal of Immunology 82, 444-447.

Bucafusco, D., Di Giacomo, S., Pega, J., Juncos, M.S., Schammas, J.M., Perez-Filgueira, M., Capozzo, A.V., 2014. Influence of antibodies transferred by colostrum in the immune responses of calves to current foot-and-mouth disease vaccines. Vaccine 32, 6576-6582.

Buetre, B., Wicks, S., Kreger, H., Millist, N., Yainshet, A., Garner, G., Duncan, A., Abdalla, A., Trestrail, C., Hatt, M., Thompson, L.-J., Symes, M. 2013. Potential socio-economic impacts of an outbreak of foot-and-mouth disease in Australia. In ABARES Research report 13.11 (Canberra, Australia, Department of Agriculture, Australia).

Bugis, S.B.A., 2018. Evaluation, awareness and use of cattle identification and traceability system in Peninsular Malaysia. University Putra Malaysia,

Burman, A., Clark, S., Abrescia, N.G., Fry, E.E., Stuart, D.I., Jackson, T., 2006. Specificity of the VP1 GH loop of foot-and-mouth disease virus for alphav integrins. Journal of Virology 80, 9798-9810.

Burrows, R., Mann, J.A., Greig, A., Chapman, W.G., Goodridge, D., 1971. The growth and persistence of foot-and-mouth disease virus in the bovine mammary gland. Journal of Hygiene 69, 307-321.

Cao, Y., Lu, Z., Liu, Z., 2016. Foot-and-mouth disease vaccines: progress and problems. Expert Review of Vaccines 15, 783-789.

Capozzo, A.V.E., Periolo, O.H., Robiolo, B., Seki, C., LaTorre, J.L., Grigera, P.R., 1997. Total and isotype humoral responses in cattle vaccinated with foot and mouth disease virus (FMDV) immunogen produced either in bovine tongue tissue or in BHK-21 cell suspension cultures. Vaccine 15, 624-630.

Caridi, F., Vazquez-Calvo, A., Sobrino, F., Martin-Acebes, M.A., 2015. The pH stability of foot-and-mouth disease virus particles is modulated by residues located at the pentameric interface and in the N terminus of VP1. Journal of Virology 89, 5633-5642.

Cartwright, B., Chapman, W.G., Brown, F., 1980. Serological and immunological relationships between the 146S and 12S particles of Foot-and mouth disease virus. Journal of General Virology 50, 369-375.

Cartwright, B., Morrell, D.J., Brown, F., 1982. Nature of the antibody-response to the foot-and-mouth-disease virus particle, its 12S protein subunit and the isolated immunizing polypeptide VP1. Journal of General Virology 63, 375-381.

Cavanagh, D., Sangar, D.V., Rowlands, D.J., Brown, F., 1977. Immunogenic and cell attachment sites of FMDV: further evidence for their location in a single capsid polypeptide. Journal of General Virology 35, 149-158.

Charleston, B., Bankowski, B.M., Gubbins, S., Chase-Topping, M.E., Schley, D., Howey, R., Barnett, P.V., Gibson, D., Juleff, N.D., Woolhouse, M.E.J., 2011. Relationship between clinical signs and transmission of an infectious disease and implication for control. Science 332, 726-728.

Chinsangaram, J., Mason, P.W., Grubman, M.J., 1998. Protection of swine by live and inactivated vaccines prepared from a leader proteinase-deficient serotype AI2 foot-and-mouth disease virus. Vaccine 16, 1516-1522.

Chong, S.K. 1979. Control of foot and mouth disease: slaughter or vaccination. In Annual Conference 24th Malaysian Veterinary Association (Kuala Lumpur).

Clavijo, A., Sanchez-Vazquez, M.J., Buzanovsky, L.P., Martini, M., Pompei, J.C., Cosivi, O., 2017. Current status and future prospects to achieve foot-and-mouth disease eradication in South America. Transboundary and Emerging Diseases 64, 31-36.

Clavijo, A., Wright, P., Kitching, P., 2004. Developments in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. Veterinary Journal 167, 9-22.

Cokcaliskan, C., Turkoglu, T., Uzunlu, E., Sareyyupoglu, B., Hanci, I., Ipek, A., Arslan, A., Babak, A., Ildeniz, G., Gulyaz, V., 2017. Influence of vaccine potency and booster administration of foot-and-mouth disease vaccines on the antibody response in calves with maternal antibodies. Journal of Veterinary Science 18, 315-322.

Cottral, G.E., Gailiunas, P. 1972. Experimental multiple infection of animals with foot-and-mouth disease viruses. In Annual meeting of the United States Animal Health Association, 441–465.

Crowther, J., Reckziegel, P.O., Prado, J.A., 1995. Quantification of whole virus-particles (146s) of foot-and-mouth-disease virus in the presence of virus subunits (12s), using monoclonal-antibodies in a sandwich ELISA. Vaccine 13, 1064-1075.

Crowther, J.R., Farias, S., Carpenter, W.C., Samuel, A.R., 1993. Identification of a fifth neutralizable site on type O foot-and-mouth disease virus following characterization of single and quintuple monoclonal antibody escape mutants. Journal of General Virology 74, 1547-1553.

Darzynkiewicz, Z., Huang, X., 2004. Analysis of cellular DNA content by flow cytometry. Current Protocols in immunology Chapter 5, Unit 5 7.

Dawe, P.S., Flanagan, F.O., Madekurozwa, R.L., Sorensen, K.J., Anderson, E.C., Foggin, C.M., Ferris, N.P., Knowles, N.J., 1994. Natural transmission of foot-and-mouth disease virus from African buffalo (Syncerus caffer) to cattle in a wildlife area of Zimbabwe. Veterinary Record 134, 230-232.

Dawe, P.S., Sorensen, K., Ferris, N.P., Barnett, I.T., Armstrong, R.M., Knowles, N.J., 1994. Experimental transmission of foot-and-mouth disease virus from carrier African buffalo (Syncerus caffer) to cattle in Zimbabwe. Veterinary Record 134, 211-215.

De Carvalho Ferreira, H.C., Pauszek, S.J., Ludi, A., Huston, C.L., Pacheco, J.M., Le, V.T., Nguyen, P.T., Bui, H.H., Nguyen, T.D., Nguyen, T., Nguyen, T.T., Ngo, L.T., Do, D.H., Rodriguez, L., Arzt, J., 2017. An Integrative Analysis of Foot-and-Mouth Disease Virus Carriers in Vietnam Achieved Through Targeted Surveillance and Molecular Epidemiology. Transboundary and Emerging Diseases 64, 547-563.

De Castro, M.P., 1964. Behavior of foot-and-mouth disease virus in cell culture: susceptibility of the IB-RS-2 swine cell line. Arquivos do Instituto Biologico Sao Paulo 31, 63-78.

De Castro, M.P., 1970. Clonal variation in the swine kidney cell line, IB-RS-2, in relation to morphology, karyotype and susceptibility to the foot-and-mouth disease virus (FMDV). Arquivos do Instituto Biologico Sao Paulo 37, 103-127.

De Castro, M.P., 1973. An infectious agent causing "spontaneous" degeneration of swine cells in vitro. 9, 8-16.

De Los Santos, T., Diaz-San Segundo, F., Rodriguez, L.L., 2018. The need for improved vaccines against foot-and-mouth disease. Current Opinion in Virology 29, 16-25.

Dekker, A., Eble, P., Stockhofe, N., Chenard, G., 2014. Intratypic heterologous vaccination of calves can induce an antibody response in presence of maternal antibodies against foot-and-mouth disease virus. BMC Veterinary Research 10, 127.

Department of Veterinary Services Malaysia. Department of Veterinary Services Malaysia.

Department of Veterinary Services Malaysia 1995. Report on foot and mouth diagnostic in Malaysia. Department of Veterinary Services Malaysia 2018. Laman rasmi Jabatan Perkhidmatan Veterinar Malaysia.

