Epitope dominance studies with serotype O Foot-and-Mouth Disease

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Thesis Abstract

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Foot-and-mouth disease virus (FMDV) is an economically devastating and highly contagious livestock pathogen. It exists as seven serotypes, comprising numerous antigenically distinct subtypes. The large amount of antigenic heterogeneity has confounded attempts at developing broadly reactive vaccines. In order to overcome this issue the fundamentals of the interactions between the virus and the host humoral immune response must first be understood. Previous work in this area using monoclonal antibody (mAb) escape mutants has identified five antigenic sites for the O serotype and efforts have been made to quantify their relative importance. However, this does not represent a complete picture of serotype O antigenicity. The work conducted in this thesis demonstrates the role of a limited number of dominant substitutions in mediating the antigenic diversity of serotype O Foot-and-Mouth disease virus. Two alternative but complementary methods for identifying epitopes were developed. The first used a mathematical model to analyse newly generated serological and sequence data from 105 viruses, cultured for this purpose (and cross-reacted to 5 reference antisera), in the context of an existing crystallographic structure to identify and quantify the antigenic importance of sites on the surface of the virus. The second approach was purely structural, using existing B cell epitope prediction tools to develop a method for predicting FMDV epitopes using existing crystallographic structures of FMDV. These techniques were validated by the use of reverse genetics, which confirmed the impact on cross reactivity of two predicted novel serotype O antigenic residues, with a further four novel residues identified by looking in depth at the interactions between two genetically close, but antigenically distant viruses. This increased knowledge of the antigenic composition of serotype O FMDV contributes to our understanding of the nature of vaccine efficacy and the breadth of protection, which, in the longer term, will aid in the goal of developing vaccines to better protect livestock from such a highly antigenically variable disease.

Declaration

I hereby declare that the research described within this thesis is my own work, unless otherwise stated, and certify that is has never been submitted for any other degree or professional qualification

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Abbreviations

°C	Degrees Celsius
µl	Microliter
Å	Angstrom
A/T/G/C	adenine/thymine/guanine/cytosine
AA	Amino acid
ABS	Adult bovine sera
ADCC	Antibody dependant cell mediated cytotoxicity
ADCVMI	Antibody dependant, cell mediated viral inhibition
ADIN	Antibody dependant intracellular neutralisation
ANOVA	Analysis of variance
ATP	Adenosine triphosphate,
BFS	British field sample
BHK	Baby hamster kidney
bp	Base pairs
BTY	Bovine thyroid
BVS	Bovine vaccinate serum
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Complementarity determining regions
CI	Confidence interval
cm	Centimetre
CMV	Compliment mediated virolysis
CO_2	Carbon dioxide
CPE	Cytopathic effect
cre	*cis*-acting replication element
CTP	Cytidine triphosphate
DC	Dendritic cells
DEFRA	Department for environment, farming and rural affairs
DNA	Deoxy-ribonucleic acid
dNTP	Deoxy-nucleotide triphosphate
ddNTP	Dideoxy-nucleotide triphosphate
DPN	Diplococcus pneumoniae
DTT	dithiothreitol
E.coli	Escherichia coli
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunsorbent assay
EMCV	Encephalomyocarditis Viruses
EMEM	Eagle's minimum essential medium
ER	Endoplasmic reticulum
Fab	Fragment, antigen binding
Fc	Fragment, crystallisable
FMD	Foot-and-Mouth disease
FMD	Foot-and-Mouth Disease

FMDV	Foot-and-mouth disease virus
gp	Glycoprotein
GTP	Guanosine triphosphate,
HIV	Human immunodeficiency virus
Hpa	Haemophilus parainfluenzae
HRV	Human rhinovirus
HS	Heparan sulphate
IBRS-2	Porcine renal cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosome entry site
IU	International units
kb	Kilobase
KOD	Pyrococcus kodakaraensis
LB	Lysogeny broth
LPBE	Liquid phase blocking ELISA
LME	Linear mixed effects
Lpro	Leader protein
M	Molar
mAb	Monoclonal antibody
MAC	Membrane attack complex
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
ml	Millilitre
mM	Millimolar
moi	Multiplicity of infection
MZ	Marginal zone
NK	Natural killer
nt	Nucleotide
O KIC	O Kaufbeuren Infectious copy virus
O KWT	O Kaufbeuren wild type virus
OD	Optical density
OIE	Office international des epizooties
ORF	Open reading frame
P	Polyprotien
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween-20
PCR	Polymerase chain reaction
PD_{50}	50% protective dose
PDB	Protein databank
PFU	Plaque forming units
pH	Potential of hydrogen
pM	Picomolar
RGD	Arginine-glycine-aspartic acid

RMSD	Root mean square distance
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAT	South African territories
SOC	Super Optimal broth with Catabolite repression
TBE	Tris Borate EDTA
TCA	Trichloroacetic acid
TCID	Tissue culture infectious dose
TI	T Independent
TLR	Toll like receptors
TRIM	Tripartite motif
TSS	Transformation and storage solution
TTP	Thymidine triphosphate,
UTR	Untranslated region
VNT	Virus neutralisation test
VP	Virion polypeptide
w/v	Weight/volume
WRL	World Reference Laboratory
xg	T imes gravity

Amino acids

Amino Acid	3-letter code	1-letter code	Side chain polarity	Side chain acidity or basicity
Alanine	Ala	A	Nonpolar	Neutral
Arginine	Arg	R	Polar	Basic (strongly)
Asparagine	Asn	N	Polar	Neutral
Aspartic acid	Asp	D	Polar	Acidic
Cysteine	Cys	C	Polar	Neutral
Glutamic acid	Glu	E	Polar	Acidic
Glutamine	Gln	Q	Polar	Neutral
Glycine	Gly	G	Nonpolar	Neutral
Histidine	His	H	Polar	Basic (weakly)
Isoleucine	Ile	I	Nonpolar	Neutral
Leucine	Leu	L	Nonpolar	Neutral
Lysine	Lys	K	Polar	Basic
Methionine	Met	M	Nonpolar	Neutral
Phenylalanine	Phe	F	Nonpolar	Neutral
Proline	Pro	P	Nonpolar	Neutral
Serine	Ser	S	Polar	Neutral
Threonine	Thr	T	Polar	Neutral
Tryptophan	Trp	W	Nonpolar	Neutral
Tyrosine	Tyr	Y	Polar	Neutral
Valine	Val	V	Nonpolar	Neutral

Chapter 1: Introduction

1.1 Foot-and-Mouth Disease.

Foot-and-Mouth disease (FMD) is a highly contagious disease that predominantly affects animals of the order artiodactyla, with the primary domestic hosts being cattle, buffalo (Bubalus bubalus), sheep, pigs and goats. FMD is globally one of the most economically important livestock diseases and is currently to be found in all parts of the world except North America, Oceania and Western Europe (Figure 1.1). Although FMD does not generally have a high mortality rate in adult animals (depending on the strain) it can have a high mortality rate in very young animals (50-80% in calves and up to 100% in piglets) and in some wildlife species (Arzt *et al.*, 2011). The disease can also lead to dramatic, long-lasting reductions in the productivity of infected herds (Doel, 2003). It seriously damages the economies of enzootically infected countries by impeding exports of livestock and livestock products, as the most common mechanism for FMD to spread is movement of infected animals/animal products (Donaldson & Alexandersen, 2001).

FMD is not only a concern to those countries where the disease is enzootic. Recent outbreaks of the disease in the Far East (Paton *et al.*, 2010) and Eastern Europe (Valdazo-Gonzalez *et al.*, 2011) demonstrate the ability of FMD to disseminate into areas previously free from disease, with major economic impact. The 2001 outbreak of FMD in the UK is estimated to have cost the treasury alone around £2.7 billion and led to the slaughter of approximately 4 million animals (Davies, 2002). Such wholesale slaughter programmes, like those that have been used in the EU since 1991, are becoming unpalatable to a population increasingly concerned about animal welfare. This has placed an increased emphasis on the need to develop new vaccines and control strategies.

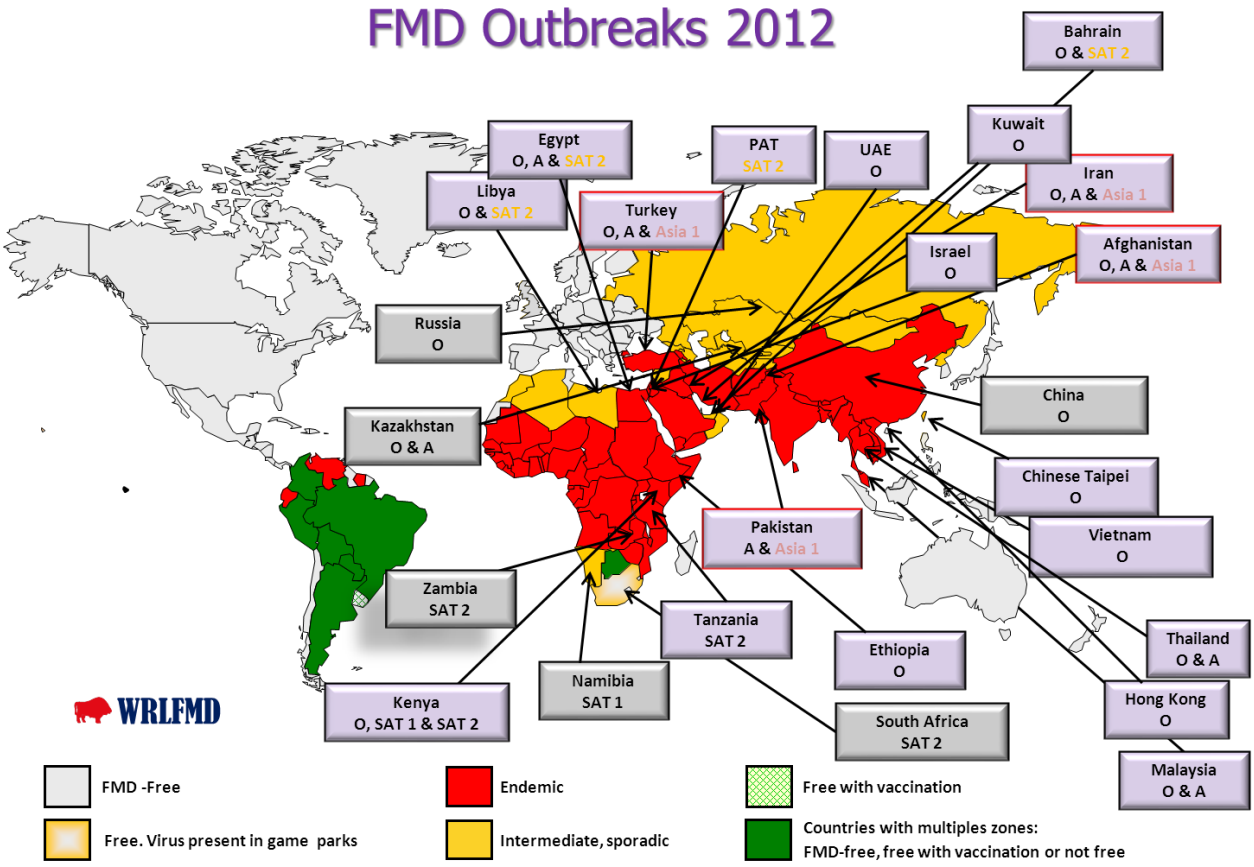


Figure 1.1 Current status of countries with regard to FMDV. Countries are coloured as stated in the key in the bottom of the figure. Outbreaks of FMDV that have been reported in 2012 are also shown, along with the serotype(s) associated with each outbreak. Figure courtesy of the World Reference Laboratory for FMDV, Pirbright, UK.

1.2 Foot-and-Mouth disease virus.

1.2.1 Introduction.

The causative agent of FMD is Foot-and-Mouth disease virus (FMDV). This virus is a member of the family *Picornaviridae* and makes up, along with Equine Rhinitis A (Li *et al.*, 1996) and Bovine Rhinitis A and B (previously named bovine Rhinovirus B) (Adams & Carstens, 2012; Hollister *et al.*, 2008), the genus *Aphthovirus,* one of the 12 genera (Figure 1.2) currently described for the Picornaviruses (Knowles, 2012). FMDV is a positive-sense single-stranded RNA virus ~8.3 kilobases in length comprising a single open reading frame (ORF) that codes for 4 structural and 8-10 non-structural proteins.

Due to the lack of proof reading ability of the FMDV RNA polymerase FMDV exhibits large amounts of genetic variation. FMDV is currently divided into 7 serotypes: A, O, C, SAT (South African territories) 1-3 and Asia-1. These serotypes share an approximately 86% amino acid identity to each other (Yang *et al.*, 2007). However, some of the capsid proteins exhibit more variation, VP1 has been reported to vary by around 30-50% (Knowles & Samuel, 2003) or as much as 74% (Carrillo *et al.*, 2005) between serotypes. Genome analysis shows that the O, A , C and Asia-1 serotypes exhibit a clearly distinct evolutionary lineage from the SAT serotypes (see figure 1.2). Strains within the SAT serotypes are also more genetically and antigenically diverse than the other serotypes, because persistently infected buffalo act as a source of antigenic and genetic variants (Vosloo *et al.*, 1996). Serotype O is the most prevalent serotype, as can be seen by its predominance in the outbreaks occurring in 2012 (see figure 1.1). Within each of these 7 serotypes there are several subtypes (now referred to as strains), with up to 61 previously described (Cottral, 1972a).

FMDV exists as a viral quasi-species (see section 1.6.1 for more detail) with any population of the virus embodying a range of genotypes and phenotypes (Mateu *et al.*, 1989). The characterisation of these viruses is increasingly utilising sequence based as opposed to serological methods to give an improved picture of the relationships between circulating viruses (Domingo *et al.*, 2002). This is

demonstrated in the use of sequence data to determine topotypes, which FMDV genotypes cluster into according to their geographical distribution, based on a sequence difference of up to 15% between VP1 sequences (Samuel & Knowles, 2001). A high level of genotypic and antigenic variation can also be observed in populations where no selective pressure is being exerted (Rowlands *et al.*, 1983; Sevilla *et al.*, 1996).

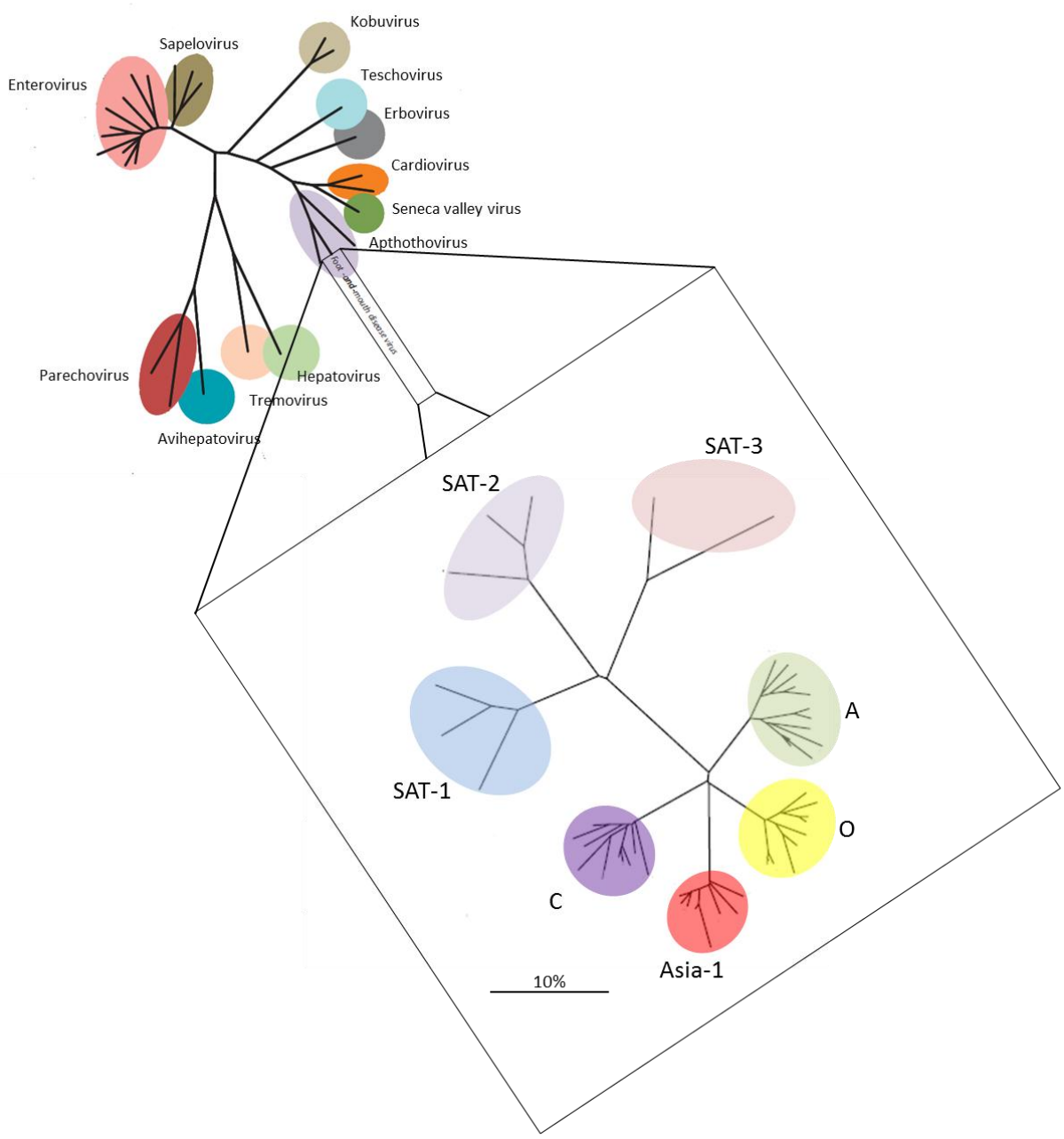


Figure 1.2 Unrooted neighbour joining tree of the Picornaviruses with a zoomed in area showing the relationships between the different serotypes of FMDV. Trees adapted from (Knowles & Samuel, 2003; Rowlands, 2010)

1.2.2 Host range and pathogenesis.

The host range of FMD is predominantly within the Artiodactyla; however, over 70 wildlife species can also become infected (Alexandersen *et al.*, 2003). African buffalo act as a reservoir, with the disease persisting in individuals for more than 5 years (Thomson *et al.*, 2003).