Doel, T.R., 2003. FMD vaccines. Virus Research 91, 81-99.

Doel, T.R., Baccarini, P.J., 1981. Thermal stability of foot-and-mouth disease virus. Archives of Virology 70, 21-32.

Doel, T.R., Chong, W.K., 1982. Comparative immunogenicity of 146S, 75S and 12S particles of foot-and-mouth disease virus. Archives of Virology 73, 185-191.

Domingo, E., Ruiz-Jarabo, C.M., Arias, A., Garcia, A.J., Escarmis, C. 2004. Quasispecies Dynamics and Evolution of foot and mouth disease virus, In: Sobrino, F., Domingo, E. (Eds.) Foot and Mouth Disease: Current Perspectives. Horizon Bioscience, Norfolk, England, 261-304.

Donaldson, A. 2004. Clinical signs of foot-and-mouth disease, In: Sobrino, F., Domingo, E. (Eds.) Foot and Mouth Disease: Current Perspectives. Horizon Bioscience, Wymondham, UK, 93-102.

Donaldson, A.I., 1975. Risks of spreading foot and mouth disease through milk and dairy products. Revue Scientifique et Technique 16, 117-124.

Donaldson, A.I. 1987. Investigations to determine the minimum aerosol doses of foot-and-mouth disease virus to infect sheep and cattle. In Aerosols. Their generation, behaviour and applications. First Conference. Held at Loughborough University of Technology, 31st March-1st April 1987., 121-123.

Donaldson, A.I., Alexandersen, S., Sorensen, J.H., Mikkelsen, T., 2001. Relative risks of the uncontrollable (airborne) spread of FMD by different species. Veterinary Record 148, 602-604.

Donaldson, A.I., Ferris, N.P., 1975. The survival of foot-and-mouth disease virus in open air conditions. Journal of Hygiene 74, 409-416.

Donaldson, A.I., Herniman, K.A., Parker, J., Sellers, R.F., 1970. Further investigations on the airborne excretion of foot-and-mouth disease virus. Journal of Hygiene 68, 557-564.

Duarte, E.A., Novella, I.S., Weaver, S.C., Domingo, E., Wain-Hobson, S., Clarke, D.K., Moya, A., Elena, S.F., de la Torre, J.C., Holland, J.J., 1994. RNA virus quasispecies: significance for viral disease and epidemiology. Infectious Agents and Disease 3, 201-214.

Edwards, J.R. 2004. Strategy for the control of foot-and-mouth disease in Southeast Asia (SEAFMD), In: Schudel, A., Lombard, M. (Eds.) Control of Infectious Animal Diseases by Vaccination. Karger, Basel, 423-431.

Elnekave. E., Dekker, A., Eble, P., Hemert-Kluitenberg, F.V., Gelman, B., Storm, N., Klement, E., 2016. The serological response against foot and mouth disease virus elicited by repeated vaccination of dairy cattle. Vaccine 34, 4920-4926.

Ellard, F.M., Drew, J., Blakemore, W.E., Stuart, D.I., King, A.M.Q., 1999. Evidence for the role of His-142 of protein 1C in the acid-induced disassembly of foot-and-mouth disease virus capsids. Journal of General Virology 80, 1911-1918.

European Commission 2013. Final Report Summary - FMD-DISCONVAC (Development, enhancement and complementation of animal-sparing, foot-and-mouth disease vaccine-based control strategies for free and endemic regions) (Belgium).

Everett, H., Salguero, F.J., Graham, S.P., Haines, F., Johns, H., Clifford, D., Nunez, A., La Rocca, S.A., Parchariyanon, S., Steinbach, F., Drew, T., Crooke, H., 2010. Characterisation of experimental infections of domestic pigs with genotype 2.1 and 3.3 isolates of classical swine fever virus. Veterinary Microbiology 142, 26-33.

FAO 2002. Animal diseases: implications for international meat trade. In Intergovernmental group on meat and dairy products (19th Session), 27-29 August 2002 (Rome, Food and Agriculture Organization of the United Nations).

FAO/OIE, 2018. The Progressive Control Pathway for Foot and Mouth Disease control (PCP-FMD) Principles, Stage Descriptions and Standards, Second Edition.

Farooq, U., Ahmed, Z., Naeem, K., Bertram, M., Brito, B., Stenfeldt, C., Pauszek, S.J., LaRocco, M., Rodriguez, L., Arzt, J., 2018. Characterization of naturally occurring, new and persistent subclinical foot-and-mouth disease virus infection in vaccinated Asian buffalo in Islamabad Capital Territory, Pakistan. Transboundary and Emerging Diseases 65, 1836-1850.

Fernandez-Sainz, I., Medina, G.N., Ramirez-Medina, E., Koster, M.J., Grubman, M.J., de Los Santos, T., 2017. Adenovirus-vectored foot-and-mouth disease vaccine confers early and full protection against FMDV O1 Manisa in swine. Virology 502, 123-132.

Ferrari, G., Paton, D., Duffy, S., Bartels, C., Knight-Jones, T., 2016. Foot and mouth disease vaccination and post-vaccination monitoring: guidelines. The Food and Agriculture Organization of the United Nations and the World Organisation for Animal Health 74 p.

Ferris, N.P., Dawson, M., 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. Veterinary Microbiology 16, 201-209.

Ferris, N.P., Donaldson, A.I., 1992. The World Reference Laboratory for Foot and Mouth Disease: a review of thirty-three years of activity (1958-1991). Revue Scientifique et Technique 11, 657-684.

Ferris, N.P., Donaldson, A.I., Barnett, I.T.R., Osborne, R.W., 1984. Inactivation, purification and stability of 146S antigen of foot-and-mouth disease virus for use as reagents in the complement fixation test.pdf. Revue Scientifique et Technique 3, 339-350.

Ferris, N.P., King, D.P., Reid, S.M., Hutchings, G.H., Shaw, A.E., Paton, D.J., Goris, N., Haas, B., Hoffmann, B., Brocchi, E., Bugnetti, M., Dekker, A., De Clercq, K., 2006. Foot-and-mouth disease virus: A first inter-laboratory comparison trial to evaluate virus isolation and RT-PCR detection methods. Veterinary Microbiology 117, 130-140.

Filho, Y.L.V., Astudillo, V., Gomes, I., Fernández, G., Rozas, C.E.E., Ravison, J.A., Alonso, A., 1993. Potency control of foot-and-mouth disease vaccine in cattle. Comparison of the 50% protective dose and the protection against generalization. Vaccine 11, 1424-1428.

Foote, M.R., Nonnecke, B.J., Beitz, D.C., Waters, W.R., 2007. Antigen-specific B-cell responses by neonatal calves after early vaccination. Journal of Dairy Science 90, 5208-5217.

Forman, S., Le Gall, F., Belton, D., Evans, B., François, J.L., Murray, G., Sheesley, D., Vandersmissen, A., Yoshimura, S., 2009. Moving towards the global control of foot and mouth disease: an opportunity for donors. Revue Scientifique et Technique 28, 883-896.

Fox, G., Parry, N.R., Barnett, P.V., McGinn, B., Rowlands, D.J., Brown, F., 1989. The cell attachment site on foot-and-mouth-disease virus includes the amino-acid sequence RGD (Arginine-Glycine-Aspartic acid). Journal of General Virology 70, 625-637.

Fry, E.E., Newman, J.W., Curry, S., Najjam, S., Jackson, T., Blakemore, W., Lea, S.M., Miller, L., Burman, A., King, A.M., Stuart, D.I., 2005. Structure of foot-and-mouth disease virus serotype A10 61 alone and complexed with oligosaccharide receptor: receptor conservation in the face of antigenic variation. Journal of General Virology 86, 1909-1920.