FMDV infection is often, but not exclusively, acquired through the respiratory tract, where the virus is thought to replicate initially in the epithelial cells of the dorsal soft palate, tonsils, and pharyngeal area (Burrows *et al.*, 1981; Zhang & Kitching, 2001). The virus is then able to migrate via the bloodstream, as a cell free viraemia, to infect secondary locations within the host including the squamous epithelium of the mouth, feet and mammary glands (Alexandersen *et al.*, 2003; Brown *et al.*, 1996). Vesicles that are characteristic of FMDV develop at these secondary sites of infection particularly in areas subjected to trauma (Zhang & Kitching, 2001). The development of symptoms can vary widely between susceptible host species. Pigs often develop severe signs of infection, which can include complete separation of the hoof wall (thimbling). This contrasts with smaller ruminants, such as sheep, that often develop very mild symptoms (Hughes *et al.*, 2002). In young animals the virus has also been associated with myocarditis (Arzt *et al.*, 2011)

A molecular basis for the virulence and host-range specificity of FMDV has been proposed. It appears that deletions within the 3A non-structural protein can generate viruses that are still virulent within pigs but that do not cause disease in cattle (Knowles *et al.*, 2001; Pacheco *et al.*, 2003) and a single amino acid substitution within this protein can also permit infection of guinea pigs (Nunez *et al.*, 2001). Additionally, the 3B protein has been implicated in the virulence of FMDV, with removal of one or more of the three copies of this protein reducing virus virulence (Pacheco *et al.*, 2003). Cattle and sheep can also become long-standing virus carriers; however, pigs are not believed to become persistently infected (Doel, 2005).

As with the development of clinical signs, the susceptibility of the various host species to FMDV varies widely. For example, it has been demonstrated that cattle can succumb to viral doses of type O_1 of as low as 12 tissue culture infectious dose $(TCID)_{50}$ (Donaldson *et al.*, 1987; Hughes *et al.*, 2002). This contrasts with Pigs which have a higher threshold for infection by aerosols. However, a single pig can excrete as much as 6.1 $\log_{10}$ $TCID_{50}$ when respiring, 60 times greater than that measured in sheep (Donaldson & Alexandersen, 2001). It has also been demonstrated that FMDV can travel for long distances on the wind, an example being the airborne spread of the virus reported from Brittany to the Isle of Wight and the Channel Islands (Donaldson *et al.*, 1982).

1.2.3 The FMDV genome

The FMDV genome is comprised of a single ORF around 7kb in length flanked by both a 5' and 3' non coding region (Fig 1.3). These regions include a number of structural elements that are essential for virus replication.

1.2.3.1 The 5' Untranslated region (UTR)

The 5' UTR of FMDV is much longer than those of other Picornaviruses, with an average length of 1300 nucleotides (Belsham, 2005). The RNA is covalently attached to the primer protein 3B (VPg) at the 5' terminus (Sangar *et al.*, 1977) and contains many structural elements. The first is a large hairpin called the S fragment (Newton *et al.*, 1985). Although the precise role of this structure is not known, a role for the S fragment in RNA replication through interactions with RNA helicase A (Lawrence & Rieder, 2009) and interactions with the 3' UTR (Serrano *et al.*, 2006) have been proposed. Such a role would be consistent with the function of a cloverleaf structure found at the same location on the Poliovirus genome (Andino *et al.*, 1990). The S fragment is followed by a long polyribocytidylic (Poly-C) tract generally between 150-200 nucleotides in length (Belsham, 2005). The length of this tract has been shown to have an impact on virus growth *in vitro* (Rieder et al., 1993); however, reports on the effects of shortening the poly C tract on virulence *in vivo* have been

contradictory (Costa Giomi *et al.*, 1984; Harris & Brown, 1977; Rieder *et al.*, 1993). The degree of the impact of the poly C length on virulence has been reported to vary markedly between cardioviruses, where a short poly-C tract leads to almost complete attenuation of Mengoviruses but has only marginal effects of the virulence of Encephalomyocarditis viruses (Hahn & Palmenberg, 1995; Osorio *et al.*, 1996). The poly C tract is followed by a series of pseudoknots (Escarmis *et al.*, 1995; Le *et al.*, 1993), a characteristic shared with other picornaviruses (Le *et al.*, 1993) which at present have no known biological function.

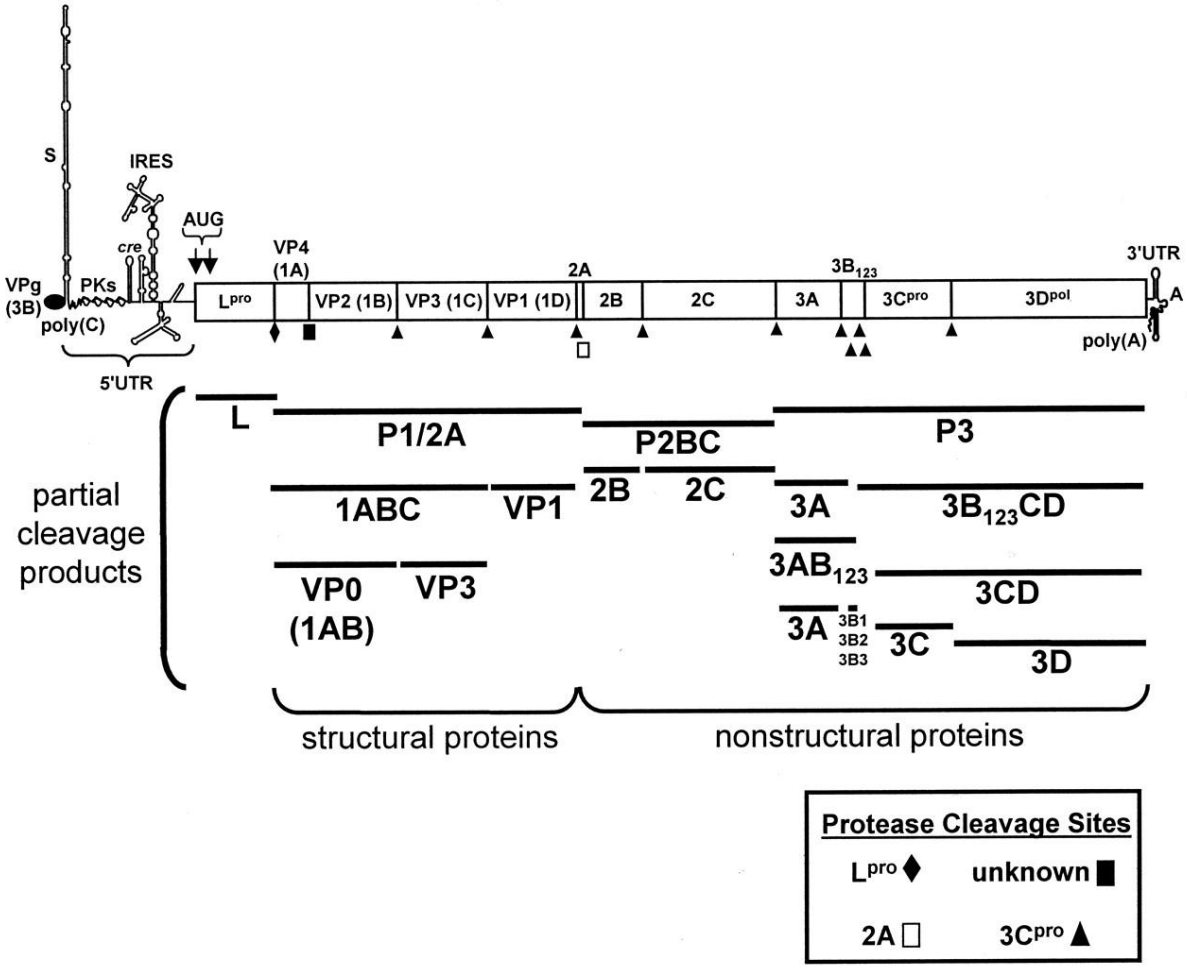


Figure 1.3 The FMDV genome arrangement. The partial cleavage products produced before the generation of the mature protiens are shown. The proteases responsible for cleavage events are also indicated. From (Grubman & Baxt, 2004).

The *cis*-acting replication element (cre- also called the 3B uridylylation site or 3BUS as it can also act in *trans* (Tiley *et al.*, 2003)) is a loop structure found downstream from the pseudoknots. This element contains a highly conserved AAACA motif and facilitates the uridylylation of the VPg (3B) primer, using two adenosine residues to guide the virus 3D polymerase to add two uridine residues to a tyrosine residue located on the primer (Steil & Barton, 2009). The cre element is conserved in many Picornaviruses; however, in contrast to FMDV, the cre is not normally located outside of the coding region of the RNA (Belsham, 2005). Mutations of this element have been shown to severely inhibit viral replication *in vitro* (Mason *et al.*, 2002). The final element of the 5'UTR is the internal ribosome entry site (IRES), a feature of all Picornaviruses. This mediates CAP-independent protein synthesis through recruitment of host cell factors and ribosomes to form an initiation complex (Martinez-Salas *et al.*, 2008), which moves down the RNA from the 5' to 3' translating the FMDV reading frame.

1.2.3.2 The Open reading frame

The ORF of FMDV is approximately 7000 nucleotides in length. Unlike other Picornaviruses, the FMDV open reading frame contains two start codons (AUG) which can produce two different versions of the leader protein , L_{ab} and L_b (Clarke *et al.*, 1985). Although some other Picornaviruses have leader proteins, several of the functions of the aphthovirus leader protein (Lpro) are unique (Hinton *et al.*, 2002). The aphthovirus Lpro is a papain-like cysteine proteinase (Guarne *et al.*, 1998) that auto-catalytically cleaves itself from the nascent polypeptide and is responsible for cleaving the host cells EIF4G protein (Medina *et al.*, 1993). In doing so, it shuts down host cell, cap-dependant mRNA translation. Absence of the Lb or an insertion into the inter AUG regions between L_{ab} and L_b results in a virus that is attenuated in cattle (Brown *et al.*, 1996; Piccone *et al.*, 2010). The Lpro also plays a prominent role in controlling the *in vivo* innate immune response, as discussed in section 1.5.1.

The P1-2A region consists of the structural proteins, VP1-4 (Figure 1.3) and the 18 amino acid long 2A peptide. This peptide possesses no known function in FMDV other than enabling the cleavage of the P1-2A element from the P2 element. This occurs at the N terminus of the protein 2B in a stop codon independent manner known as 'stop-carry on' recoding, using a conserved 2A C terminal NPGP motif (Donnelly *et al.*, 2001; Sharma *et al.*, 2012). This function for 2A is shared with some other members of the Picornavirus genera, although others have different functions. The enterovirus 2A is a proteinase responsible for cleaving EIF4G, a function performed by Lpro in FMDV (Palmemberg, 2010). Once the P1-2A region has been cleaved off the polyprotein, further cleavage takes place, mediated by the viral 3C proteinase with the final products being VP0, VP3, VP1 and 2a. VP0 is presumed to auto-catalytically cleave itself into VP2 and VP4. This occurs late in infection during the process of virus assembly, possibly mediated by a conserved histidine at position 145 of VP2 (Curry *et al.*, 1997).

The P2 precursor (2BC) and the mature proteins 2B and 2C (cleaved by 3Cpro (Belsham, 2005)) have been shown, when co-expressed, to have an inhibitory effect on the transport of vesicles between the endoplasmic reticulum and the Golgi apparatus (Moffat *et al.*, 2005; Moffat *et al.*, 2007); a similar function is carried out by the 3A protein in Poliovirus (Doedens *et al.*, 1997). This could explain the down regulation of MHC class 1 reported on the surface of FMDV-infected cells (Sanz-Parra *et al.*, 1998) and thus implicates 2BC and 2B/2C as important modulators of the immune response to FMDV. FMDV utilises the cellular autophagy pathway during replication (O'Donnell *et al.*, 2011) and a role for autophagosomes in FMDV cell entry, but not in providing membranes for replication, has been described (Berryman *et al.*, 2012). 2C, along with VP1, 3A and 3D have been seen to co-localise with membranous vesicles formed by the re arrangement of host cell membranes at the site of the degraded ER and Golgi apparatus, which are believed to provide platforms for viral replication, also known as replication complexes (Knox *et al.*, 2005; Monaghan *et al.*, 2004). Recently, a role for 2C in preventing the binding of auto-phagosomes to lysosomes, by interaction

with host cell factors, has been identified that may result in the prevention of virus degradation (Gladue *et al.*, 2012).

The P3 precursor is also cleaved by 3Cpro into the proteins 3A, 3 copies of 3B, 3C and 3D, with many intermediate products formed. As discussed in section 1.2.2, deletions in the sequence of 3A can alter host specificity (Knowles *et al.*, 2001). 3A has been shown to localise at FMDV replication complexes and a role has been suggested for 3A and its precursor 3AB in RNA replication (Rosas *et al.*, 2008), perhaps performing a similar role in replication to that of Poliovirus 3AB (Towner *et al.*, 1996). The FMDV virus 3B (Vpg) is unique among the Picornaviruses, including the other members of the *Aphthovirus* family (Hollister *et al.*, 2008; Li *et al.*, 1996) in that each virion has three copies of this 23-24 amino acid long protein (Forss & Schaller, 1982). It is still unclear why FMDV has three copies of this protein, however deletion of one or more of these copies results in impaired virus replication (Pacheco *et al.*, 2003), suggesting the presence of all three is essential in maintaining replication competence. 3B acts as the primer for syntheisis of both the positive and negative RNA strands and, as discussed previously, is uridylylated by the *cre* in a mechanism that has been reported to also involve the 3CD precursor and the 3D polymerase (Nayak *et al.*, 2005).

The viral 3C protease is related in structure and function to the trypsin-like serine proteinases (excepting that the serine is replaced by a cysteine in the active site) and is responsible for all the cleavage events, excluding the cleavage of L_{pro}, VP0 and P1-2A (Bablanian & Grubman, 1993; Grubman *et al.*, 1995). This contrasts with Poliovirus, where 3CD is required for some cleavages ((Ypma-Wong *et al.*, 1988). FMDV 3C also cleaves many host cell proteins, including the histone H3 (Tesar & Marquardt, 1990), EIF4G and EIF4A. The cleavage of the latter two happens late in the infection cycle and has been postulated to result in a switch from translation to packaging (Belsham *et al.*, 2000). The 3C protease has also been reported to interact with the microtubule system of both FMDV and Poliovirus infected cells and hence may alter cell morphology (Armer *et al.*, 2008; Joachims *et al.*, 1995). The final protein that makes up the ORF is 3D, an RNA dependant RNA

polymerase, which as mentioned previously (section 1.2.3.1) is localised at the replication complexes and is dependent on the primer function of the cre and 3B protein for initiation of RNA synthesis.

1.2.3.3 The 3' UTR

The FMDV 3' UTR comprises a heterogeneous nucleotide sequence of approximately 100 nucleotides, which has been modelled as forming two stable stem loops, followed by a poly-A tail (Serrano *et al.*, 2006). Deletion of the 3' UTR abrogates viral infectivity in FMDV (Saiz *et al.*, 2001) but not in both Rhinovirus and Poliovirus (Todd *et al.*, 1997). This region has been demonstrated to enhance FMDV IRES activity (Lopez de Quinto *et al.*, 2002) and results in increased translation of the viral genome. Enhancement of cellular mRNA translation is brought about by the circularization of the genome through interaction with the EIF4G and PABP (Tarun & Sachs, 1996); however, the region that PABP uses to interact with EIF4G is cleaved off by the FMDV Lpro. It has been demonstrated that the 3' UTR interacts with the 5' UTR at sites both on the S-fragment and the IRES, while also interacting with a host cell factor that also binds to the S fragment, suggesting a role for these interacting elements in circularising the genome (Serrano *et al.*, 2006). The 3'UTR does not bind simultaneously to the IRES and S fragment suggesting different roles for each interaction, with the interaction with the IRES being responsible for the enhancement of translation observed and the interaction with the S fragment occurring during replication. This circularization is also a process required for Poliovirus RNA replication using the cloverleaf structure located in the same place on the FMDV genome as the S fragment (Herold & Andino, 2001).