Geering, W.A., Forman, A.J., Nunn, M.J. 1995. Foot-and-mouth disease, In: Exotic diseases of animals: a field guide for Australian veterinarians., 112-131.

Gleeson, L.J., 2002. A review of the status of foot and mouth disease in South-East Asia and approaches to control and eradication. Revue Scientifique et Technique 21, 465-475.

Golde, W.T., Pacheco, J.M., Duque, H., Doel, T., Penfold, B., Ferman, G.S., Gregg, D.R., Rodriguez, L.L., 2005. Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: Use in emergency outbreak response. Vaccine 23, 5775-5782.

Goris, N., De Clercq, K. 2008. Keynote: Vaccine quality tests: The value of alternative methods? In the Global control of FMD - Tools, ideas and ideals – 14-17 October 2008 (Erice, Italy, FAO), Appendix 24, 159-168.

Goris, N., Merkelbach-Peters, P., Diev, V.I., Verloo, D., Zakharov, V.M., Horst-Peter, K., De Clercq, K. European Pharmacopoeia foot-and-mouth disease vaccine potency test: between test variability, Appendix 32, 220-228.

Goris, N., Willems, T., Diev, V.I., Merkelbach-Peters, P., Vanbinst, T., Van der Stede, Y., Kraft, H.P., Zakharov, V.M., Borisov, V.V., Nauwynck, H.J., Haas, B., De Clercq, K., 2008. Indirect foot-and-mouth disease vaccine potency testing based on a serological alternative. Vaccine 26, 3870-3879.

Grant, C.F., Carr, B.V., Kotecha, A., van den Born, E., Stuart, D.I., Hammond, J.A., Charleston, B., 2017. The B Cell Response to foot-and-mouth disease virus in cattle following sequential vaccination with multiple serotypes. Journal of Virology 91, e02157-02116.

Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clinical Microbiology Reviews 17, 465-493.

Guerin, B., Pozzi, N., 2005. Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. Theriogenology 63, 556-572.

Guzylack-Piriou, L., Bergamin, F., Gerber, M., McCullough, K.C., Summerfield, A., 2006. Plasmacytoid dendritic cell activation by foot-and-mouth disease virus requires immune complexes. European Journal of Immunology 36, 1674-1683.

Haas, B., Ahl, R., Bohm, R., Strauch, D., 1995. Inactivation of viruses in liquid manure. Revue Scientifique et Technique 14, 435-445.

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

Harmsen, M.M., De Haard, H.J., 2007. Properties, production, and applications of camelid single-domain antibody fragments. Applied Microbiology and Biotechnology 77, 13-22.

Harmsen, M.M., Fijten, H.P.D., Westra, D.F., Coco-Martin, J.M., 2011. Effect of thiomersal on dissociation of intact (146S) foot-and-mouth disease virions into 12S particles as assessed by novel ELISAs specific for either 146S or 12S particles. Vaccine 29, 2682-2690.

Harmsen, M.M., Seago, J., Perez, E., Charleston, B., Eble, P.L., Dekker, A., 2017. Isolation of single-domain antibody fragments that preferentially detect intact (146S) particles of foot-and-mouth disease virus for use in vaccine quality control. Frontiers in Immunology 8, 960.

Hayama, Y., Osada, Y., Oushiki, D., Tsutsui, T., 2017. An economic assessment of foot and mouth disease in Japan. Revue Scientifique et Technique 36, 207-215.

Hayer, S.S., VanderWaal, K., Ranjan, R., Biswal, J.K., Subramaniam, S., Mohapatra, J.K., Sharma, G.K., Rout, M., Dash, B.B., Das, B., Prusty, B.R., Sharma, A.K., Stenfeldt, C., Perez, A., Delgado, A.H., Sharma, M.K., Rodriguez, L.L., Pattnaik, B., Arzt, J., 2018. Foot-and-mouth disease virus transmission dynamics and persistence in a herd of vaccinated dairy cattle in India. Transboundary and Emerging Diseases 65, e404-e415.

Heath, L., van der Walt, E., Varsani, A., Martin, D.P., 2006. Recombination patterns in aphthoviruses mirror those found in other picornaviruses. Journal of Virology 80, 11827-11832.

Heininger, U., Bachtiar, N.S., Bahri, P., Dana, A., Dodoo, A., Gidudu, J., Santos, E.M., 2012. The concept of vaccination failure. Vaccine 30, 1265-1268.

Henderson, W.M., 1978. Historical review of control of foot and mouth disease. British Veterinary Journal 134, 3-9.

Hingley, P.J., Pay, T.W.F., 1987. Sources of variability in Foot-and-mouth-disease vaccine potency estimates based on serum neutralizing antibody-assay. Journal of Biological Standardization 15, 127-142.

Horsington, J., Nfon, C., Bittner, H., Durr, P.A., Singanallur, N., Alexandersen, S., Vosloo, W., 2018. The protective capacity of high payload FMDV A22 IRQ vaccine in sheep against direct-contact challenge with a heterologous, contemporary FMDV A strain from South East Asia. PLoS One 13, e0195302.

House, J.A., House, C., Llewellyn, M.E., 1988. Characteristics of the porcine kidney-cell line IB-RS-2 clone D10 (IB-RS-2D10) which is free of Hog cholera virus. In Vitro Cellular and Developmental Biology 24, 677-682.

Hughes, G.J., Mioulet, V., Kitching, R.P., Woolhouse, M.E., Alexandersen, S., Donaldson, A.I., 2002. Foot-and-mouth disease virus infection of sheep: implications for diagnosis and control. Veterinary Record 150, 724-727.

Hugh-Jones, M.E., Tinline, R.R., 1976. Studies on the 1967-68 foot and mouth disease epidemic: incubation period and herd serial interval. Journal of Hygiene 77, 141-153.

Jamal, A.S.M., Bouma, A., van den Broek, J., Stegeman, A., Chenard, G., Dekker, A., 2008. Foot-and-mouth disease vaccine potency testing: The influence of serotype, type of adjuvant, valency, fractionation method, and virus culture on the dose-response curve in cattle. Vaccine 26, 6317-6321.

Kalmar, E., Peleg, B.A., Modan, M., 1972. Non-specific neutralizing activity of bovine sera against various types of foot and mouth disease virus II Genetic control of non-specific neutralizing activity in calf sera. Refuah Veterinarith 2991-102, 91-102.

Kärber, G., 1931. Beitrag zur Kollektiven Behandlung Pharmakologischer Reihenversuche. Naunyn-Schmiedebergs Archiv für Experimentelle Pathologie und Pharmakologie 162, 480-483.

Kitching, R.P., 1998. A recent history of foot-and-mouth disease. Journal of Comparative Pathology 118, 89-108.

Kitching, R.P., 2002. Clinical variation in foot and mouth disease: cattle. Revue Scientifique et Technique 21, 499-504.

Kitching, R.P. 2004. Diagnosis and Control of Foot-and-Mouth Disease, In: Sobrino, F., Domingo, E. (Eds.) Foot and Mouth Disease: Current Perspectives. Horizon Bioscience, Wymondham, Norfolk, England, 411-424.

Kitching, R.P., Hutber, A.M., Thrusfield, M.V., 2005. A review of foot-and-mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. Veterinary Journal 169, 197-209.

Kitching, R.P., Knowles, N.J., Samuel, A.R., Donaldson, A.I., 1989. Development of foot-and-mouth disease virus strain characterization - a review. Tropical Animal Health and Production 21, 153-166.

Kitching, R.P., Rendle, R., Ferris, N.P., 1988. Rapid correlation between field isolates and vaccine strains of foot-and-mouth-disease virus. Vaccine 6, 403-408.

Kitson, J.D.A., McCahon, D., Belsham, G.J., 1990. Sequence-analysis of monoclonal antibody resistant mutants of tyoe O foot-and-mouth disease-evidence for the involvement of the 3 surfaces exposed capsid proteins in 4 antigenic sites. Virology 179, 26-34.