1.2.4 Attachment and entry.

FMDV, like a number of other picornaviruses (Tuthill *et al.*, 2010) uses integrins as host cell attachment molecules (Jackson *et al.*, 2002; Jackson *et al.*, 2000; Jackson *et al.*, 2004; Jackson *et al.*, 1997) of which Integrin αvβ6 and αvβ3 are believed to be the most important *in vivo* in cattle (O'Donnell *et al.*, 2009). Integrin αvβ6 and αvβ3 (to a lesser extent) have also been shown to be important for infection of wildlife species by the SAT viruses (Maree *et al.*, 2011a), while integrin

αvβ8 can be used to infect porcine cells (Johns *et al.*, 2009). Binding to integrin is mediated by the conserved arginine-glycine-aspartic acid (RGD) motif located on the GH loop of VP1 (Fox *et al.*, 1989).

During *in vitro* replication, FMDV can change receptor to binding cells via heparan sulphate (HS). This involves substitution of one or more amino acid(s), on the outer surface of the virion, with a change at VP3 56 from histidine to arginine being the key substitution in serotype O and A (Fry *et al.*, 1999; Jackson *et al.*, 1996). However, the SAT viruses appear to use a different part of the capsid around the fivefold axis of symmetry to interact with HS (Maree *et al.*, 2010). The structure of Heparin (a structural mimic of HS) bound to both serotype O and A has been described and the interactions with the surface were nearly identical. In addition to VP3 56, other capsid residues involved in this interaction were shown to include VP1 193 (for type A) 195 (for type O), VP2 134, 135 and 138, VP3 59, 60, 87 and 88 for both serotypes (Fry *et al.*, 1999; Fry *et al.*, 2005b; Jackson *et al.*, 1996). Viruses that carry the VP3 56 adaptation are attenuated *in vivo* (Borca *et al.*, 2012; Sa-Carvalho *et al.*, 1997) and the arginine at VP3 56 rapidly reverts back to histidine within infected cattle (Wright *et al.*, 2011). However, it has been suggested that the host immune response may drive the virus to modulate receptor use *in vivo* (Fry et al., 1999) and both receptors are located at known neutralising antigenic sites *in vitro* (see section 1.8). Additional evidence suggests that there may also be a third, as yet undefined virus receptor (Baranowski et al., 2000).

Once the virus has become attached it enters the cell via clathrin-mediated endocytosis (Berryman *et al.*, 2005; Johns *et al.*, 2009; Martin-Acebes *et al.*, 2009; O'Donnell *et al.*, 2005) or Calveolin-mediated endocytosis when binding HS (O'Donnell *et al.*, 2008). FMDV is very acid labile (Brown & Cartwright, 1961) and when in the endosome the low pH triggers capsid disassembly and release of the viral RNA which translocates into the cytosol by an unknown mechanism (Baxt & Bachrach, 1980). In contrast, many other picornaviruses release their RNA from the endosome via a change in capsid conformation through an altered (A) intermediate particle which involves interactions of VP1 and VP4 with the endosomal membrane, resulting in the loss of VP4 from the

intermediate particle and release of the viral RNA into the cytoplasm leaving behind an empty capsid in the endosome (Hogle, 2002). Recent evidence for enteroviruses suggests that the viral RNA is likely to exit the capsid at the twofold axis and not the fivefold axis of symmetry as previously hypothesised (Bostina *et al.*, 2011; Garriga *et al.*, 2012; Wang *et al.*, 2012b).

A transient intermediate (A) particle for the closely related Aphthovirus, Equine Rhinitis A virus, which is also acid labile at mild pH has now been identified (Tuthill *et al.*, 2009) and recently, evidence has begun to emerge for the formation of a transient FMDV intermediate particle (Gold., 2012 Personal communication) which may release the viral RNA prior to dissociation into pentamers. This recent evidence obtained for both ERAV and FMDV suggests there may be a unified mechanism for Picornavirus RNA release via an altered conformation particle, either as an intermediate before capsid dissociation or on the way to becoming a stable empty particle.

1.2.5 Translation, replication, assembly and exit from the cell.

Upon entry into the cell, the VPg that is covalently attached to the 5' UTR is cleaved off by VPg unlinkase, which has recently been identified for Polio and Rhinoviruses as the DNA repair enzyme 5'–tyrosyl–DNA phosphodiesterase–2 (Virgen-Slane *et al.*, 2012). After this cleavage event RNA translation commences. The viral positive sense RNA acts as a template for CAP-independent translation via the IRES. The polyprotein is processed and interacts with the host cell, as described in section 1.2.3. The input genome must act as a template for both protein synthesis (translation) and transcription to generate negative strands that are subsequently used to make more positive strand genomes. However, it has been demonstrated that the passage of ribosomes along the viral RNA prevents RNA replication (Barton *et al.*, 1999; Gamarnik & Andino, 1998) and therefore a mechanism to switch from translation to negative strand synthesis is necessary. For Poliovirus, this is thought to be mediated by the circularization of the genome and the formation of ribonucleoprotein (RNP) complexes inhibiting the binding of ribosomes and therefore switching from translation to replication (Barton *et al.*, 2001). However, the mechanism for FMDV may be slightly different. It has been

suggested that the 3'UTR may be responsible for this change by switching from specifically binding to the IRES to binding to the S fragment, as discussed previously (Serrano *et al.*, 2006).

Once circularized, the VPg primer can act as a template for RNA replication, which occurs at membranous vesicles (replication complexes) as discussed previously. The first step of RNA synthesis is the generation of the negative sense RNA strand. The Uridylylated VPg linked to the RNA polymerase (3D) is translocated to the 3'UTR and the replicative (double stranded) form of the RNA is generated (Grubman & Baxt, 2004). From this, positive-sense genomes are generated. Replication of RNA then commences asymmetrically, with many more copies of the positive strand generated than the negative strand. For Poliovirus, the ratio of positive to negative strands has been reported to be around 50:1 (Novak & Kirkegaard, 1991).

Little is known about the mechanism of picornavirus assembly. As discussed, the structural proteins are initially processed to VP1, VP3 and the intermediate VP0. These then form protomers, five protomers self-associate to form a pentamer and 12 pentamers self-associate to form the complete capsid (see figure 1.5). The precise mechanism of RNA encapsidation is unclear. There is evidence for Poliovirus that RNA interacts with the pentamer before the capsid is assembled (Verlinden *et al.*, 2000). However, recent evidence for Bovine enterovirus suggests that empty capsids are formed as precursors and the RNA added at a later stage through an unknown mechanism (Li *et al.*, 2012). The final step in capsid maturation is the cleavage of VP0 to VP2 and VP4, which, as discussed previously, is believed to cleave itself auto-catalytically through a conserved histidine on VP2 (Curry *et al.*, 1997). For Poliovirus, it has been suggested that RNA is required for this cleavage to occur (Basavappa *et al.*, 1994); however, for FMDV, empty capsids with no RNA associated, have been shown to cleave VP0 to VP2 and VP4 (Curry *et al.*, 1997). This paper also reports that both this cleavage and the addition of RNA play a role in stabilising the mature capsid.

FMDV is a lytic virus and therefore the newly formed virions accumulate within the cytoplasm until the host cell lyses, resulting in release into the surrounding environment.

Interestingly, however, FMDV has been shown to develop into a non-cytolytic virus when subjected to selective pressures *in vitro* (Escarmis *et al.*, 2008).

1.3 FMDV Structure

FMDV is a small icosahedral virus, approximately 30nm in diameter, exhibiting a pseudo T=3 symmetry. It comprises 60 copies of each of the four 4 structural proteins VP1-4. VP1, 2 and 3 are on the surface of the virus and are comprised essentially of eight anti-parallel β strands linked to each other by loop structures to form a β barrel, whereas VP4 is much smaller in size and has little secondary structure (Acharya *et al.*, 1989). As mentioned previously, one copy of each of the virion polypeptides combine to form a protomer (the biological protomer is shown in figure 1.4 (a) and a triangular (icosahedral) protomer, used in the subsequent chapters for ease of interpretation is shown in figure 1.4(b)), five protomers form a pentamer and 12 pentamers form the complete capsid (Figure 1.4- shows the complete capsid and β barrel).

The pentameric subunit is held together by disulphide bridges between the cysteine residues at residue 7 of VP3 (Acharya *et al.*, 1989), and by hydrophobic and ionic attractions between the N termini of VP3 at the 5 fold axis forming a β annulus (and leaving a hole at this point) while the stability between pentamers is mediated by six β strands; two from VP2 and four from VP3 at the two fold axis of symmetry, and VP2-VP2 interactions at the three fold axis (Fry *et al.*, 1990). VP4 is also myristoylated and this may play a role in stabilising VP3 (Fry *et al.*, 2005a). FMDV is highly acid labile and dissociates into pentamers at a pH of below 6.8 (Brown & Cartwright, 1961). It has been suggested that histidine clusters at the pentamer-pentamer interface may be responsible for this dissociation (Acharya *et al.*, 1989; van Vlijmen *et al.*, 1998).

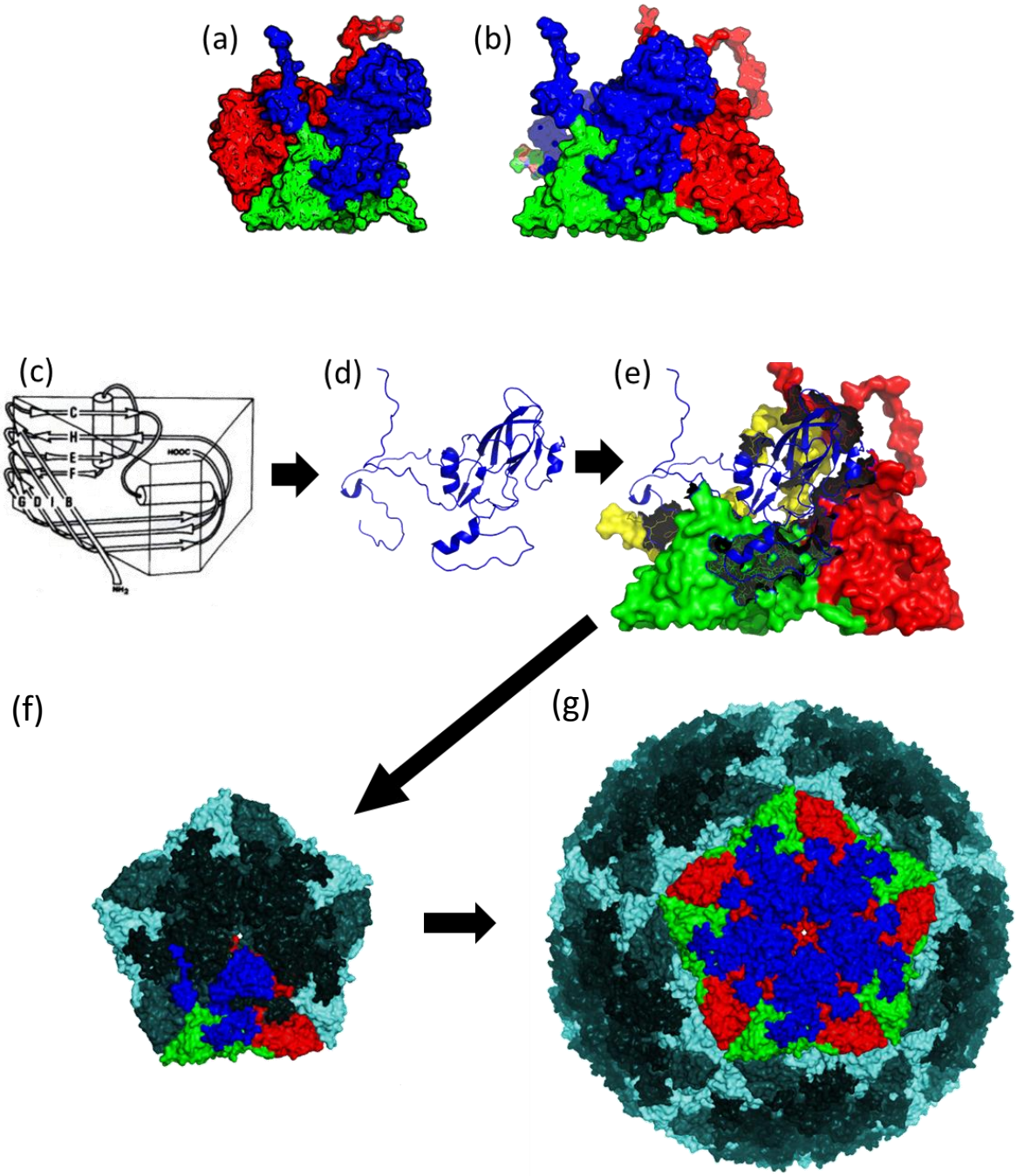


Figure 1.4: FMDV Structure and assembly. (a) and (b) represent the molecular surfaces of a biological protomer and triangular protomer respectively. (c) shows a schematic of the β barrel structure that VP1, 2 and 3 form. (d) shows a ribbon drawing of the actual structure of a representative β barrel, in this case VP1 (blue). (e) Shows the position of the VP1 ribbon within an assembled triangular protomer, with the other elements VP2 (green), VP3 (red) and VP4 (yellow) shown as the molecular surface. (f) Shows the molecular surface of a constructed pentamer with a single protomer coloured as (e). (g) shows the molecular surface of a complete capsid made up of 12 pentamers, with a single pentamer overlaid, capsid proteins coloured as (f). All pictures with the exception of (c) were made using Pymol (Schrödinger LLC) and PDB file 1FOD (Reduced O BFS). picture (c) adapted from (Kitson *et al.*, 1990).

The structure of FMDV serotype O was the first to be described by crystallography (Acharya *et al.*, 1989). Following the resolution of the structure for type O, structures for serotypes A, C and SAT-1 have also been described (Curry *et al.*, 1996; Lea *et al.*, 1994; Reeve *et al.*, 2010). These serotypes have a strong structural homology with that observed for serotype O. In contrast to several other Picornaviruses, the FMDV capsid is thinner and does not possess any canyon features (Hogle *et al.*, 1985; Rossmann *et al.*, 1985) thought to shield the receptor binding sites. It was observed that the VP1 C terminus of each protomer overlaps the adjacent protomer, coming into close proximity with the GH loop on VP1 of the adjacent protomer.

One part of the surface that could not originally be resolved by X-ray crystallography was the highly disordered and extended VP1 GH loop of FMDV. This loop structure contains the RGD motif that is used by the virus to bind to integrin *in vivo* (Fox *et al.*, 1989). It was proposed that this loop could exist in two conformations: "up" and "down" (Parry *et al.*, 1990). Acharya and colleagues (Acharya *et al.*, 1989) had described a disulphide bridge between two cysteine's at positions 134 of VP1 and 130 of VP2 and postulated that this may influence the conformation of the GH loop. When this disulphide bridge was disrupted by reducing the O_1 BFS with DTT, the GH loop was resolved in the more ordered "down" conformation (Fig 1.5) lying across the surface of VP2 (Logan *et al.*, 1993). Observation of this reduced structure revealed that the RGD motif was in an exposed position at the turn of a loop, in a similar conformation to that found on γ II Crystallin, which also binds integrin (Wistow *et al.*, 1983). This disulphide bridge between VP1 & 2 of type O, which is suggested to hold the GH loop in an "up" conformation (Parry *et al.*, 1990), is also present in the serotype SAT-1 structure but absent in both the A and C structures.

The first evidence for the "up" conformation came from the determination of the structure of a monoclonal antibody bound to a serotype C capsid (Hewat *et al.*, 1997). This showed the GH loop protruding from the surface. These findings were confirmed 2 years later (Verdaguer *et al.*, 1999) and it was suggested that this change in conformation from up to down is brought about by

hinged rotation about the base of the loop. Both the "up" and "down" conformations are displayed in Figure 1.5. Type C FMDV was used to demonstrate the "up" conformation, where the GH loop is 4 amino acids shorter than that found on type O.