Knight-Jones, T.J., Bulut, A.N., Gubbins, S., Stark, K.D., Pfeiffer, D.U., Sumption, K.J., Paton, D.J., 2015. Randomised field trial to evaluate serological response after foot-and-mouth disease vaccination in Turkey. Vaccine 33, 805-811.

Knight-Jones, T.J., Gubbins, S., Bulut, A.N., Stark, K.D., Pfeiffer, D.U., Sumption, K.J., Paton, D.J., 2016. Mass vaccination, immunity and coverage: modelling population

protection against foot-and-mouth disease in Turkish cattle. Scientific Reports 6, 22121.

Knight-Jones, T.J., Rushton, J., 2013. The economic impacts of foot and mouth disease - what are they, how big are they and where do they occur? Preventive Veterinary Medicine 112, 161-173.

Knight-Jones, T.J.D., McLaws, M., Rushton, J., 2017. Foot-and-mouth disease impact on smallholders - what do we know, what don't we know and how can we find out more? Transboundary and Emerging Diseases 64, 1079-1094.

Knowles, N.J. 2018. Picornaviridae website.

Knowles, N.J., Samuel, A.R., 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Research 91, 65-80.

Knowles, N.J., Samuel, A.R., Davies, P.R., Midgley, R.J., Valarcher, J.F., 2005. Pandemic strain of foot-and-mouth disease virus serotype O. Emerging Infectious Diseases 11, 1887-1893.

Knowles, N.J., Wadsworth, J., Bachanek-Bankowska, K., King, D.P., 2016. VP1 sequencing protocol for foot and mouth disease virus molecular epidemiology. Revue Scientifique et Technique 35, 741-755.

Kotecha, A., Seago, J., Scott, K., Burman, A., Loureiro, S., Ren, J.S., Porta, C., Ginn, H.M., Jackson, T., Perez-Martin, E., Siebert, C.A., Paul, G., Huiskonen, J.T., Jones, I.M., Esnouf, R.M., Fry, E.E., Maree, F.F., Charleston, B., Stuart, D.I., 2015. Structure-based energetics of protein interfaces guides foot-and-mouth disease virus vaccine design. Nature Structural and Molecular Biology 22, 788-794.

Kotecha, A., Zhang, F., Juleff, N., Jackson, T., Perez, E., Stuart, D., Fry, E., Charleston, B., Seago, J., 2016. Application of the thermofluor PaSTRy technique for improving foot-and-mouth disease virus vaccine formulation. Journal of General Virology 97, 1557-1565.

Laporte, J., Grosclaude, J., Wantyghem, J., Bernard, S., Rouze, P., 1973. Neutralization in cell-culture of infectiousness of Aphthous fever virus by serum from immunised pigs using purified viral protein. Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D 276, 3399-3401.

Lavoria, M.A., Di-Giacomo, S., Bucafusco, D., Franco-Mahecha, O.L., Perez-Filgueira, D.M., Capozzo, A.V., 2012. Avidity and subtyping of specific antibodies applied to the

indirect assessment of heterologous protection against foot-and-mouth disease virus in cattle. Vaccine 30, 6845-6850.

Lee, F., Jong, M.H., Yang, D.W., 2006. Presence of antibodies to non-structural proteins of foot-and-mouth disease virus in repeatedly vaccinated cattle. Veterinary Microbiology 115, 14-20.

Leforban, Y., Gerbier, G., 2002. Review of the status of foot and mouth disease and approach to control/eradication in Europe and Central Asia. Revue Scientifique et Technique 21, 477-492.

Li, F., Browning, G.F., Studdert, M.J., Crabb, B.S., 1996. Equine rhinovirus 1 is more closely related to foot-and-mouth disease virus than to other picornaviruses. Proceedings of the National Academy of Sciences of the United States of America 93, 990-995.

Li, Z., Yi, Y., Yin, X., Zhang, Y., Liu, M., Liu, H., Li, X., Li, Y., Zhang, Z., Liu, J., 2012. Development of a foot-and-mouth disease virus serotype A empty capsid subunit vaccine using silkworm (Bombyx mori) pupae. PLoS One 7, e43849.

Lombard, M.F., Schermbrucker, C.G. 1994. Vaccines for control of foot-and-mouth disease worldwide: production, selection and field performance. In Diagnosis and epidemiology of foot-and-mouth disease in Southeast Asia: international workshop held September 6-9, 1993 (Lampang, Thailand), 16-20.

Lubroth, J., Rweyemamu, M.M., Viljoen, G., Diallo, A., Dungu, B., Amafu, W., 2007. Veterinary vaccines and their use in developing countries. Revue Scientifique et Technique 26, 179-201.

Ludi, A.B., Horton, D.L., Li, Y., Mahapatra, M., King, D.P., Knowles, N.J., Russell, C.A., Paton, D.J., Wood, J.L.N., Smith, D.J., Hammond, J.M., 2014. Antigenic variation of foot-and-mouth disease virus serotype A. Journal of General Virology 95, 384-392.

Ludi, A.B., Mioulet, V., J., K.N., King, D.P. 2017. Laboratory diagnostic methods to support the surveillance and control of foot-and-mouth disease, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-mouth Disease Virus: Current Research and Emerging Trends. Caister Academic Press, Madrid, 275-285.

Lyons, N.A., Ludi, A.B., Wilsden, G., Hamblin, P., Qasim, I.A., Gubbins, S., King, D.P., 2017. Evaluation of a polyvalent foot-and-mouth disease virus vaccine containing A Saudi-95 against field challenge on large-scale dairy farms in Saudi Arabia with the emerging A/ASIA/G-VII viral lineage. Vaccine 35, 6850-6857.

Lyons, N.A., Lyoo, Y.S., King, D.P., Paton, D.J., 2016. Challenges of generating and maintaining protective vaccine-induced immune responses for foot-and-mouth disease virus in pigs. Frontiers in Veterinary Science 3, 102.

Mackay, D.K.J., Forsyth, M.A., Davies, P.R., Berlinzani, A., Belsham, G.J., Flint, M., Ryan, M.D., 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine 16, 446-459.

Mackowiak, C., Fontaine, J., Camand, R., Petermann, H.G., Lang, R., 1962. Relationship between neutralising antibody titre and protection in animals immunised against foot-and-mouth disease. Annales De L Institut Pasteur 103, 252-261.

MacLachlan, N.J. 2017. Picornaviridae, In: N. James MacLachlan, Dubovi, E.J. (Eds.) Fenner's Veterinary Virology. 478 495.

Mahapatra, M., Aggarwal, N., Cox, S., Statham, R.J., Knowles, N.J., Barnett, P.V., Paton, D.J., 2008. Evaluation of a monoclonal antibody-based approach for the selection of foot-and-mouth disease (FMD) vaccine strains. Veterinary Microbiology 126, 40-50.

Mahy, B.W.J. 2005. Introduction and history of foot-and-mouth disease virus, In: Foot-And-Mouth Disease Virus. Springer, Berlin, 1-8.

Mansley, L.M., Dunlop, P.J., Whiteside, S.M., Smith, R.G.H., 2003. Early dissemination of foot-and-mouth disease virus through sheep marketing in February 2001. Veterinary Record 153, 43-50.

Maradei, E., La Torre, J., Robiolo, B., Esteves, J., Seki, C., Pedemonte, A., Iglesias, M., D'Aloia, R., Mattion, N., 2008. Updating of the correlation between lpELISA titers and protection from virus challenge for the assessment of the potency of polyvalent aphtovirus vaccines in Argentina. Vaccine 26, 6577-6586.

Maree, F.F., Blignaut, B., Esterhuysen, J.J., de Beer, T.A., Theron, J., O'Neill, H.G., Rieder, E., 2011. Predicting antigenic sites on the foot-and-mouth disease virus capsid of the South African Territories types using virus neutralization data. Journal of General Virology 92, 2297-2309.