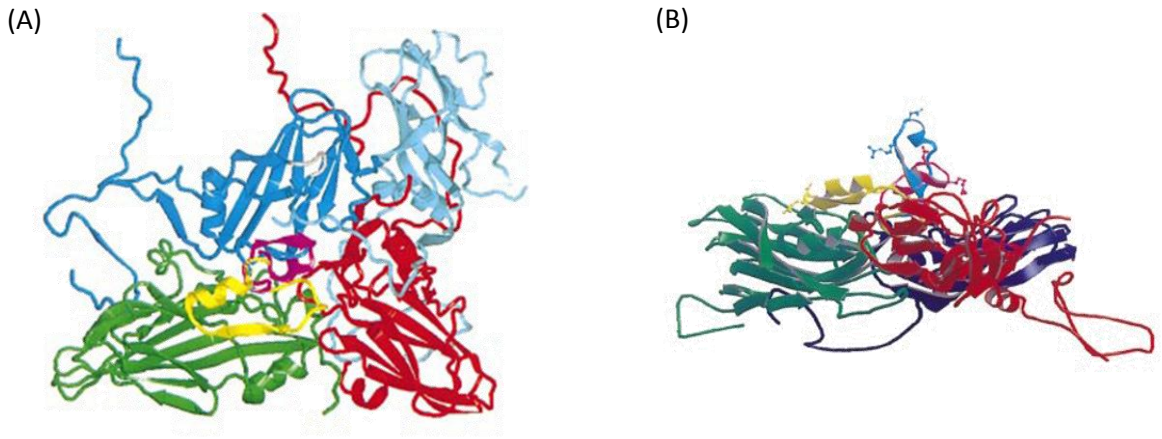


Figure 1.5. The up and down conformations of the GH loop of FMDV: Figure A shows the location of the GH loop in the up (magenta) and down conformation (yellow). Figure B shows a side view of the GH loop in the up (blue as in magenta on a) and down (yellow) conformations. Pictures from (Hewat *et al.*, 1997; Verdaguer *et al.*, 1999).

1.4 An overview of the humoral response to viral infection.

1.4.1 B cells as the source of antibody

Humoral immunity is one of the components of the adaptive immune response mediated by antibody, produced by B cells. In mice and humans, B cell development and diversification occurs in the bone marrow; however, in pigs, cattle and sheep this process happens in the ileal peyers patches (Andersen *et al.*, 1999; Yasuda *et al.*, 2006). The B cell repertoire expands through rearrangements of the variable (V), diversity (D- only on heavy chains) and joining (J) gene regions, with the constant (C) gene region remaining unchanged. During B cell maturation the immunoglobulin (Ig) heavy chain genes undergo rearrangement of their V, D and J genes first during the pro B cell phase of development, after which no more gene rearrangements of these elements occur. The light chain V and J genes then undergo rearrangement in the pre B cells, which develop through the immature B cell into a mature naive B cell (Murre, 2007).

Immunoglobulins are comprised of two sections, the Fc region and Fab region. The Fc region forms most of the constant domain of an immunoglobulin and is responsible for interactions with other components of the host immune system; while the Fab region contains the variable regions responsible for binding to a specific epitope (see section 1.8 for more detail). The constant domain of the immunoglobulin heavy chain indicates the specific isotype. There are five different immunoglobulin heavy chain isotypes in most mammals: α, δ, ε, γ, and μ. These correspond to the IgA, IgD, IgE, IgG, and IgM immunoglobulins, respectively. These have all been identified in cattle, sheep (Yasuda *et al.*, 2006) and pigs (Butler & Brown, 1994), however, the number of sub-classes of these isotypes differ between species. Humans have 7 families of immunoglobulin variable heavy gene (Li *et al.*, 2002) and this contrasts with cattle, sheep and pigs who each have a single immunoglobulin heavy chain gene family (Dufour *et al.*, 1996; Sun *et al.*, 1994). Like humans, cattle, pigs and sheep have two different light chain isotypes known as λ and κ, although the levels of expression of each of these varies markedly between species (Sun *et al.*, 2012).

There are several types of B cells, contributing differently to the humoral immune response. In Cattle, B-1 B cells are found in the spleen and peripheral blood but not in other secondary lymphoid tissues (Naessens & Williams, 1992). These cells are considered to be more primitive than B-2 B cells and constitutively produce low affinity, polyreactive IgM Immunoglobulin, which can act to either directly neutralise virus or to activate the complement cascade (Ochsenbein & Zinkernagel, 2000). These cells, along with B cells found in the marginal zone (MZ B cells) of the spleen are classified as innate B lymphocytes, as they act as a first line of defence against pathogens prior to the onset of the more specific responses (Carey *et al.*, 2008; Kearney, 2005). Unlike follicular B cells, these cells express CD9 on their surface (Won & Kearney, 2002).

Conventional B cells (also called B2 or follicular B cells) reside in the secondary lymphoid tissue of mammals and in mice they have been demonstrated to survive for several months (Forster & Rajewsky, 1990). These cells are associated with T cell dependent antibody responses. When a B

cell encounters a T dependent antigen it is internalised and presented on MHC II molecules on the B cell surface (Cambier *et al.*, 1994). Upon interaction with a primed CD4+ helper T cell, co-stimulatory molecules are up-regulated (June *et al.*, 1994) and these interact with T cells expressing CD28 which in turn leads to CD154 expression on the activated T cell. Interaction between CD154 and CD40 on the B cell leads to proliferation, isotype switching and the production of antibodies (Armitage *et al.*, 1992). These activated B cells proliferate into two different cell types, short lived antibody producing plasma cells and long lived memory B cells (Bernard *et al.*, 2005). Somatic hypermutation also occurs and helps to increase the affinity of the B cells for the pathogen (McHeyzer-Williams, 2003). It has also been suggested that interactions of pathogens with B cell toll like receptors (TLR's) are essential for optimisation of antibody responses (Booth *et al.*, 2011).

B cells can also be activated to produce antibodies in processes that do not require T cell activation. There are two different types of T cell independent (TI) response. Type 1 TI responses are brought about in response to mitotic agents that can activate B cells without direct signalling through the B cell receptor, for example through B cell TLR ligands (Swanson *et al.*, 2012). TI 2 responses are brought about by proteins made up of highly organised repetitive elements that are able to directly cross-link B cell receptors (Swanson *et al.*, 2012) and virus capsids have been associated with this response (Bachmann & Zinkernagel, 1996). Several viruses are known to act as TI antigens, including vesicular stomatitis virus (VSV) (Bachmann *et al.*, 1995), influenza virus (Lee *et al.*, 2005) and rotavirus (Franco & Greenberg, 1997). T independent responses to FMDV infection have also been described in mice (Ostrowski *et al.*, 2007) and cattle, where class switching and complete protection were observed in the absence of a T cell dependent response (Juleff *et al.*, 2009). In mice, the MZ and B1 B cells were described as being the key component of the early production of antibodies (Ostrowski *et al.*, 2007), consistent with the idea that this T independent response predominantly generated by MZ and B1 cells in the spleen is crucial in containing infection in the early stages before the onset of an adaptive immune response (Martin *et al.*, 2001)

1.4.2 Neutralisation of viruses by antibody

Neutralisation of viruses by antibody is seen as a crucial mechanism in the immune response to infection and generally levels of neutralising antibody have been demonstrated to correspond well with protection observed post vaccination (Neurath, 2008). However there is still considerable debate as to how antibodies achieve direct neutralisation, whether it is only by antibodies that are able to bind to certain regions (Dimmock, 1993) or whether simple occupancy of a given area of the virion surface by a given number of antibodies, of a high enough affinity, is effectively the means to neutralise virus (Klasse & Sattentau, 2002; Parren & Burton, 2001). The latter essentially also argues that non-neutralising antibodies are simply low affinity antibodies that, as opposed to binding different regions of the capsid surface to neutralising antibodies, cannot bind strongly enough to occupy a sufficient amount of the virion surface and therefore cannot neutralise.

The paper by Parren and Burton that argues for the latter demonstrated that a linear correlation could be observed between the size of a virus particle and the number of antibodies required for neutralisation *in vitro* and suggested that the area of virus covered by these antibodies is enough to effectively coat the molecule. This ranged from just 4 antibodies for Poliovirus (Icenogle *et al.*, 1983), 70 for Influenza (Taylor *et al.*, 1987) and an average of 225 for Rabies (Flamand *et al.*, 1993). However, the same rabies paper also found that one virus was resistant to neutralisation, even at levels of virion saturation (1080 IgG/virion). In addition this does not hold for adenoviruses where just 1.4 neutralising antibodies have been demonstrated to effectively neutralise virus (Wohlfart, 1988) whilst a second study on Polio suggested that a single antibody could neutralise virus (Wetz *et al.*, 1986). Recent evidence has shown a direct link between levels of the intracellular high affinity Ig receptor, TRIM-21 and the number of bound antibodies needed to neutralise adenovirus (McEwan *et al.*, 2012). This study found that ablation of TRIM-21 activity *in vitro* meant that virus could readily infect cells, even in the presence of saturating concentrations of antibody and, conversely, that with high TRIM-21 expression viruses would be neutralised by 1.6 antibodies per molecule. This mechanism has been attributed to controlling the level of the persistent fraction

(the level of non-neutralised virus present even at high neutralising antibody concentrations) that has previously been associated with aggregation of virus, among other mechanisms (Klasse & Sattentau, 2002; Parren & Burton, 2001). The above evidence would suggest that a simple occupancy model, although likely to be an important mechanism may not be sufficient to be a unifying model to explain neutralisation and indeed probably such a unifying model may not exist.

Several mechanisms for direct neutralisation of viruses without the aid of effector mechanisms have been described, including binding of antibodies to virus surfaces that sterically hinder interactions with virus receptors. For some Picornaviruses, a limited number of antibodies have been demonstrated to reduce the number of infectious viruses significantly, which may be attributable to blocking receptor binding (for the relative size of a picornavirus capsid and an antibody molecule see figure 1.6), for example five to six copies of a monoclonal antibody (mAb) that bound on a Rhinovirus capsid near the canyon (the location of the receptor binding site for a major group of Rhinoviruses) reduced virus infectivity by 50% (Smith *et al.*, 1993). For Poliovirus, only 4 monoclonal antibodies, bound to the virion surface, were described as being sufficient for neutralisation (Icenogle *et al.*, 1983). This mechanism of neutralisation has also been reported for enveloped viruses, including HIV (Posner *et al.*, 1991) and Newcastle disease virus (Iorio *et al.*, 1989).

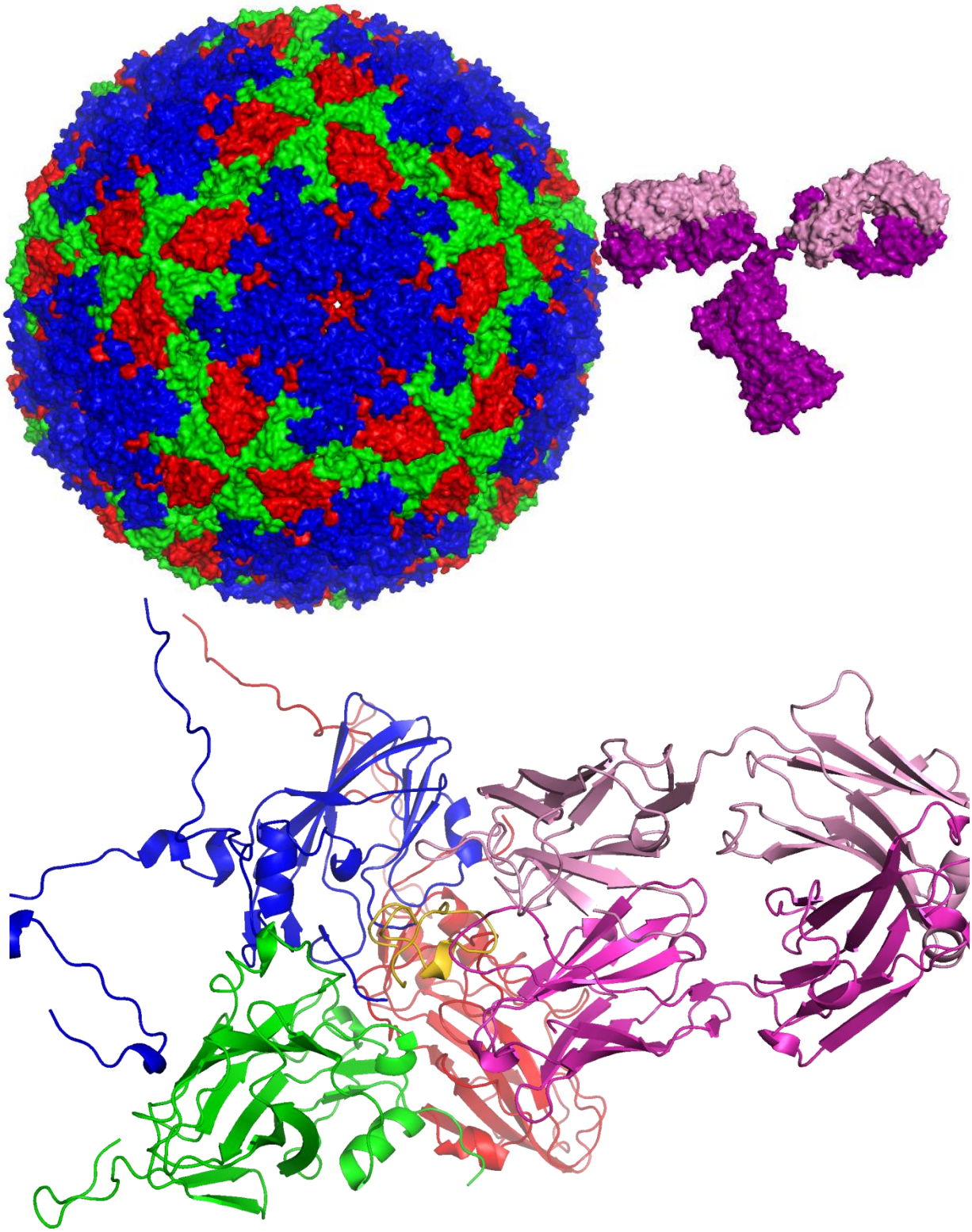


Figure 1.6: (a) Comparison of the size of an FMDV capsid and an Immunolobulin. The capsid is coloured as per the convention with VP1 Blue, VP2 green and VP3 red. The Immunogloulin light chains are pink with the heavy chains represented in purple. The molecular surface of both is shown. (b) cartoon representation of a FMDV protomer bound to an immunoglobulin (coloured as (a)). Also shown is the GH loop in an up conformation (gold). Picture (a) generated using PDB files 1FOD and 1IGT, (b) was generated using PDB file 1QGC. Picture made using Pymol (Schrödinger LLC).

For Poliovirus, it has been observed that antibodies can cross link two virions to form large aggregates of virus capsids, the formation of which is directly correlated to loss of infectivity (Brioen *et al.*, 1983; Icenogle *et al.*, 1983; Thomas *et al.*, 1985; Thomas *et al.*, 1986); this has also been proposed as a mechanism for the neutralisation of adenovirus (Wohlfart, 1988). Another mechanism for Poliovirus neutralisation involves the permanent alteration of the capsid structure, with the release of RNA (Delaet & Boeye, 1993). However this only occurred at "fever" temperatures and not at 37°C. A similar mechanism has also been described for FMDV, where a rapid destabilisation of the capsid occurred (after only 30 seconds of contact) upon interaction with a mAb, leading to openings in the capsid and partial externalisation of the virus RNA (McCullough *et al.*, 1987); however, unlike Poliovirus, this occurred at 37°C. A role in conformational alteration of virus glycoproteins on the surface of enveloped viruses leading to neutralisation has also been observed for Sindbis virus (Hernandez *et al.*, 2008) and suggested for rabies virus (Irie & Kawai, 2005).

Neutralising antibodies are also able to inhibit fusion and entry of viruses into cells even after the virus has attached itself to the appropriate receptor. The binding of a broadly neutralising HIV monoclonal antibody to the metastable fusogenic protein gp41 has been shown to halt fusion by preventing the conformational changes to gp41 that enable membrane fusion (Barbato *et al.*, 2003). A broadly neutralizing antibody against Influenza also inhibits fusion by binding to the stem of the viral haemagglutinin receptor glycoprotein, again blocking conformational changes (dependant on pH) that bring about fusion of the virus membrane with the host cell membrane (Ekiert *et al.*, 2009). A mechanism for the blocking of viral fusion to an endosome once internalised has been proposed for adenovirus (Wohlfart, 1988) and may also be an alternative mechanism for the neutralisation of Poliovirus by just four antibodies (Icenogle *et al.*, 1983), where cross linking of viruses by bivalently bound antibodies reported to be bridging across pentamers may prevent the structural rearrangements required (as discussed in section 1.2.4) for RNA release.

A method for IgA to neutralise intracellularly through uptake via the polymeric immunoglobulin receptor (pIgR) found on the basolateral membranes of mucosal epithelial cells usually used for antibody transcytosis across epithelium has also been described. This was first proposed for Sendai virus (Mazanec *et al.*, 1995b) and has subsequently been described for influenza (Mazanec *et al.*, 1995a), HIV (Huang *et al.*, 2005; Wright *et al.*, 2006), measles (Zhou *et al.*, 2011) and the non-enveloped rotavirus (Corthesy *et al.*, 2006). This mechanism has been proposed to work by binding virus proteins within the cell, effectively preventing virus assembly. Another mechanism of neutralising virus is signalling across the virus membrane via CD4 interactions to inhibit HIV replication (Peleraux *et al.*, 1998), although this is unlikely to be important in natural infection. It has also been reported that some antibodies to influenza appear to neutralise by blocking transcription (Armstrong & Dimmock, 1992; Rigg *et al.*, 1989) and virus release from the cell (Gerhard, 2001). Antibodies can also play a role in preventing the cell-to-cell transmission of both HIV (Pantaleo *et al.*, 1995) and Herpes simplex virus (Burioni *et al.*, 1994).