Marquardt, O., Adam, K.H., 1990. Foot-and-mouth disease virus subtyping by sequencing VP1 genes. Veterinary Microbiology 23, 175-183.

Martinez, M.A., Hernandez, J., Piccone, M.E., Palma, E.L., Domingo, E., Knowles, N., Mateu, M.G., 1991. Two mechanisms of antigenic diversification of foot-and-mouth disease virus. Virology 184, 695-706.

Martinez-Salas, E., Belsham, G.J. 2017. Genome Organisation, Translation and Replication of Foot-and-mouth Disease Virus RNA, In: Sobrino, F., Domingo, E. (Eds.) Foot-and-Mouth Disease Virus: Current Research and Emerging Trends. 13-42.

Mason, P.E., Piccone, M.E., Mickenna, S.C., Chinsangaram, J., Grubman, M.J., 1997. Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. Virology 227, 96-102.

Mason, P.W., Grubman, M.J., Baxt, B., 2003. Molecular basis of pathogenesis of FMDV. Virus Research 91, 9-32.

Maswati, M.A., Azri, A., Zalina, M.Z., Naheed, M.H., Mokhtar, A., Kushairi, W.A. 2000. Outbreaks of foot and mouth disease in Selangor and Negeri Sembilan. In The 12th Veterinary Association Malaysia Scientific Congress, 1-4 September 2000, Johara, M.Y., Chandrasekaran, S., Muniandy, N., Sharifah, S.H., eds. (Kuantan, Pahang, Veterinary Association Malaysia), 47-49.

Mateu, G.M. 2017. The Foot-and-mouth Disease Virion: Structure and Function, In: Domingo, F.S.a.E. (Ed.) Foot-and-mouth Disease Virus: Current Research and Emerging Trends. Centro de Biologa Molecular'Servero Ochoa' (CSIC-UAM), Madrid, Spain, 61-105.

Mateu, M.G., 1995. Antibody recognition of Picornaviruses and escape from neutralization - a structural view. Virus Research 38, 1-24.

Mateu, M.G., Martinez, M.A., Capucci, L., Andreu, D., Giralt, E., Sobrino, F., Brocchi, E., Domingo, E., 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. Journal of General Virology 71, 629-637.

Mattion, N., Goris, N., Willems, T., Robiolo, B., Maradei, E., Beascoechea, C.P., Perez, A., Smitsaart, E., Fondevila, N., Palma, E., De Clercq, K., La Torre, J., 2009. Some guidelines for determining foot-and-mouth disease vaccine strain matching by serology. Vaccine 27, 741-747.

McColl, K.A., Westbury, H.A., Kitching, R.P., Lewis, V.M., 1995. The persistence of foot-and-mouth disease virus on wool. Australian Veterinary Journal 72, 286-292.

McCullough, K.C., Bruckner, L., Schaffner, R., Fraefel, W., Muller, H.K., Kihm, U., 1992. Relationship between the anti-FMD virus-antibody reaction as measured by different assays, and protection in-vivo against challenge infection. Veterinary Microbiology 30, 99-112.

McCullough, K.C., De Simone, F., Brocchi, E., Capucci, L., Crowther, J.R., Kihm, U., 1992. Protective immune-response against foot-and-mouth-disease. Journal of Virology 66, 1835-1840.

McCullough, K.C., Ruggli, N., Summerfield, A., 2009. Dendritic cells--at the front-line of pathogen attack. Veterinary Immunology and Immunopathology 128, 7-15.

McCullough, K.C., Sáiz, M., Summerfield, A. 2017. Innate to Adaptive: Immune Defence Handling of Foot-and-mouth Disease Virus, In: Foot-and-Mouth Disease Virus: Current Research and Emerging Trends. 211-274.

McVicar, J.W., Sutmoller, P., 1969. The epizootiological importance of foot-and-mouth disease carriers. II. The carrier status of cattle exposed to foot-and-mouth disease following vaccination with an oil adjuvant inactivated virus vaccine. Archiv für die gesamte Virusforschung 26, 217-224.

Meloen, R.H., Briaire, J., 1980. A study of the cross-reacting antigens on the intact foot-and-mouth disease virus and its 12S subunits with antisera against the structural proteins. Journal of General Virology 51, 107-116.

Meloen, R.H., Rowlands, D.J., Brown, F., 1979. Comparison of the antibodies elicited by the individual structural polypeptides of foot-and-mouth-disease and polio viruses. Journal of General Virology 45, 761-763.

Miguel A. Martinez, Nuria Verdaguer, Mauricio G. Mateu, Domingo, E., 1997. Evolution subverting essentiality: Dispensability of the cell attachment Arg-Gly-Asp motif in multiply passaged foot-and-mouth disease virus. Proceedings of the National Academy of Sciences of the United States of America 94, 6798-6802.

Mohd Nor, M.N., Gan, C.H., Ong, B.L., 2000. Nipah virus infection of pigs in peninsular Malaysia. Revue Scientifique et Technique 19, 160-165.

Morris, R.S., Sanson, R.L., Stern, M.W., Stevenson, M., Wilesmith, J.W., 2002. Decision-support tools for foot and mouth disease control. Revue Scientifique et Technique 21, 557-567.

Mulcahy, G., Gale, C., Robertson, P., Iyisan, S., DiMarchi, R.D., Doel, T.R., 1990. Isotype responses of infected virus vaccinated and peptide vaccinated cattle to FMDV.pdf. Vaccine 8, 249-256.

Muroga, N., Hayama, Y., Yamamoto, T., Kurogi, A., Tsuda, T., Tsutsui, T., 2012. The 2010 foot-and-mouth disease epidemic in Japan. Journal of Veterinary Medical Science 74, 399-404.

Nampanya, S., Khounsy, S., Abila, R., Young, J.R., Bush, R.D., Windsor, P.A., 2016. Financial Impacts of Foot-and-Mouth Disease at Village and National Levels in Lao PDR. Transboundary and Emerging Diseases 63, e403-e411.

Newman, J.F.E., Rowlands, D.J., Brown, F., 1973. Physicochemical sub-grouping of mammalian picornaviruses. Journal of General Virology 18, 171-180.

Newman, J.F.E., Rowlands, D.J., Brown, F., Goodridge, D., Burrows, R., Steck, F., 1977. Physicochemical characterization of 2 serologically unrelated equine rhinoviruses. Intervirology 8, 145-154.

Nicholls, M.J., Black, L., Rweyemamu, M.M., Genovese, J., Ferrari, R., Hammant, C.A., de Silva, E., Umehara, O., 1984. The effect of maternally derived antibodies on the response of calves to vaccination against foot and mouth disease. Journal of Hygiene 92, 105-116.

Niedbalski, W., Haas, B., 2003. Differentiation of infection from vaccination by detection of antibodies to the non-structural protein 3ABC of foot-and-mouth disease virus. Bulletin of the Veterinary Institute in Pulawy 47, 51-60.

Oh, Y., Fleming, L., Statham, B., Hamblin, P., Barnett, P., Paton, D.J., Park, J.H., Joo, Y.S., Parida, S., 2012. Interferon-gamma induced by in vitro re-stimulation of CD4+ T-cells correlates with in vivo FMD vaccine induced protection of cattle against disease and persistent infection. PLoS One 7, e44365.

OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

OIE. 2012. Chapter 2.1.5, O.I.E Terrestrial manual, In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

OIE, 2016. SEACFMD Roadmap 2016: A strategic framework to control, prevent and eradicate foot-and-mouth disease in Southeast Asia and China 2016 2020, 3rd Edition. OIE, Paris, France, 83 p.

OIE. 2017. Chapter 2.1.8 O.I.E terrestrial manual-foot-and-mouth disease, In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

OIE 2018. Foot and Mouth Disease (FMD).

OIE 2018. OIE 2018 FMD Status.

Olson, N.H., Kolatkar, P.R., Oliveira, M.A., Cheng, R.H., Greve, J.M., McClelland, A., Baker, T.S., Rossmann, M.G., 1993. Structure of a human rhinovirus complexed with its receptor molecule. Proceedings of the National Academy of Sciences of the United States of America 90, 507-511.

Orsel, K., Bouma, A., Dekker, A., Stegeman, J.A., de Jong, M.C., 2009. Foot and mouth disease virus transmission during the incubation period of the disease in piglets, lambs, calves, and dairy cows. Preventive Veterinary Medicine 88, 158-163.

Orsel, K., de Jong, M.C.M., Bouma, A., Stegeman, J.A., Dekker, A., 2007. The effect of vaccination on foot and mouth disease virus transmission among dairy cows. Vaccine 25, 327-335.

Ouldridge, E.J., Barnett, P.V., Parry, N.R., Syred, A., Head, M., Rweyemamu, M.M., 1984. Demonstration of neutralizing and non-neutralizing epitopes on the trypsin-sensitive site of Foot-and-mouth-disease virus. Journal of General Virology 65, 203-207.

Ozawa, Y. 1994. Strategy options for the control of foot-and-mouth disease in Southeast Asia. In Diagnosis and epidemiology of foot-and-mouth disease in Southeast Asia: international workshop held September 6-9, 1993, Copland, J.W., Gleeson, L.J., Chamnanpood, C., eds. (Lampang, Thailand, Australian Centre for International Agricultural Research), 79-84.

Parker, J., 1971. Presence and inactivation of foot-and-mouth disease virus in animal faeces. Veterinary Record 88, 659-662.

Paton, D.J., de Clercq, K., Greiner, M., Dekker, A., Brocchi, E., Bergmann, I., Sammin, D.J., Gubbins, S., Parida, S., 2006. Application of non-structural protein antibody tests in substantiating freedom from foot-and-mouth disease virus infection after emergency vaccination of cattle. Vaccine 24, 6503-6512.

Paton, D.J., Gubbins, S., King, D.P., 2018. Understanding the transmission of foot-and-mouth disease virus at different scales. Current Opinion in Virology 28, 85-91.

Paton, D.J., Valarcher, J.F., Bergmann, I., Matlho, O.G., Zakharov, V.M., Palma, E.L., Thomson, G.R., 2005. Selection of foot and mouth disease vaccine strains - a review. Revue Scientifique et Technique 24, 981-993.

Pay, T.W.F., Hingley, P.J., 1987. Correlation of 140S antigen dose with the serum neutralizing antibody-response and the level of protection induced in cattle by Foot-and-mouth-disease vaccines. Vaccine 5, 60-64.

Pereira, H.G., 1976. Subtyping of foot-and-mouth disease virus. Developments in Biological Standardization 35, 167-174.

Pluimers, F.H., Akkerman, A.M., van der Wal, P., Dekker, A., Bianchi, A., 2002. Lessons from the foot and mouth disease outbreak in The Netherlands in 2001. Revue Scientifique et Technique 21, 711-721.

Porta, C., Kotecha, A., Burman, A., Jackson, T., Ren, J., Loureiro, S., Jones, I.M., Fry, E.E., Stuart, D.I., Charleston, B., 2013. Rational engineering of recombinant picornavirus capsids to produce safe, protective vaccine antigen. PLoS Pathogens 9, e1003255.

Qiu, Y., Abila, R. 2017. FMD current status in Southeast Asia and China.

Ramanoon, S.Z., Robertson, I.D., Edwards, J., Hassan, L., Isa, K.M., 2013. Outbreaks of foot-and-mouth disease in Peninsular Malaysia from 2001 to 2007. Tropical Animal Health and Production 45, 373-377.

Ramanoon, S.Z.B., 2016. The epidemiology of foot and mouth disease in Malaysia. Murdoch University,

Randrup, A., 1954. On the stability of bovine foot-and-mouth disease virus dependent on Ph - investigations on the complement fixing and the immunizing antigen as well as on the infective agent. Acta Pathologica Et Microbiologica Scandinavica 35, 388-395.

Ranjan, R., Biswal, J.K., Subramaniam, S., Dash, B.B., Singh, K.P., Arzt, J., Rodriguez, L.L., Pattnaik, B., 2018. Evidence of subclinical foot-and-mouth disease virus infection in young calves born from clinically recovered cow under natural condition. Tropical Animal Health and Production 50, 1167-1170.

Rao, M.G., Butchaiah, G., Sen, A.K., 1994. Antibody-response to 146S particle, 12S protein subunit and isolated VP1 polypeptide of Foot-and-mouth-disease virus type Asia 1. Veterinary Microbiology 39, 135-143.

RCoreTeam 2017. R: A language and environment for statistical computing. . In R Foundation for Statistical Computing.

Reading, S.A., Dimmock, N.J., 2007. Neutralization of animal virus infectivity by antibody. Archives of Virology 152, 1047-1059.

Reeve, R., Blignaut, B., Esterhuysen, J.J., Opperman, P., Matthews, L., Fry, E.E., de Beer, T.A.P., Theron, J., Rieder, E., Vosloo, W., O'Neill, H.G., Haydon, D.T., Maree, F.F., 2010. Sequence-based prediction for vaccine strain selection and identification of

antigenic variability in foot-and-mouth disease virus. Plos Computational Biology 6, 13.

Reeve, R., Borley, D.W., Maree, F.F., Upadhyaya, S., Lukhwareni, A., Esterhuysen, J.J., Harvey, W.T., Blignaut, B., Fry, E.E., Parida, S., Paton, D.J., Mahapatra, M., 2016. Tracking the antigenic evolution of foot-and-mouth disease virus. PLoS One 11, e0159360.

Robinson, L., Windsor, M., McLaughlin, K., Hope, J., Jackson, T., Charleston, B., 2011. Foot-and-mouth disease virus exhibits an altered tropism in the presence of specific immunoglobulins, enabling productive infection and killing of dendritic cells. Journal of Virology 85, 2212-2223.

Robiolo, B., Grigera, P.R., Periolo, O.H., Seki, C., Bianchi, T., Maradei, E., La Torre, J.L., 1995. Assessment of foot and mouth disease vaccine potency by liquid-phase blocking ELISA: a proposal for an alternative to the challenge procedure in Argentina. Vaccine 14, 1346-1352.

Robiolo, B., La Torre, J., Maradei, E., Perez Beascoechea, C., Perez, A., Seki, C., Smitsaart, E., Fondevila, N., Palma, E., Goris, N., De Clercq, K., Mattion, N., 2010. Confidence in indirect assessment of foot-and-mouth disease vaccine potency and vaccine matching carried out by liquid phase ELISA and virus neutralization tests. Vaccine 28, 6235-6241.

Rossmann, M.G., 1989. The Canon hypothesis - hiding the host-cell receptor attachment site on a viral surface from immune surveillance. Journal of Biological Chemistry 264, 14587-14590.

Rowlands, D.J., Sangar, D.V., Brown, F., 1975. A comparative chemical and serological study of the full and empty particles of foot-and mouth disease virus. Journal of General Virology 26, 227-238.

Rueckert, R.R., Wimmer, E., 1984. Systematic nomenclature of picornavirus proteins. Journal of Virology 50, 957-959.

Ruggli, N., Tratschin, J.D., Schweizer, M., McCullough, K.C., Hofmann, M.A., Summerfield, A., 2003. Classical swine fever virus interferes with cellular antiviral defense: evidence for a novel function of Npro. Journal of Virology 77, 7645-7654.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J.F., Knowles, N.J., Saraiva, V., 2008. Epidemiological patterns of foot-and-mouth disease worldwide. Transboundary and Emerging Diseases 55, 57-72.