As mentioned previously, another novel mechanism for intracellular neutralisation has recently been described called antibody dependant intracellular neutralisation (ADIN). This may require a re-evaluation of theories of direct neutralisation of viruses (McEwan *et al.*, 2011). The mechanism proposed involves the high affinity, highly specific, intracellular immunoglobulin Fc receptor TRIM-21, expressed in many tissues, to target virus/antibody (both IgG and IgM) complexes for degradation of viruses in the proteasome (Mallery *et al.*, 2010). As discussed above, this is a potent regulator of virus neutralisation *in vitro* (McEwan *et al.*, 2012), and has been suggested to be a mechanism for neutralisation of other non-enveloped viruses. It was also noted that interferon activation of cells leads to more efficient neutralisation by TRIM21. Synergy between antibody and interferon has been reported for non-enveloped viruses, including Adenovirus, and a number of Picornaviruses (Langford *et al.*, 1983) and ADIN may possibly explain this synergy. However, it should be noted that this synergy was observed for some enveloped viruses also and ADIN is unlikely to neutralise enveloped viruses. It will also be interesting to see if this mechanism can interfere with

Picornavirus replication, where the genome is released by fusion with the endosome, presumably leaving the capsid and any bound antibody inside the vesicle.

1.4.3 The roles of non-neutralising antibodies in viral infection.

As opposed to the actions of neutralising antibodies, that can directly block the infectivity of a pathogen, non-neutralising antibodies rely on interactions with other components of the immune system to clear pathogens. These antibodies can aid in the elimination of viral infections via several mechanisms, primarily though Fc mediated interactions and interactions with complement. A list of some of these methods with a brief description follows:

1. antibody dependant cell mediated cytotoxicity (ADCC): ADCC is a mechanism whereby antibodies bound to viral elements on the cell surface target cells for Natural Killer (NK) cell mediated apoptosis
2. antibody dependant, cell mediated viral inhibition (ADCVMI): ADCVMI is the effect of antibody Fc mediated functions on the virus output from infected cells
3. complement mediated virolysis (CMV): Where virus is destroyed through binding of the complement component C1 to the Fc of antibody bound to virus and the subsequent formation of a membrane attack complex (MAC)
4. Opsonisation enhanced phagocytosis: the uptake and destruction of viruses by phagocytes.

Many of these mechanisms are not applicable to non-enveloped viruses, with the exception of opsonisation enhanced phagocytosis, which has been described as an important mechanism in the antibody response to FMDV and is discussed in more detail in section 1.5.2.1. Over the next few paragraphs, the described roles of non-neutralising antibodies in response to viral infections will be discussed.

Protection has been associated with non-neutralising antibodies in flavivirus infections, against both dengue virus (Henchal *et al.*, 1988) and Yellow fever virus (Schlesinger *et al.*, 1985) following passive transfer of antibodies to non-structural proteins expressed on the surface of

infected cells, but not on complete virions. The mechanism of action for these antibodies could potentially be through ADCC. Non-neutralising antibodies to vesicular stomatitis virus elicited protection via Fc mediated effector mechanisms (Lefrancois, 1984) and are also important in limiting virus spread in the early stages of lymphocytic choriomeningitis infection (Hangartner *et al.*, 2006). For sendai virus, non-neutralising antibodies may have roles in phagocytosis, ADCC and CMV (Mochizuki *et al.*, 1990). CMV has also been reported for interactions with non-neutralising antibodies with the closely related Parainfluenza virus (Vasantha *et al.*, 1988). Work conducted with the *alphavirus* prototype, Sindbis virus, demonstrated that non-neutralising antibodies were able to prevent lethal infection in the absence of neutralising antibodies through complement mediated cell lysis (Schmaljohn *et al.*, 1982). This study also implicated non-neutralising antibodies in the cross-protective immunity seen between Alphaviruses.

ADCC, ADCVI and complement mediated inactivation have all been observed as important mechanisms in the humoral response to HIV infection, with roles for non-neutralising antibodies previously described (Aasa-Chapman *et al.*, 2005; Florese *et al.*, 2009; Forthal & Moog, 2009; Hidajat *et al.*, 2009). Additionally, a subset of antibodies termed non-neutralising inhibitory antibodies have also been described to limit the replication of HIV in both macrophages and immature dendritic cells (DC's), through Fc receptor mediated opsonisation leading to enhanced phagocytosis and degradation within these cells (Holl *et al.*, 2006).

Some viruses are known to make use of antibodies produced during virus infections to gain entry to target cells or use other mechanisms for antibody dependant viral enhancement of infection. Whether this is mediated by non-neutralising antibodies or levels of antibody occupancy not sufficient to neutralise is still debatable The infection of macrophages and DC's can occur through either Fc or complement receptor mediated endocytosis and this has been demonstrated *in vitro* with a particular set of non-neutralising antibodies that appeared to aid the entry of Dengue virus into cells (Halstead & O'Rourke, 1977). A similar enhancement of infection has also been seen in

HIV (Stoiber, 2009), measles (Iankov *et al.*, 2006) and RSV in young infants (Osiowy *et al.*, 1994). Interestingly, there is an also an association between enhancement of infection by non-protective antibodies and vaccination with formalin inactivated vaccines (Ubol & Halstead, 2010). A further example of a negative role played by non-neutralising antibodies is provided by human cytomegalovirus infection, where non-neutralising antibodies can be seen competing with neutralising antibodies to bind to antigenic domain 1 in a 50/50 ratio (Speckner *et al.*, 1999).

So far, the literature on non-neutralising antibodies seems to point to a role mainly in complement mediated pathways regarding enveloped viruses. To date, apart from the work performed on FMDV (see section 1.5.2.1) no other data has been published regarding the role of non-neutralising antibodies to non-enveloped viruses.

1.5 The immune response to FMDV

1.5.1 Innate immunity.

The innate immune system has been reported to play a role in the immune response to FMDV. However this component of the host response to infection has, as yet, not received a significant amount of research. The innate response can be mediated by the complement system, by virus infected cells (through the release of cytokines) and by those cells of the immune system that can uptake and destroy the virus or stimulate the apoptosis of infected cells.

In both cattle and swine it has been demonstrated that animals administered an emergency vaccine can be protected from viral challenge, before detection of FMDV specific antibodies (Barnett *et al.*, 2002; Doel *et al.*, 1994), and type 1 interferon (IFN) has been suggested as a mechanism for controlling early stage viraemia in pigs (Nfon *et al.*, 2010). Type 1 IFN is also observed to increase in cattle during infection, although at much lower levels than in pigs (Reid *et al.*, 2011). FMDV is highly sensitive to type 1 IFNs *in vitro* (Chinsangaram et al., 2001), however, the FMDV Leader proteinase (Lpro) and most recently 3C have been shown to block the expression of type 1 IFN *in vitro* through

various mechanisms (de Los Santos *et al.*, 2007; de Los Santos *et al.*, 2006; Wang *et al.*, 2011b; Wang *et al.*, 2012a). It has been suggested that this initial down-regulation may provide the virus with enough time to replicate and disseminate effectively (Nfon *et al.*, 2010). As discussed earlier, the viral Lpro functionality is unique to the aphthovirus genome (Hinton *et al.*, 2002), deletion of which leads to attenuation both *in vitro* and *in vivo* (Brown et al., 1996). Lpro interference with host protein synthesis has been proposed as an important immune evasion technique, counteracting the innate immune response (de los Santos *et al.*, 2009)

Type 2 IFN has been described to play a role in the clearance of virus in persistently infected animals (Zhang *et al.*, 2002) and levels of type 2 IFN have also been shown to correlate well with *in vivo* protection post vaccination (Oh *et al.*, 2006). Recently it has been reported that type 3 IFN can delay the onset of disease in cattle and therefore may play a critical role in the innate immune response (Perez-Martin *et al.*, 2012). However it has been reported that the FMDV Lpro can also inhibit this pathway *in vitro* (Wang *et al.*, 2011a)

Macrophages are cells of the immune system that act as part of the interface between the innate and adaptive immune system by phagocytising pathogens and acting as antigen presenting cells. Macrophages can be infected with FMDV and have been implicated as having a role in the acute infection (Baxt & Mason, 1995), possibly as infectious carriers, disseminating virus to other parts of the body, as observed in pigs (Rigden *et al.*, 2002). However, although macrophages can be infected, infection is cleared within 10-14 hours *in vitro* (Rigden *et al.*, 2002). Macrophages are also much more likely to play a beneficial role in the immune response through opsonisation and subsequent destruction of virus, as discussed later in section 1.4.7.1.

Dendritic cells (DC) are the major group of professional antigen presenting cells providing a key interface between the innate and adaptive immune response. DC's are made up of two distinct cell types: - myeloid (moDC) and plasmocytoid (pDC). It has been demonstrated that immune complexed FMDV can infect porcine pDC's and undergo an abortive replication cycle with the

production of high levels of IFN-α (Guzylack-Piriou *et al.*, 2006). Nfon and colleagues reported that FMDV infection is associated with transient loss of pDC function accompanied by an increase in IFN-α in pigs which, as mentioned above, they suggest controls viremia prior to the antibody response (Nfon *et al.*, 2010). In cattle, the expression of high levels of type 1 IFN has also been associated with immune complexed FMDV, however unlike in pigs, it was postulated that as this response comes after the development of the antibody response it probably plays a minor role in control of infection (Reid *et al.*, 2011).

Examination of bovine moDC found that infection with immune complexed FMDV leads to productive infection with high levels of moDC death along with a reduced ability of these cells to induce FMDV-specific T-lymphocyte proliferation (Robinson *et al.*, 2011). In addition, it has been demonstrated that during acute FMDV infection in pigs, moDC's secrete interleukin (IL)-10 which leads to a reduction in T lymphocyte responses, possibly linked to the observed T lymphocyte depletion during infection in pigs (Diaz-San Segundo *et al.*, 2009; Diaz-San Segundo *et al.*, 2006). In contrast, cattle produce much less IL-10 during infection and no lymphopenia is observed (Windsor *et al.*, 2011).

Very little is published with respect to the role of natural killer (NK) cells in the immune response to FMDV, primarily because NK cells have only recently been identified and characterised in ruminants (Storset *et al.*, 2004). However, it has been reported that FMDV can interfere with the cytotoxic activity of pig NK cells (Toka *et al.*, 2009). The recent characterisation of NK cells in ruminants and the availability of antibodies against these cells should lead to more detailed studies of the function of NK cells in FMDV pathogenesis (Storset *et al.*, 2004).

Gamma delta (γδ) T cells can account for up to 75% of the lymphocyte population in calves and 10% in adult ruminants (Wyatt *et al.*, 1994), with a comparable number to ruminants also found in pigs (Takamatsu *et al.*, 2006). Although their precise role in the immune system remains unclear, a wide range of functions have been identified in which these cells participate. In cattle, is has been

suggested that these cells have a role as a link between the innate and adaptive immune response to intracellular infections (Pollock & Welsh, 2002). As with NK cells, very little is known about the role of these cells in the immune response to FMDV, although antigen specific proliferation of this cell type has been reported in porcine cells *in vitro* (Takamatsu et al., 2006)

1.5.2 Adaptive immunity.

1.5.2.1 The humoral response

The humoral immune response is the dominant factor in the host response to FMDV infection (Alexandersen *et al.*, 2003; Juleff *et al.*, 2009) and generally levels of neutralising antibody correlate well with protection observed *in vivo* (Pay & Hingley, 1987; Van Bekkum, 1969), even if this is not always the case (McCullough *et al.*, 1992).

In infected cattle, IgA and IgM can be observed around 3-4 days post infection, followed by IgG 1 and 2, which can be detected at 4-5 days post infection. This can coincide with the emergence of neutralising antibody. IgM and IgA titres peak at 8 days post infection before levelling off at day 15 with the IgG antibodies reaching a plateau at around 17 days (Abu Elzein & Crowther, 1981). Antibody is produced against both the capsid and non-structural proteins. As discussed previously, the antibody response to infection has been reported to be T-cell independent in both mice (Borca *et al.*, 1986; Ostrowski *et al.*, 2007) and cattle (Juleff *et al.*, 2009). Nevertheless, in cattle, T-cell independent antibodies to the immunogenic GH loop (antigenic site 1- See section 1.8) were not reported and it was proposed that these are T cell dependant. This T cell independent response in mice has been reported to come about through the interaction of DC's with splenic B lymphocytes expressing CD9, modulated by the cytokines IL-6 and IL-10. A T dependant response may be more important in stimulating an effective antibody response to vaccination (Glass & Millar, 1994).

The antibody response is effective at clearing the virus from circulation quickly, followed by clearance from the epithelium of virus within 10-14 days (Oliver *et al.*, 1988). However, the antibody

response is inefficient at clearing virus from the oesophageal-pharyngeal tract of cattle and sheep and it has been shown that virus can persist in this region even in the presence of high levels of circulating neutralising antibodies (Salt, 1993). It has been demonstrated that immunity to infection can last over a prolonged period of many years (Cunliffe, 1964; Garland, 1974).

As discussed, the levels of neutralising antibody correlate well with the protection observed *in vivo*. Although means of direct neutralisation of FMDV have been reported, for example in altering the capsid conformation (McCullough *et al.*, 1987), it has been suggested that the mechanisms underlying the correlation are not fully understood.

It has previously been demonstrated that antibody is able to passively protect mice *in-vivo* at much lower concentrations than those that are required for *in-vitro* neutralisation. When *in vivo* opsonisation to clear virus was removed by either silica treatment of mice or pepsin treatment to remove the Fc region of antibodies, this enhanced *in-vivo* protection was abrogated (McCullough *et al.*, 1986). It has also been demonstrated that FMDV can effectively infect cells expressing Fc receptors (Mason *et al.*, 1993). A recent study also implicated non-neutralising antibodies (or at least sub-neutralising virus titres) in the early response to an FMDV vaccination in mice through opsonisation via macrophages (Quattrocchi *et al.*, 2011). These antibodies again appeared to have been generated in a T cell independent manner. It has also been demonstrated *in vitro* that antigen/antibody complexes enhanced the ability of FcR+ cells to phagocytise FMDV over just virus alone (McCullough *et al.*, 1988). Further to this, a study using llama single domain antibodies to passively protect Guinea pigs identified a lack of correlation between *in vivo* protection and *in vitro* neutralisation titres and concluded that this was down to opsino-phagocytosis (Harmsen *et al.*, 2007). Macropinocytosis has also been shown to be the most effective route of entry of FMDV into macrophages (Baxt & Mason, 1995; Rigden *et al.*, 2002), demonstrating the efficiency of virus entry through Fc mediated pathways.

The role suggested for opsonisation places an increased emphasis on understanding the role of the whole antibody response to FMDV as those antibodies that opsonise may not necessarily be those that can also neutralise. Recently, it has also been reported that opsonising antibody titres for FMDV correlate well with neutralising antibody titres and that these antibodies appear to be more broadly cross reactive (Lannes *et al.*, 2012). Understanding whether the correlation observed between the neutralisation and opsonisation titres is due to antibody binding to the same regions of the virus capsid is an important question and suggests that knowledge of neutralizing antigenic sites alone, as discussed in section 1.8 may not give a complete picture of the overall antigenicity of FMDV.

1.5.2.2 The adaptive cellular response.

T-cell responses to FMDV are still relatively poorly understood, although FMDV specific CD4+ T-cell Proliferative responses have been observed following vaccination (Sharma *et al.*, 1985; Windsor *et al.*, 2011) and partial protection has been observed in pigs post vaccination with a recombinant adenovirus vector expressing the FMDV P1, where a T-cell response but no antibodies were observed (Sanz-Parra *et al.*, 1999). However, no FMDV specific T-cell proliferation has been observed after natural infection, even after 32 days (Windsor *et al.*, 2011). In contrast to the capsid epitopes identified for neutralising antibodies, T-cell epitopes located on VP3 can be cross-reactive between serotypes of FMDV (Collen & Doel, 1990). T-cell epitopes to non-structural proteins are also cross-serotype reactive and may enhance vaccine efficacy (Blanco *et al.*, 2001; Glass & Millar, 1994). A role for T-cell dependant antibody responses have also been described (Collen *et al.*, 1989). However as already discussed, effective T-cell independant antibody responses appear to be the main effector mechanism for the antibody response and the T-cell responses in pigs (but not cattle) are supressed upon infection by IL10, which along with the lack of a proliferative T-cell response post infection suggests a limited role for T cells in natural infection. CD4+ T-cells may play a more important role in vaccine induced immunity and in the memory response to FMDV, and memory T-cells have been detected up to 20 months post vaccination (Collen & Doel, 1990).