Rweyemamu, M.M., 1984. Antigenic variation in foot-and-mouth-disease - studies based on the virus neutralization reaction. Journal of Biological Standardization 12, 323-337.

Rweyemamu, M.M., Booth, J.C., Head, M., Pay, T.W.F., 1978. Micro-neutralization tests for serological typing and subtyping of Foot-and-mouth-disease virus strains. Journal of Hygiene 81, 107-123.

Rweyemamu, M.M., Hingley, P.J., 1984. Foot and mouth-disease virus-strain differentiation - Analysis of the serological data. Journal of Biological Standardization 12, 225-229.

Rweyemamu, M.M., Pay, T.W.F., Parker, M.J. 1977. Serological differentiation of foot-and-mouth disease virus strains in relation to selection of suitable vaccine viruses. In Proceedings, International Symposium on Foot-and-Mouth Disease (II), Lyon, France, 5-8 October 1976, Mackowiak, C., Regamey, R.H., eds. (Karger), 205-214.

Salt, J. 2004. Persistence of foot-and-mouth disease virus, In: Sobrino, F., Domingo, E. (Eds.) Foot and Mouth Disease: Current Perspectives. Horizon Bioscience, Wymondham, UK, 103-143.

Saraiva, V., 2004. Foot-and-mouth disease in the Americas: epidemiology and ecologic changes affecting distribution. Annals of the New York Academy of Sciences 1026, 73-78.

Sasaki, M. 1994. Patterns of National and International Livestock Movement in Southeast Asia: Implication for a Regional Foot and Mouth Disease Control Program. In Diagnosis and epidemiology of foot-and-mouth disease in Southeast Asia: international workshop held September 6-9, 1993 (Lampang, Thailand, Australian Centre for International Agricultural Research), 75-79.

Schijven, J., Rijs, G.B., de Roda Husman, A.M., 2005. Quantitative risk assessment of FMD virus transmission via water. Risk Analysis 25, 13-21.

Sellers, R.F., 1971. Quantitative aspects of the spread of foot and mouth disease. Veterinary Bulletin 41, 431-439.

Sellers, R.F., Donaldson, A.I., Herniman, K.A.J., 1970. Inhalation, persistence and dispersal of foot-and-mouth disease virus by man Journal of Hygiene 68, 565-573.

Sellers, R.F., Parker, J., 1969. Airborne excretion of foot-and-mouth disease virus. Journal of Hygiene 67, 671-677.

Senawi, J.B., 2012. Epidemiology of Foot and Mouth Disease in Cattle In Pahang, Malaysia. Murdoch University

Siegrist, C.-A. 2013. Vaccine Immunology, In: Plotkin, S.A., Orenstein, W.A., Offit, P.A. (Eds.) Vaccines. Elsevier, 14-32.

Sobrino, F., Domingo, E., 2017. Foot-and-mouth disease virus: current research and emerging trends. Caister Academic Press, Norfolk, UK, 431 p.

Sorensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J., Mackay, D.K.J., 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Archives of Virology 143, 1461-1476.

Spearman, C., 1908. The method of "right and wrong cases" ("constant stimuli") without Gauss's formulae. British Journal of Psychology 2, 227-242.

Spitteler, M.A., Fernandez, I., Schabes, E., Krimer, A., Regulier, E.G., Guinzburg, M., Smitsaart, E., Levy, M.S., 2011. Foot and mouth disease (FMD) virus: quantification of whole virus particles during the vaccine manufacturing process by size exclusion chromatography. Vaccine 29, 7182-7187.

Stenfeldt, C., Eschbaumer, M., Rekant, S.I., Pacheco, J.M., Smoliga, G.R., Hartwig, E.J., Rodriguez, L.L., Arzt, J., 2016. The foot-and-mouth disease carrier state divergence in cattle. Journal of Virology 90, 6344-6364.

Stenfeldt, C., Pacheco, J.M., Brito, B.P., Moreno-Torres, K.I., Branan, M.A., Delgado, A.H., Rodriguez, L.L., Arzt, J., 2016. Transmission of foot-and-mouth disease virus during the incubation period in pigs. Frontiers in Veterinary Science 3, 105.

Studdert, M.J., Gleeson, L.J., 1978. Isolation and characterisation of an equine rhinovirus. Zentralblatt fur Veterinarmedizin 25B, 225-237.

Sumption, K., Domenech, J., Ferrari, G., 2012. Progressive control of FMD on a global scale. Veterinary Record 170, 637-639.

Suseno, P.P., Wongsathapornchai, K. 2004. Preliminary epidemiological study of foot and mouth disease in MTM zones. In: 10th meeting of the OIE sub-commission for foot and mouth disease in Southeast Asia, Luang Prabang, Lao PDR.

Swanepoel, F.J.C., Stroebel, A., Nesamvuni, E. 2002. Key functions of livestock of smallholder farming system: A South African case study. In Australian Society of Animal Production, 237-240.

Tekleghiorghis, T., Weerdmeester, K., van Hemert-Kluitenberg, F., Moormann, R.J.M., Dekker, A., 2014. Comparison of test methodologies for foot-and-mouth disease virus serotype A vaccine matching. Clinical and Vaccine Immunology 21, 674-683.

Thomas, A.A., Woortmeijer, R.J., Puijk, W., Barteling, S.J., 1988. Antigenic sites on foot-and-mouth-disease virus type-A10. Journal of Virology 62, 2782-2789.

Thuraisingham, S. 1977. The control and eradication of foot and mouth disease in Malaysia. In Association of Veterinary Surgeons, Malaysia and Australian Association of Cattle Veterinarians (Kuala Lumpur), 122-132.

Turton, J. 2004. Minimum standard definitions and rules for control and eradication of foot and mouth disease in the MTM Peninsula Campaign for Foot and Mouth Disease Freedom (Bangkok, Thailand).

Van Bekkum J., Frenkel H., Frederiks H. & Frenkel S. Observations on the carrier state of cattle exposed to foot- and-mouth disease virus. Tijdschrift voor Diergeneeskunde 84, 1159–1164 (1959).

Van Maanen, C., Terpstra, C., 1989. Comparison of a liquid-phase blocking sandwitch ELISA and a serum neutralisation test to evaluate immunity in potency tests of foot-and-mouth-disease vaccines. Journal of Immunological Methods 124, 111-119.

Wallace, W.R. 1936. Foot and mouth disease situation, In: Annual report of the veterinary department S.S. and F.M.S. for the state. Federated Malay States Government Press, Kuala Lumpur.

Wallace, W.R., 1939. Report on the Veterinary Departments, Malaya. Federated Malay States Government Press, Kuala Lumpur.

Walter, T.S., Ren, J.S., Tuthill, T.J., Rowlands, D.J., Stuart, D.I., Fry, E.E., 2012. A plate-based high-throughput assay for virus stability and vaccine formulation. Journal of Virological Methods 185, 166-170.

Waters, R., Ludi, A.B., Fowler, V.L., Wilsden, G., Browning, C., Gubbins, S., Statham, B., Bin-Tarif, A., Mioulet, V., King, D.J., Colenutt, C., Brown, E., Hudelet, P., King, D.P., 2018. Efficacy of a high-potency multivalent foot-and-mouth disease virus vaccine in cattle against heterologous challenge with a field virus from the emerging A/ASIA/G-VII lineage. Vaccine 36, 1901-1907.

Wongsathapornchai, K., Salman, M.D., Edwards, J.R., Morley, P.S., Keefe, T.J., Van Campen, H., Weber, S., 2008. Assessment of the likelihood of the introduction of foot-and-

mouth disease through importation of live animals into the Malaysia-Thailand-Myanmar peninsula. American Journal of Veterinary Research 69, 252-260.

Woodbury, E.L., 1995. A review of the possible mechanisms for the persistence of foot-and-mouth disease virus. Epidemiology and Infection 114, 1-13.

Wright, C.F., Gloster, J., Mazelet, L., Paton, D.J., Ryan, E.D., 2010. Short-lived carriage of foot-and-mouth disease virus in human nasal cavities after exposure to infected animals. Veterinary Record 167, 928-931.

WRLFMD 2018. Annual OIE/FAO FMD Reference Laboratory Network Reports.

WRLFMD 2018. WRL website.

Wutz, G., Auer, H., Nowotny, N., Grosse, B., Skern, T., Kuechler, E., 1996. Equine rhinovirus serotypes 1 and 2: Relationship to each other and to aphthoviruses and cardioviruses. Journal of General Virology 77, 1719-1730.

Xie, Q.-C., McCahon, D., Crowther, J.R., Belsham, G.J., McCullough, K.C., 1987. Neutralization of foot-and-mouth disease virus can be mediated through any of at least three separate antigenic sites. Journal of General Virology 68, 1637-1647.

Yang, M., Goolia, M., Xu, W.H., Bittner, H., Clavijo, A., 2013. Development of a quick and simple detection methodology for foot-and-mouth disease virus serotypes O, A and Asia 1 using a generic RapidAssay Device. Virology Journal 10, 125.

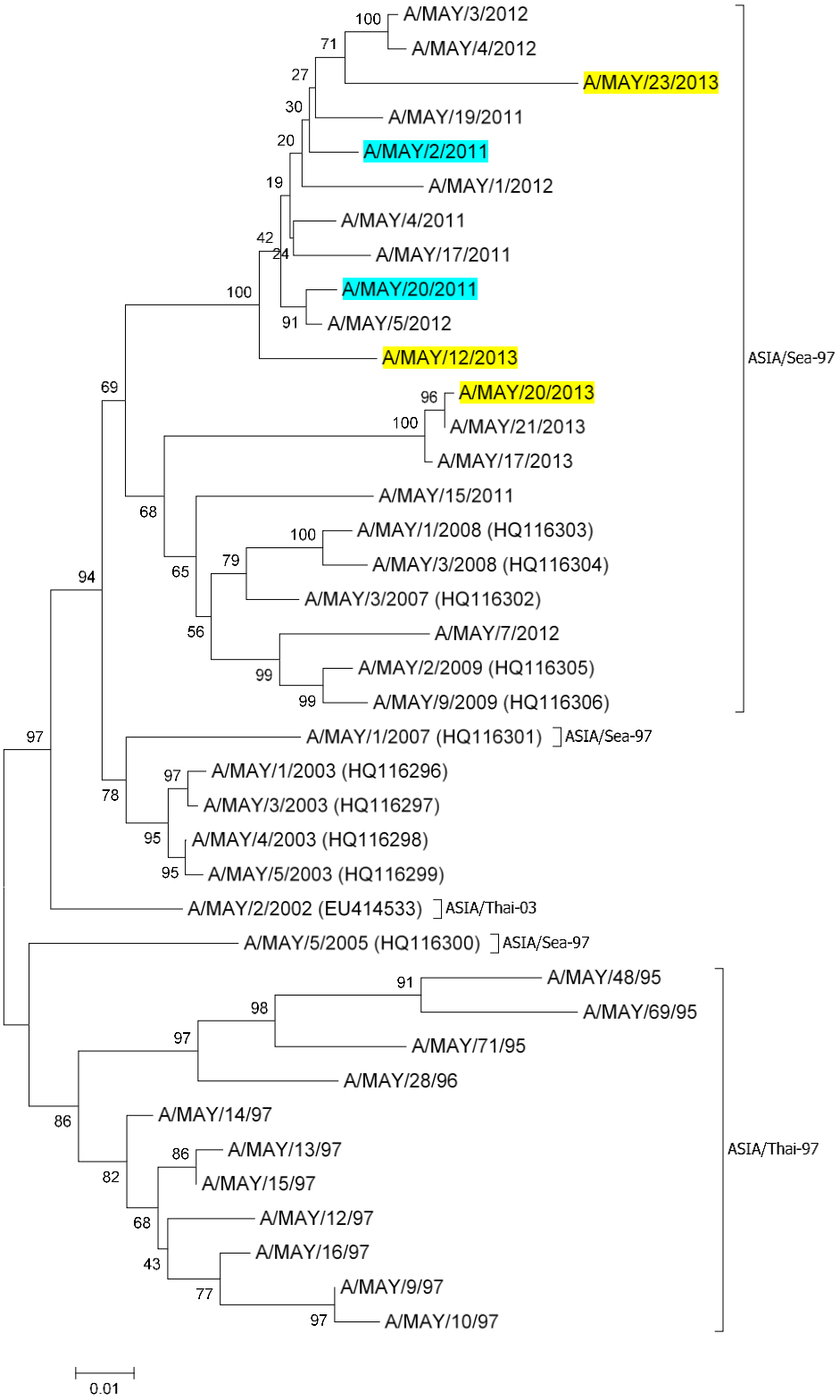
Yang, Y., Li, H., Li, Z., Zhang, Y., Zhang, S., Chen, Y., Yu, M., Ma, G., Su, Z., 2015. Size-exclusion HPLC provides a simple, rapid, and versatile alternative method for quality control of vaccines by characterizing the assembly of antigens. Vaccine 33, 1143-1150.

Yang, Y., Zhao, Q., Li, Z., Sun, L., Ma, G., Zhang, S., Su, Z., 2017. Stabilization study of inactivated foot and mouth disease virus vaccine by size-exclusion HPLC and differential scanning calorimetry. Vaccine 35, 2413-2419.

Young, J.R., Suon, S., Rast, L., Nampanya, S., Windsor, P.A., Bush, R.D., 2016. Benefit-cost analysis of foot and mouth disease control in large ruminants in Cambodia. Transboundary and Emerging Diseases 63, 508-422.

Appendix i

Phylogenetic tree for lineage A/ASIA/Sea-97 viruses isolated in P. Malaysia. Midpoint-rooted Neighbour-joining phylogenetic tree were constructed based on RNA sequences and visualised using MEGA 6.06. Bootstrap values are displayed next to branches.



Appendix ii

List of FMDV isolates selected

Virus	Lineage	Country of Origin	Passage history
A/May-97	A/ASIA/Sea-97	Boehringer Ingelheim*	BHK1
A/Iran-05	A/ASIA/Iran-05	Boehringer Ingelheim*	BHK1
MAY/2/2011	A/ASIA/Sea-97	Malaysia	BTy1
VIT/10/2015	A/ASIA/Sea-97	Vietnam	BTy1
VIT/13/2015	A/ASIA/Sea-97	Vietnam	BTy1
TAI/4/2017	A/ASIA/Sea-97	Thailand	BTy1
VIT/14/2014	A/ASIA/Sea-97	Vietnam	BTy1
MAY/15/2014	A/ASIA/Sea-97	Malaysia	BTy1
MAY/20/2011	A/ASIA/Sea-97	Malaysia	BTy1
MAY/7/2012	A/ASIA/Sea-97	Malaysia	BTy1
CAM/2/2015	A/ASIA/Sea-97	Cambodia	BTy1
TUR/3/2012	A/ASIA/Iran-05	Turkey	BTy1
IRN/55/2011	A/ASIA/Iran-05	Iran	BTy1
AFG/140/2010	A/ASIA/Iran-05	Afghanistan	BTy1
IRN/24/2012	A/ASIA/Iran-05	Iran	BTy1
IRN/33/2012	A/ASIA/Iran-05	Iran	BTy1
AFG/69/2011	A/ASIA/Iran-05	Iran	BTy1
IRN/36/2011	A/ASIA/Iran-05	Iran	BTy1

BHK: Baby hamster kidney cell BTy: Bovine thyroid cells

*These are vaccine viruses and the full passage histories are not known; however, prior to use in these experiments the virus was passaged in BHK. Their origin is marked as Boehringer Ingelheim to indicate the commercial source from which the virus was derived.