The role of cytotoxic CD8+ T-cells in FMDV immunity is also poorly understood. Proliferation of the CD8+ T-cells has been observed, starting at 10-14 days post vaccination and continuing for 3-4 weeks (Childerstone *et al.*, 1999) and recently an MHC restricted CD8+ T-cell response has been demonstrated in animals vaccinated with whole inactivated virus (Guzman *et al.*, 2008). FMDV restricted CD8+ T-cell induced killing has also been shown (Patch *et al.*, 2011). However, the role of CD8+ immune responses could be limited due to the rapid decrease in MHC 1 expression that occurs with FMDV infection. This has been demonstrated *in vitro* with MHC-1 expression down to about 53% that of normal at 6 hours post infection (Sanz-Parra *et al.*, 1998).

1.6 Genetic and antigenic variation of viruses.

1.6.1 Genetic variation

Many viruses, and in particular those with RNA genomes exhibit extensive genetic and antigenic variability. In RNA viruses, this is due primarily to the high error rate of the RNA dependent RNA polymerases these viruses use to copy their genomes. Typically, a mutation is introduced into the genome at a rate of around 10^{-4}-10^{-6} per nucleotide incorporated (Sanjuan *et al.*, 2010). These high error rates, along with the large number of virus progeny generated from an infected cell give rise to a diverse and dynamic virus population called a quasispecies (Domingo, 2010). Viral quasispecies have been defined as:

"a cloud of diverse variants that are genetically linked through mutation, interact cooperatively on a functional level, and collectively contribute to the characteristics of the population"(Lauring & Andino, 2010).

Viral quasispecies enable RNA viruses to evolve rapidly in response to selective pressures, such as the host immune response, as has been seen with FMDV (Mateu *et al.*, 1989) and other RNA viruses which produce large numbers of antigenic variants (Holland *et al.*, 1992). Changes to the Poliovirus polymerase that increase the fidelity, reducing the number of variants has been shown to lead to an attenuated phenotype (Vignuzzi *et al.*, 2006) and suggests an evolutionary advantage for diversity in virus pathogenesis. However, the level of error that can be tolerated in any viral

population is limited (defined as the error threshold) beyond which error catastrophe occurs, resulting in virus extinction (Domingo *et al.*, 2005).

Another method of genetic variation used by viruses is recombination, where chimeric genomes are created. Homologous recombination is where part of the genome of a donor sequence replaces that of an acceptor sequence at a site that is homologous between the sequences either by a replicative (the switching of the polymerase between two RNA templates) or non-replicative (the covalent joining of two pieces of RNA) model. This form of recombination has been documented in many Picornaviruses including Poliovirus, Rhinovirus, other Enteroviruses and FMDV (Haydon *et al.*, 2004; Huang *et al.*, 2009; King *et al.*, 1982; Pringle, 1965; Santti *et al.*, 1999; Savolainen-Kopra & Blomqvist, 2010).

In addition to homologous recombination, viruses with segmented genomes can also undergo reassortment of their genes during a co-infection between two different strains by swapping gene segments. Recently, it has been suggested that the emerging Schmallenberg virus is derived from a reassortment between two other members of the Orthobunyavirus genus (Yanase *et al.*, 2012). Further examples of gene reassortment can be seen in the reoviridae, notably Rotaviruses and bluetongue viruses (He *et al.*, 2010; Kirkwood, 2010) and Influenza viruses (Greenbaum *et al.*, 2012). Such reassortment events are linked to the evolution of novel virus serotypes and can lead to antigenic shift, which will be discussed in more detail below.

1.6.2 Antigenic variation.

Viruses undergo two types of antigenic variation: Antigenic drift involves minor antigenic changes which are caused by the accumulation of amino acid changes over time. Antigenic shift is a process whereby large changes in the antigenicity of a virus occur abruptly, primarily due to recombination or reassortment events. These events are important in the formation of new pandemic strains of influenza, as observed recently in the 2009 swine Influenza pandemic H1N1

virus, which contained genome segments from strains derived from humans, birds and pigs (Shapshak *et al.*, 2011).

Antigenic drift, through the generation of antigenically diverse populations, provides challenges for the control of viral disease through vaccination. This can be observed in FMD, where vaccines are poorly cross-reactive (see section 1.9.1) and in several other diseases including Influenza, where the vaccine strain for seasonal flu must be updated regularly (Yewdell, 2011) and for HIV, where the high levels of heterogeneity have, in a small part, confounded attempts at vaccine development (Saunders *et al.*, 2012). In HIV, the level of genetic variation of the env gene has been reported to be the same in an individual 6 years post infection as the level of genetic variation in the Influenza HA gene seen globally over a year (Ndung'u & Weiss, 2012). Antigenic drift has been observed within individuals infected with HIV, with the substitution rate at antigenic sites increasing with the efficiency of the immune response (Sasaki & Haraguchi, 2000). Similar reports of antigenic drift within individuals have been reported in influenza, with the level of antigenic change within an individual was equivalent to that seen over the population over the same amount of time (McMinn *et al.*, 1999). These within-host demonstrations provide an excellent example of antigenic drift where genetic and antigenic variation occurs in response to the pressure exerted by humoral immunity.

The antigenic drift of influenza has also been observed globally over previous decades, with changes at four known antigenic sites linked to disease incidence (Wiley *et al.*, 1981). Through the use of antigenic cartography a very close correlation between the level of genetic variation and the antigenic drift was observed (Smith *et al.*, 2004). However, it was noted that although the genetic change was continuous, the antigenic drift was more punctuated into clusters, which reflects the idea that several genetic changes take place away from sites of antigenic importance and it is only the accumulation of changes within antigenic sites that cause antigenic drift. For example, the same single amino acid substitution was observed as key for cluster transition on two separate occasions. Upon site directed mutagenesis, this single mutation was noted to reduce the binding of the serum

by 80%. The idea of single amino acid substitutions playing a large role in the drift of Influenza has been observed in single site mutations within the immunodominant antigenic site (Sb) of haemagglutinin, where single mutations were noted to reduce the binding of polyclonal serum by greater than 50%. These mutations also coincided with changes in receptor binding avidity and it has been suggested that this may also drive virus antigenic drift (Hensley *et al.*, 2009). The canyon hypothesis, initially proposed for Rhinoviruses, suggested that Picornavirus receptor binding sites are location within a deep canyon in order to conserve key residues for cellular attachment in the face of antigenic selection pressures, with the residues surrounding the canyon able to change rapidly in response to these selective pressures (Rossmann, 1989). However, contrary to this finding the FMDV integrin binding RGD motif is located on the most exposed region of the capsid, the extended GH loop. FMDV appears to utilise a hyper-variable region (Fox *et al.*, 1989) on the GH loop, just prior to this RGD motif to effectively camouflage the receptor binding site. This hyper-variable region is also antigenic on all serotypes of FMDV (section 1.8)

In FMDV, it has been observed that phylogenically distantly-related viruses can be antigenically very closely related, with some viruses even exhibiting higher antibody binding affinity than the homologous virus to which serum had been raised (Barnett *et al.*, 2001). These results contrast with other studies where very few mutations have been demonstrated to have a large effect on virus antigenicity. One study on serotype O FMDV demonstrated that a five-site antigenic mutant with only a 1.17% percentage nucleotide difference was able to completely escape neutralisation by cattle antiserum elicited by both infection and vaccinaton (Crowther *et al.*, 1993b). Moreover, this paper also identified that in one animal, a single amino acid substitution at antigenic site 5 was enough to escape neutralisation, with a reduction in titre of over 2 $\log_{10}$/ml reported. A further study with serotype O demonstrated that two viruses ostensibly called O Lausanne had very different reactivity profiles (Rweyemamu & Ouldridge, 1982) and studies on serotype C found that, as opposed to the general accumulation of amino acid residue changes it was the fluctuations between a small number of residues that drove antigenic diversity (Martinez *et al.*, 1992). It has also been

demonstrated that changes in a small number of residues can amplify antigenic variation by rearranging the GH loop conformation (Parry *et al.*, 1990). The above evidence suggests that as in influenza, the antigenic drift of FMDV does not occur through the gradual accumulation of amino acid changes across the entire surface of the virus but through changes in a small number of immunodominant residues within antigenic sites. It has previously been demonstrated that only a small number of amino acid side chains are involved in binding affinity of an interaction between epitopes and paratopes at so called 'hot spots' on a protein's surface (Clackson & Wells, 1995). However, unlike influenza, the level of correlation between the accumulation of genetic changes and virus antigenicity is not observed at the level of the capsid sequence or at the antigenic sites, meaning that any correlations are likely to be more difficult to identify. However, recent work conducted with the SAT viruses did identify sub sequences across the capsid that correlated with changes observed in sero-reactivity (Reeve *et al.*, 2010).

It is interesting that some viruses exhibit large amounts of antigenic heterogeneity while other similar viruses do not. For example, Poliovirus has only 3 serotypes while Rhinovirus has over 100 different serotypes, yet these two viruses share a very similar genome and capsid structure. Influenza exhibits large antigenic variability, while parainfluenza, which is similar in many ways to Influenza, does not (Yewdell, 2011).

1.7 Epitope-paratope interactions.

The interaction of immunoglobulins with viruses and other pathogens has been extensively studied. Immunoglobulin specificity comes from the hypervariable regions of the Fab region. These are termed complementarity determining regions (CDR's) and comprise part of the antigen binding site. There are 6 CDR's on each Fab region (Polonelli *et al.*, 2008), with 3 on each variable domain generally averaging between 10-17 amino acids long, although the central CDR3 can vary greatly in length from 4-26 amino acids (Padlan, 1994; Padlan *et al.*, 1995). Recent evidence has demonstrated that up to 20% of residues that make up an antigen binding site are located outside these CDRs

(Kunik *et al.*, 2012). However only a small subset of these residues are actually likely to make up the functional epitope (Clackson & Wells, 1995; Cunningham & Wells, 1993). The entire antigen binding site forms the paratope that interacts with the corresponding epitope of an antigen.

The terms epitope and paratope were first used in 1960, with epitopes being defined as "*surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas"* and paratope meaning *"combining sites, reactive segments, complementary cavities, specific receptors"* (Jerne, 1960). For Poliovirus, an antigenic site has been defined as a region of the capsid composed of amino acids that induces and binds to immunoglobulin, while an epitope is the conformation of amino acids within an antigenic site that will recognise and bind immunoglobulin (Wimmer *et al.*, 1984).

Spatial adaptive complementarity, hydrogen bonds, van der Vaals forces and electrostatic interactions, but not covalent interactions all contribute to binding affinity (the strength of the interaction between an individual Fab region and the epitope) of an immunoglobulin to an epitope (Braden & Poljak, 1995). This study also identified many water molecules playing a role in the binding of epitopes and paratopes. Induced fit (conformational alterations that occur during binding) of the epitope and the paratope may also lead to a stronger interaction between the epitope and paratope (Van Regenmortel, 1996). The affinity of this binding directly influences the effectiveness of an immunoglobulin as it correlates with the equilibrium constant of antigen antibody interaction. That is, the higher the binding affinity, the longer virus will remain in complex with the antibody before dissociating and hence the less free antigen remains. Another important measure of binding is the avidity of an immunoglobulin, which could be expressed as the sum of the affinities of each Fab region of an immunoglobulin.

Epitopes have traditionally been considered as either continuous (sequential) or discontinuous (conformational). Continuous epitopes are made up of a linear arrangement of peptides on a sequence while discontinuous epitopes are comprised of amino acid residues of distant

parts of the sequence that are brought together by protein folding (Atassi & Smith, 1978). Discontinuous epitopes are believed to comprise over 90% of the total number of epitopes (Van Regenmortel, 1996). It has been argued that continuous epitopes are not completely independent of conformation (Van Regenmortel, 2006), usually forming part of a larger discontinuous epitope (Van Regenmortel, 1989) and that the term epitope should be reserved for regions that are recognised by antibody towards the natively folded protein (Laver *et al.*, 1990).

There have been attempts to look at what makes a good epitope. Not surprisingly there has been shown to be a good correlation between surface exposure and the location of B cell epitopes (Novotny *et al.*, 1986; Thornton *et al.*, 1986) and it has been reported that there are correlations between hydrophilicity and backbone flexibility, among others, and antigenicity (Hopp & Woods, 1981; Westhof *et al.*, 1984). Through analysis of 53 epitope-paratope structures it was observed that 75% of epitopes covered an area made up of between 15-25 amino acids and were mainly located on convex protruding loop structures (Rubinstein *et al.*, 2008). This paper also noted that the amino acids tyrosine and tryptophan were found in a significantly higher frequency than in other regions. Epitopes were also found to have a higher proportion of polar and charged amino acids than non-epitope surfaces and several amino acid pairs are also overrepresented, with each pair identified containing either tyrosine or proline. This study contrasts slightly with other findings using a dataset of 76 epitope/paratope structures that found asparagine and proline, in addition to charged amino acids to be overrepresented in epitopes (Haste Andersen *et al.*, 2006). This study did find a similar number of amino acids acids making up the binding region (14-19 for 60% of interactions). The amino acid composition of paratopes has also been studied and it has been noted that serine and tyrosine residues are overrepresented, along with other aromatic residues (Lea & Stuart, 1995). In the case of epitope/paratope interactions it has been observed that polar and aromatic amino acids contribute by far the greatest amount of interaction energy (Jackson, 1999). It should also be noted that for FMDV, allosteric changes in sequence can affect the structure of an epitope (Parry *et al.*, 1990).

Immunoglobulins have historically been associated with having unique specificity to the epitope the immunoglobulin was generated against. However it has been proposed that Immunoglobulins can have multiple specificities and are generally cross-reactive (Van Regenmortel, 2006). Usually, a paratope will bind to the homologous epitope it was raised against with a higher affinity than to an heterologous epitope, although it is not uncommon for a paratope to bind more strongly to other related epitopes, a phenomenon known as heterospecificity or heteroclitic activity (Van Regenmortel, 1998). A heteroclitic immunoglobulin is one that binds antigens not used to generate antiserum as well as or better than the original antigen (Chitarra *et al.*, 1993). A recent study on a heteroclitic antibody found that this antibody bound to two structures with little or no predicted structural similarity and suggested that an increase in antibody avidity was involved in this activity by binding repeated oligomers within the variable regions of immunoglobulin (Gjelstrup *et al.*, 2011); thus, if true, this may have implications for heteroclitic activity towards viruses where the repeated nature of the capsid structure may induce such responses. Other reports have suggested that these reactions have been suggested to be particularly significant when the two antigens are closely related (Underwood, 1985). Both of these findings may explain the high levels of cross reactivity observed with FMDV where in some case there is a large phylogenetic distance between viruses (see section 1.5.2.). A further item to note regarding the cross reactive binding of paratopes is mimotopes, which mimic the target of the paratope but may use different amino acids than those that comprise the original epitope (Geysen *et al.*, 1986). Again this could be explained by the limited number of side chains from an epitope that are actually involved in this interaction (Clackson & Wells, 1995), meaning that only these side chains are in the right configuration to bind antibody.

1.8 Antigenicity of serotype O FMDV.

As previously discussed, the antibody response to FMDV has been shown to be a dominant mechanism for protection *in vivo* for the control of FMDV and a good correlation between protection *in vivo* and the level of neutralising/opsonising antibodies has been reported (see section 1.5.2.1). In addition, the antigenic drift that is seen in FMDV appears to be modulated by a few changes in

immunodominant locations (see section 1.6.2). Over the preceding decades, several studies have been conducted to identify these antigenic sites on the different serotypes of FMDV, mainly using monoclonal antibody escape mutants to define regions of capsid that bind virus neutralising antibodies *in vitro*.

For serotype O, five antigenic sites have been described (see figure 1.7) (Crowther *et al.*, 1993c; Kitson *et al.*, 1990). The first is believed to be a linear epitope within the G-H loop (positions 141-160) and C terminus of VP1, with the residues critical for monoclonal antibody binding being positions 144, 148, 154 and 208. It is not surprising that the G-H loop should be a critical site bearing in mind its role in host cell attachment (Fox *et al.*, 1989). The C terminus forms the conformational epitope with the G-H loop of the adjacent protomer.

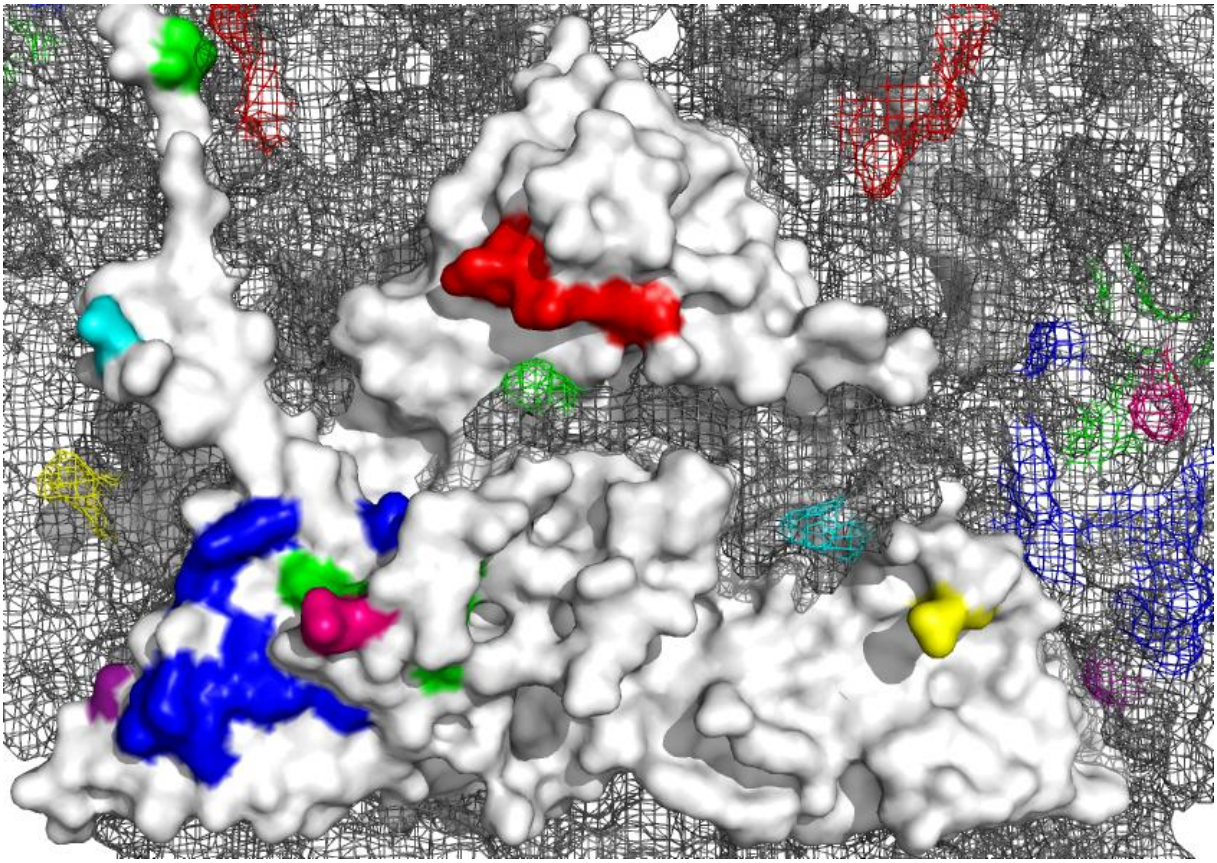


Figure 1.7: FMDV serotype O antigenic sites: A picture showing the molecular surface of the capsid, with a central protomer (in a reduced conformation with the GH loop present) represented as a solid and surrounding protomers as a mesh. Antigenic site one is coloured green, site two is blue, site three is red, site four is yellow and site five is pink. The additional residues identified from other studies are coloured purple (VP2 188) and teal (VP1 198)

Site 2 is located on VP2 at residues, 70-73, 75, 77 and 131. Residues 79 and 134 have also been proposed as making up part of this site (Mahapatra *et al.*, 2008). Site 3 resides on the B-C loop of VP1 at residues 43-45 and 48. Both sites 2 and 3 are located in regions where single amino acid substitutions in the area underlying the GH loop can result in downstream impacts on G-H loop conformation, for example residues at positions 82 of VP2 and residues 43, 48 or 59 of VP 1(Curry *et al.*, 1996; Parry *et al.*, 1990). So it may be that these sites operate in escape from neutralisation by altering the conformation of the GH loop.

Site four is located at position 58 of VP3. This site is very close to the amino acid changes associated with heparan sulphate binding of FMDV *in vitro* and this region has also been described as having antigenic features. When the structures of two serotype O viruses containing substitutions at VP3 56 and 60 were compared it was noted that large perturbations occurred on the surface of the virus that interfered with binding of a site 4 mAb (Lea *et al.*, 1995). VP3 56 was also implicated in decreasing mAb binding in several other studies (Kitson *et al.*, 1990; Sa-Carvalho *et al.*, 1997). Another study (Jensen & Moore, 1993) reported a drop in neutralisation cross reactivity of 0.64 $\log_{10}$ using bovine convalescent sera between a virus with the single mutation at VP3 56 from histidine to arginine.

A fifth site that overlaps with site one at residue 149 has also been described. This site does not interact with site 1 mAb's and it is believed access to this site is dependent on the conformation of the GH loop of VP1 (Crowther *et al.*, 1993c). Recently, a similar epitope has been described for serotype A, which is again dependant on the GH loop conformation (Mahapatra *et al.*, 2011).

Residues away from these antigenic sites have also been reported to have a role in escape from mAb neutralisation for serotype O FMDV. VP1 198 was described on O Manisa (Aktas & Samuel, 2000), this is also an epitope on serotype SAT-1, which although numbered as VP1 204 occupies the same location on the structure (Grazioli, 2006). Residue VP2 188 was identified using bovine mAb's

(Barnett *et al.*, 1998) and the authors suggested that this residue may span both antigenic sites two and four.

The relative importance of these sites within natural hosts has been observed to some extent. In cattle, it has been reported through the use of virus neutralisation and enzyme linked immunosorbent assays that antigenic site two is immunodominant, in some cattle 60% of the polyclonal neutralising response were directed to this site (Samuel, 1997). These findings have subsequently been confirmed for cattle and the immunodominance of site 2 was also identified in pigs and sheep (Mahapatra *et al.*, 2012). However, another study in pigs concluded that none of the antigenic sites were immuno-dominant (Aggarwal & Barnett, 2002). This study also found that pigs did not produce a significant immune response to the C terminus of VP1 that comprises part of site one.

Sites one, two and four of serotype O are analogous to sites identified by neutralising monoclonal antibodies on serotypes A, C, SAT-2 (only site 1) and Asia 1 (Baxt *et al.*, 1989; Bolwell *et al.*, 1989; Crowther *et al.*, 1993a; Fry *et al.*, 2005b; Grazioli *et al.*, 2004; Lea *et al.*, 1994; Saiz *et al.*, 1991; Thomas *et al.*, 1988). Site 3 is also a site of potential antigenic importance in the Asia-1, SAT-1 and SAT-2 serotypes (Grazioli *et al.*, 2004).

The locations of the neutralising epitopes of serotype O FMDV are also often implicated as being sites for neutralising epitopes on many other *Picornavirus*es. Site 1 on the G-H loop of VP1 is in an equivalent position as an epitope of Poliovirus 1 and 2 and Hepatitis A virus (Minor, 1986; Ping & Lemon, 1992). Equine Rhinitis A virus also has an epitope that is architecturally very similar to antigenic site 1 of FMDV type O and includes the C terminus. However, the other part of the epitope resides on the E-F loop of VP1 (Kriegshauser *et al.*, 2003). The B-C loop of VP1 (site 3) is antigenic in all three serotypes of Poliovirus, in both the major (HRV 14) and minor (HRV 2) group Rhinoviruses and in Mengovirus (Appleyard *et al.*, 1990; Boege *et al.*, 1991; Minor, 1986; Sherry *et al.*, 1986). Site 4 on the β-B Knob of VP3 is an antigen on all Poliovirus serotypes, minor group Rhinoviruses,

Mengovirus, Hepatitis A virus and part of a conformational epitope of Coxsackie B virus. The second location of the conformational epitope of Coxsackie B viruses is to be found on the E-F loop of VP2, the same location as part of site 2 for Serotype O. This region is also immunogenic for Poliovirus, Rhinovirus and Mengovirus (Appleyard *et al.*, 1990; Minor, 1986; Sherry *et al.*, 1986).

It is evident that many of these viruses share epitope locations, usually on the loop structures linking β strands. This is not surprising, as these loops are the most exposed points on the surface of the capsid and therefore the most open to antibody interaction and have been shown to be the most likely locations of epitopes (discussed in section 1.7).

For both Polio and Rhinovirus a neutralising epitope located at the N terminal regions of both VP1 and VP4 has been described (Katpally *et al.*, 2009; Li *et al.*, 1994). Other normally internalised residues have also been shown to be immunogenic in other *Picornavirus*es (Jimenez-Clavero *et al.*, 2000; Pulli *et al.*, 1998; Sauter *et al.*, 2008). These sites are only exposed in certain conditions; this supports the idea that *Picornavirus* capsids can undergo conformational changes known as 'breathing'. Additionally, the neutralising epitope at the end of VP1 and VP4 described for Rhinoviruses (Katpally *et al.*, 2009) has been found to be pan-serotypic. These neutralising antibodies to Polio and Rhinovirus may operate by altering the conformation of the capsid and preventing the uncoating process that involves the irreversible externalisation of both VP1 and VP4 (Hogle, 2002).

Although not much work has been conducted looking for non-neutralising epitopes of FMDV there has been some progress. Studies utilising peptide scanning techniques have described a linear epitope located at the N-terminus of VP2 (Freiberg *et al.*, 2001; Rana & Bagchi, 2008; Yang *et al.*, 2007). This site could be important as it has been found that monoclonal antibodies (mAbs) raised against this site have cross-reactivity against all serotypes of FMDV (Yang *et al.*, 2007). As such, this monoclonal antibody could be utilised to develop rapid and broad spectrum diagnostic kits. Yang and colleagues also identified a further conformational epitope that is cross-reactive between serotypes (Yang *et al.*, 2007). Unfortunately the location of this epitope has yet to be defined. Additionally,

there appears to have been little in the way of research into this topic on other *Picornavirus*es. However, work with a set of Poliovirus non-neutralising antibodies has also demonstrated that in some cases these are cross-reactive between serotypes (Vrijsen *et al.*, 1984)

The concept of capsid breathing has not been demonstrated in FMDV. It would be interesting to see whether FMDV alters its conformation dependant on physiological conditions and the subsequent impact this could have on the antigenicity of the capsid. Perhaps this is an idea that could explain the inter species differences in antigen recognition seen (Aggarwal & Barnett, 2002).

The structure and neutralising epitopes of FMDV, and in particular type O, have been well characterised. However, it has been reported that there may well be other antigenic sites across the surface of the virus. Examination of a five site neutralisation escape mutant of serotype O virus in one study found residual neutralisation values were still observed with sera developed against the parental virus (Dunn *et al.*, 1998), contrasting with earlier results that demonstrated complete escape from a polyclonal serum response by the five site mutant (Crowther *et al.*, 1993b). However, the study by Dunn and colleagues also noted from passive transfer studies that *in vivo* protection was observed in the absence of a response to the known antigenic sites. This suggests that there are several regions of the capsid away from the known neutralising antigenic sites that are playing a role in the antigenicity of FMDV. The identification of these sites may be important in increasing cross reacitivity. For example it has been shown, in serotype C, that removing two immunodominant antigenic sites increased the cross-reactivity of serum in mice (Frimann *et al.*, 2007).

1.9 Novel methods for identifying epitopes

Our understanding of the antigenicity of FMDV and the majority of other viruses comes from mapping sequence changes that allow the virus to “escape” neutralisation by a monoclonal antibody or from peptide scanning. However, as previously discussed this does not necessarily present a complete picture of the epitopes that stimulate a protective B cell response in FMDV.

Novel methods have recently been developed to correlate amino acid changes on the capsid and serological changes for multiple virus pairs for FMDV (Maree *et al.*, 2011b; Reeve *et al.*, 2010) in order to try and identify epitopes, with some promising results. However, these methods rely on amino acid variation within the capsid sequences to identify epitopes, thereby negating any possibility of identifying epitopes that are completely conserved. Such epitopes, similar to those identified as targets for broadly neutralising antibodies against both Influenza and HIV are particularly interesting as a potential route to more broadly reactive vaccines (Burton *et al.*, 2012; Corti *et al.*, 2011).

Other computational methods that have been used in the past to try and identify epitopes have relied on the analysis of sequence data alone. Several methods have been described that utilize various sequence scoring algorithms to predict novel epitopes (Jameson & Wolf, 1988; Odorico & Pellequer, 2003). However, an evaluation of the predictive power of these programs suggested that none could reliably determine epitopes (Blythe & Flower, 2005), which is perhaps not a surprise as these programs would have no way of determining solvent exposure. However, it has been suggested that using a consensus of the results from B cell epitope prediction programs may increase confidence in the predictions made (Yang & Yu, 2009). In addition, these programs are limited to predicting continuous epitopes.

Recently, several web based servers (Haste Andersen *et al.*, 2006; Liang *et al.*, 2010; Ponomarenko *et al.*, 2008; Rubinstein *et al.*, 2009; Sun *et al.*, 2009; Sweredoski & Baldi, 2008) have become available that utilize both structural and sequence information to identify conformational epitopes on the surface of a protein. As these servers use a single set of structural and sequence data they can, in principle, detect epitopes that are completely conserved in sequence both within, and between serotypes. The algorithms assign each residue a score based on the likelihood of it forming part of a conformational epitope. The performance of the algorithm is then tested using databases of known epitopes from available structural data (i.e. the Epitome database-

http://www.rostlab.org/services/epitome/). These methods have each reported much improved predictive power than those based on sequence data alone, however, none of the programs have been tested for their performance in identifying virus epitopes.

1.10 Current FMDV Vaccines and vaccine selection.

1.10.1 Current vaccines.

Currently FMD vaccines consist of purified whole virus preparations that are chemically inactivated. The high level of antigenic variation in FMDV means that currently there is no universal vaccine against all serotypes of virus. Indeed, current vaccines fail to cross-protect even between some strains of the same serotype (Paton *et al.*, 2005). However, it is worth noting that although serotype O is the most prevalent it is not the most antigenically diverse of the serotypes (Doel, 2003). Vaccines currently consist of varying formulations of inactivated viruses of different serotypes. Unfortunately, unlike the broadening of protection that has been witnessed following multiple challenges (Cottral, 1972b), vaccination with many different vaccine serotypes has no effect of broadening the protection conferred by each vaccine individually (Black *et al.*, 1986).

Current vaccines have been used successfully to aid in the eradication of the virus from many areas of the globe, including Europe and parts of South America (Sobrino *et al.*, 2001). However, these vaccines require the growth of large quantities of virus (requiring high containment facilities) and must be adapted constantly to keep the vaccine strains current to those in circulation. These vaccines also only provide a short lived protection (4-6 months after one dose), although antibodies have been detected in multiply vaccinated animals over 6 years after the termination of vaccination in Europe (Remond *et al.*, 1998). These vaccines also require cold chain delivery, which makes them very expensive. Additionally, studies have demonstrated that the virus can persist in the epithelium of the pharynx in over 50% of cattle exposed to the virus, even in immunised animals (Kitching *et al.*, 2006).

The 2001 outbreak in the UK and the Netherlands brought about a change in the policy of the EU and it is now possible for a country to be declared free from disease 6 months after an outbreak that has utilised a vaccinate-to-live policy (EU directive 2003/85/EC). This, taken with development of non-structural protein ELISAs that are able to determine the difference between infected and vaccinated cattle (Paton *et al.*, 2006) means that it may become the favoured policy to utilise current vaccines as emergency vaccines. This could potentially save a country hit by infection billions of pounds sterling and reduce the wholesale slaughter of animals

Ideally, future vaccines should aim to fulfil a wide range of criteria, being able to provide a universal protection against all strains of FMD with long lasting immunity. Additionally, these vaccines should also be cheap to produce and delivered in a cost effective manner. However any vaccine that could improve upon those currently available would be advantageous.

1.10.2 Vaccine selection methods

Due to the emergence of novel subtypes and recombinant viruses that are inadequately covered by vaccines, like the emergence of a new serotype A lineage in 2003 (Knowles *et al.*, 2009) or the recombinant Asia-1/A virus, with the external capsid protiens and 2A from Asia-1, with remainder of the genome from serotype A, currently circulating in Pakistan and Afganistan (Jamal *et al.*, 2011) for which current vaccines deployed for use in the region were found to be ineffective, continued surveillance of circulating viruses must be maintained to ensure that effective vaccine strains are available to control new outbreaks.

To ensure that the most appropriate vaccine is used during an outbreak, a representative strain from the outbreak is submitted for vaccine matching, often to the World Reference Laboratory (WRL), Pirbright, UK. Currently two vaccine matching tests that are recommended by the office international des epizooties (OIE), the Virus neutralisation test (VNT) and liquid phase blocking ELISA (LPBE) are carried out at the WRL. The virus neutralisation test, which measures the neutralising component of the antibody response, was the first to be developed (Rweyemamu *et al.*, 1978)

followed by the LPBE, which measures the response of the whole antibody component (both neutralising and non-neutralising), the serum titres of which were found to correlate well with the results for the VNT and be more reproducible (Hamblin *et al.*, 1986a; Hamblin *et al.*, 1986b; Hamblin *et al.*, 1987; Kitching *et al.*, 2006; Van Maanen & Terpstra, 1989). Recent evidence has suggested that the VNT is the more reliable method for determining vaccine match based on comparisons with *in vivo* studies (Mattion *et al.*, 2009; Robiolo *et al.*, 2010). However, the results from different laboratories can vary markedly (Barnett *et al.*, 2003). It has also been reported that the vaccine serum titre can have an effect on the reliability of these *in vitro* tests and should be taken into consideration (Mattion *et al.*, 2009).

Vaccine match is evaluated by comparison of the serum titre of a field strain to that of the vaccine strain from which the serum was prepared. An r_1 value is a reciprocal value determined by simply dividing the serum titre of the field strain by that of the vaccine strain. A vaccine is considered an appropriate match if the r_1 value is greater than 0.3 in the VNT, depending on the number of repeats (Rweyemamu, 1983, 1984). In the LPBE, an r_1 value of 0.4 is considered indicative of a good vaccine match with an r_1 value between 0.2 and 0.4 showing significant differences from the vaccine strain but with possibly a good enough level of cross protection depending on the potency of the vaccine (Ferris & Donaldson, 1992).

As mentioned above, a link with vaccine efficacy and the potency of vaccine has been reported, although there are some contradictory reports on the issue. One study conducted on serotype A FMDV demonstrated that, by increasing the potency of the vaccines (which normally contain between 3-6 times the amount that gives a PD_{50} of one), the range of protection elicited by vaccination can be increased, with vaccines containing 32 times (or greater) the level of antigen associated with a single 50% protective dose (PD_{50}) against the homologous virus preventing virus challenge in all animals tested with r_1 values as low as 0.04 in the VNT (Brehm *et al.*, 2008). This study also concluded that there was a clear link between the levels of neutralising antibody and the

protection observed. A study conducted on the O serotype came to similar conclusions and suggested that using a high potency vaccine in all circumstances was more appropriate than using closely related vaccines identified through vaccine matching for outbreak use (Barteling, 1996). The reliability of the matching tests have also been found to be not as accurate when using serum of low titres (Mattion *et al.*, 2009). However, other reports appear to contradict these findings. A study on cattle using a heterologous challenge model for serotype O found that several animals were not protected against challenge with a slightly heterologous O Campos virus at a PD_{50} of 28.78 and 9.44 against the homologous O Manisa virus, even though an r_1 value indicative of protection (0.62 and 0.64 in the VNT) was reported (Nagendrakumar *et al.*, 2011). The above investigations indicate that although the *in vitro* tests deployed to predict an effective vaccine match are generally a good indicator of a vaccine match, the results obtained can, in some instances, lead to the use of ineffective or inappropriate vaccines. This places an increased emphasis on finding a vaccine match that is as close as possible to the heterologous strain involved in an outbreak, as although high potency vaccines may work more broadly in some instances, the chance of a poor choice leading to severe economic consequences cannot be ruled out.

The methods for vaccine matching described above are currently the gold standard tests for FMDV used by the FMD World Reference Laboratory, Pibright, UK and, in addition to the expected percentage of protection test, make up the methods currently recommended by the World organisation for animal health (OIE, 2008). However, these serological tests can be expensive, time consuming and are not always reliable, which is not ideal in a situation where a rapid response and deployment of an appropriate vaccine is needed. Recent work has shown that sequencing and serological data can be used to build a model that predicts vaccine match by quantifying the importance of amino acid changes at specific capsid regions that are implicated in the loss in cross-reactivity. The final model was able to predict vaccine match better than serology (specifically VNT data) for the SAT-1 serotype (Reeve *et al.*, 2010) and it would be interesting to see if this approach can be applied to other serotypes of FMDV.

1.11 Objectives of thesis

The overall goal of this study is to increase our understanding of the antigenicity of serotype O FMD by identifying novel epitopes and to evaluate the dominance of these new and already recognised epitopes. It is also hoped that the data generated will form the basis for a novel method of vaccine matching for serotype O FMD using sequence data alone, providing a cheaper and more rapid method for identifying the correct vaccine for use in an outbreak situation. There is also the potential for identification of some broadly cross reactive epitopes, which could be beneficial in the design of novel vaccines.

The specific aims are:

1. To generate a set of sequence and serological matching data representative of the O serotype.
2. To compare the two current serological tests used in vaccine matching.
3. To expand on work previously published with the SAT serotype viruses (Reeve *et al.*, 2010) and develop a model based on both the VNT and LPBE data that can be used to predict vaccine match based on sequence data alone.
4. To use a variation of the vaccine matching model to predict immunodominant epitopes using the generated sequence and serology data.
5. To evaluate the performance of a number of structure-based B cell epitope prediction programs at identifying already known *Picornavirus* epitopes and use a consensus of the results from the best performing programs as an independent method for prediction of novel epitopes of FMDV.
6. To test the predictions made by the two independent methods (above), and to examine any further interesting results from the serological analysis, by generating mutant viruses using a reverse genetics approach and analysing the antigenic impacts of the mutations through serological methods.

Chapter 2: General Methods

2.1 A note on the organisation of methods

The general methods, used in more than one chapter, are described in this chapter. Each individual chapter has a methods section that contains a summary of the methods that were used as well as details of methods that are absolutely specific to that chapter

2.2 Virus strain and cell selection.

2.2.1 Cells and viruses.

The 105 serotype O viruses included in this study were obtained from the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (United Kingdom). These viruses were selected to give the broadest geographical and temporal distribution and were based on previous one way serological relationship ("r_1 value") data against other viruses closely related to O Kaufbeuren (i.e. O Lausanne and O BFS) and on VP1 nucleotide sequence phylogenic data. For more information on the viruses selected see appendix 1.

Baby hamster kidney 21 clone 13 (BHK) cells were used to grow the homologous O Kaufbeuren infectious copy (O KIC) virus. Porcine renal (IBRS-2) cells were used to grow the remaining homologous and heterologous (wild type) viruses. All cells and viruses were grown on Eagle's minimum essential medium supplemented with 10% adult bovine sera (ABS), 100 IU/ml Penicillin and 100µg/ml Streptomycin (EMEM).

Confluent monolayers of cells were infected with ~200µl of virus inoculum and placed in an incubator set to 37°C with 5% CO_2 for approximately 12-24 hours until >90% cytopathic effect (CPE) was observed. Flasks were frozen at -20°C to release any virus still localised within the cells. The supernatant was harvested and clarified by centrifugation at 1140xg for 10 minutes. Cellular debris were discarded and the virus suspension was aliquoted into 1ml volumes and stored at -80°C until use. All heterologous viruses were grown to a maximum of passage 3 on IBRS-2 cells.

2.3 Serological and cell based methods

2.3.1 Antisera.

Pooled antisera (either day 21 or day 31 post vaccination from five animals- recommended by the OIE) had been raised in cattle against seven serotype O vaccine antigens; O1 BFS 1860, O UKG 2001, O1 Kaufbeuren (OKIC), O Kenya 1978 O Manisa, O Turkey 2009 and O Uganda 2002 (Panasia II lineage) For details see table 2.1.

Serum name	Vaccine raised against	location generated	Days post vaccination	number of animals
O KIC	Pirbright O KIC Antigen	Pirbright	31	5
O UKG	O UKG 34/2001	Pirbright	21	5
O BFS	O BFS 1966	Pirbright	21	5
O Uga 2002	O Uga 2002	Pirbright	21	5
O Ken 1978	O Ken 77/1978	Intervet	21	5
O Manisa	O Man/	Intervet	21	5
O Tur 2009	O Tur 05/2009	Intervet	21	5

Table 2.1: Details for the sera used in this study. For country abbreviations see appendix 1.

2.3.2 Titration of viruses.

Viruses were titrated in 96 well flat bottom microtitre plates (Nunclon surface, Nunc, Denmark). 50µl of EMEM was added to all wells with the exception of column 1, to which 100µl of a 10^{-1} dilution of virus was added and titrated at 0.3 $\log_{10}$ intervals down the plate to column 11. Column 12 was left as a cell only control. After titration, wells were overlaid with a further 50µl of medium and 50µl of a $1x10^4$ IBRS-2 cells/ml suspension in EMEM. Plates were sealed and placed in an incubator set to 37°C with 5% CO_2 for 72 hours and then rinsed in 0.2% (w/v) citric acid (VWR, UK) in PBS, blotted dry and stained with 50µl 0.2% (w/v) naphthalene blue (powder from Sigma, UK) in 0.85% saline for 30 minutes before the stain was rinsed off. The titre of each virus was calculated using the Karber method (Karber, 1931) to express the 50% $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$/ml. Viruses of a titre of 2.5 $\log_{10}$ $TCID_{50}$/ml or greater were used for the virus neutralisation test (VNT).

2.3.3 Virus neutralisation test.

This was used to compare the antigenic relationship of each heterologous virus (a virus which the bovine vaccinate serum (BVS) was not raised against) to the homologous virus (the vaccine strain which the BVS was raised against) based on the neutralising component of antibody present in the BVS; serum neutralisation titres (the titre at which the antibody present in the serum is able to neutralise the infectivity of a virus) for each of the selected viruses were generated as previously described (OIE, 2008; Rweyemamu, 1983, 1984; Rweyemamu & Hingley, 1984; Rweyemamu *et al.*, 1978).

Briefly, 50µl of pooled bovine vaccinate serum (either 21 or 31 days post vaccination) was titrated at 0.3 $\log_{10}$ intervals in EMEM down the columns of a 96 well flat bottom microtitre plate (Nunclon surface, Nunc, Denmark). One plate was prepared per virus to be tested. Five 0.5 $\log_{10}$ dilutions of both the homologous and heterologous viruses were made and 50µl of each dilution was added to two columns of the above microtitre plates. Plates were then incubated for 1 hour at room temperature. The plates were then overlaid with a suspension of $1x10^4$ cells/ml of IBRS-2 or ZZ-R 127 foetal goat tongue cells (the latter used for the testing of the O KIC mutants) in EMEM. Concurrently to this, the lowest dilution of virus was titrated following the method described in section 2.3. The neutralisation and titration plates were placed in an incubator set to 37°C with 5% CO_2 for 72 hours and stained as described in section 2.3.

After staining, the plates were analysed and the virus titre ($\log_{10}$ $TCID_{50}$/ml) was calculated for each of the virus dilutions using the Karber method (Karber, 1931). Additionally, the $\log_{10}$ serum neutralisation (SN) titre was calculated for each dilution using the Karber method (Karber, 1931). Wells where more than 50% of the cell monolayer remained were treated as positive for neutralisation. A regression curve was generated for serum titre against virus titre using Minitab 15.1 statistical software. The $\log_{10}$ serum neutralising titre at 100 $TCID_{50}$/ml of virus was calculated for the

homologous and heterologous viruses (run on all tests as a control). These values were used to calculate the r_1 values (Rweyemamu & Hingley, 1984) by this equation:

Heterologous 100 $TCID_{50}$ log_{10} SN titre – Homologous 100 $TCID_{50}$ log_{10} SN titre= *x*

r_1= Reciprocal log_{10} of *x*

Each test was repeated at least twice and the mean r_1 value was determined by averaging both the heterologous and the homologous serum titres from the assay runs where the heterologous data were generated. If the homologous serum titre was more than 0.3 log_{10} from the running mean then the test was deemed invalid. The two repeats of each heterologous virus's serum neutralising titre must also be within 0.3 log_{10} of each other to be deemed valid. In addition the virus dilution range also had to encompass 100 $TCID_{50}$/ml across its range to be valid. Viruses that give an r_1 value of 0.3 or higher are generally considered to be closely related enough to the homologous strain to confer protection *in vivo* (Paton *et al.*, 2005).

2.3.4 Virus titration ELISA.

To determine the dilution of each virus used in this study that would give an optical density (OD) reading of between 0.8-2.0 at λ 490nm, the optimal range for the strain differentiation liquid phase blocking ELISA (LPBE), a virus titration ELISA was performed as described previously (Hamblin *et al.*, 1986a; Hamblin *et al.*, 1986b; OIE, 2008). Briefly, each well of a 96 well Nunc Maxisorb ELISA plate (Nunclon surface, Nunc, Denmark) was coated with 100µl of polyclonal Rabbit anti O Phi 95 sera diluted 1/5000 in 0.05M carbonate/bicarbonate buffer and incubated overnight at 4°C. The following day, the plates were washed 3 times with PBS and 50µl of PBS containing 0.05% (v/v) tween-20 and 2% (v/v) phenol red (PBST) was added to all wells. 50µl of neat virus was added to the first well of two rows and then titrated down the plate in 0.3 log_{10} intervals down to the bottom of each row, with the remaining 50µl discarded. The plates were then incubated at 37°C for one hour on an orbital shaker. After the hour, the plates were washed 3 times with PBS and then each well

was coated with 50µl of polyclonal guinea pig anti O Phi 95 sera diluted 1/1000 in blocking buffer (PBST with 5% w/v Marvel skimmed milk powder) and incubated for 1 hour at 37°C. Plates were then washed 3 times with PBS and 50µl of rabbit anti guinea pig horse radish peroxidase (HRP) conjugate was added to all wells and incubated for a further 1 hour at 37°C. The plates were then washed and 50µl of ortho-phenylenediamine dihydrochloride (10mg in 18.3ml of 0.05M phosphate citrate buffer with 9.15µl of 30%H_2O_2) was added and left to develop at room temperature for 15 minutes before the addition of 50µl of 1M H_2SO_4 to stop the reaction. The absorbance was then read at λ490nm and the dilution of virus that gave an optical density (OD) of ~1.5 was selected for testing in the LPBE.

2.3.5 Strain differentiation Liquid phase blocking ELISA.

This was used to compare the antigenic relationship of each heterologous virus to the homologous virus based on the whole antibody compliment present in the BVS, LPBE titres (the titre at which sufficient virus is bound to antibody in the test serum to reduce the OD value of virus bound an ELISA plate coated with antisera by 50%) for each of the selected viruses. The method used was as previously described (Hamblin *et al.*, 1986a; Hamblin *et al.*, 1986b; OIE, 2008).

Briefly, each well of a 96 well Nunc Maxisorb ELISA plate (Nunclon surface, Nunc, Denmark) was coated with 100µl of polyclonal Rabbit anti O Phi 95 sera diluted 1/5000 in 0.05M carbonate/bicarbonate buffer and incubated overnight at 4°C. In parallel a 96 well U bottom carrier plate (Sterilin Ltd, UK) was set up as follows: 50 µl of PBS containing 0.05% (v/v) tween-20 and 2% (v/v) phenol red (PBST) was added to all wells followed by 50µl of antisera to columns 3,4,7,8,11,&12 and then titrated down the plate in 0.3 $\log_{10}$ intervals down to the bottom of each row, with the remaining 50µl discarded. For each virus to be tested an appropriate dilution was made based on the results obtained in section 2.2.5 and each virus was overlaid onto 4 columns of the 96 well U bottom plate. The first four columns (1-4) were used for the homologous virus on each plate prepared with the remaining 8 columns divided between two heterologous viruses. In each case the first 2 columns

acted as a virus only control and the second two columns formed the test wells. The carrier plates were then incubated overnight at 4°C on an orbital plate shaker.

The following day, the Nunc Maxisorb ELISA plates were washed 3 times with PBS before 50µl from every well of the carrier plate was added to the corresponding well on the washed ELISA plate. After the hour, the plates were washed 3 times with PBS and then each well was coated with 50µl of polyclonal guinea pig anti O Phi 95 sera diluted 1/1000 in blocking buffer (PBST with 5% w/v Marvel skimmed milk powder) and incubated for 1 hour at 37°C. Plates were then washed 3 times with PBS and 50µl of rabbit anti guinea pig horse radish peroxidase (HRP) conjugate was added to all wells and incubated for a further 1 hour at 37°C. The plates were then washed and 50µl of Ortho-phenylenediamine dihydrochloride (10mg in 18.3ml of 0.05M phosphate citrate buffer with 9.15µl of 30%H_2O_2) was added and left to develop at room temperature for 15 minutes before the addition of 50µl of 1M H_2SO_4 to stop the reaction. The absorbance was then read at λ490nm.

In order to calculate the LPBE titre for each virus the average OD for the virus only control wells was calculated and the first dilution of serum from the titration wells that gave an OD reduced by greater than 50% was taken as the titre. For each virus the $\log_{10}$ value of this titre was used to calculate the r_1 values against the homologous virus (run on every plate as a control) titre from the same plate (Rweyemamu & Hingley, 1984) by this equation:

Heterologous SB $\log_{10}$ titre – Homologous LPBE $\log_{10}$ titre= *x*

r_1= Reciprocal $\log_{10}$ of *x*

Each test was repeated at least twice and the mean r_1 value was determined by averaging both the heterologous and the homologous LPBE titres from the assay runs where the heterologous data were generated. If the homologous serum binding titre from a plate was more than 0.3 $\log_{10}$ from the running median then the test was deemed invalid. The two repeats of each heterologous virus's serum binding titre must also be within 0.3 $\log_{10}$ of each other to be deemed valid. Additionally the

virus-only control wells average OD had to fall between 0.8-2 for the result to be valid. Viruses that give r_1 values of 0.4 or higher in the LPBE are generally considered to be closely related enough to the homologous strain to confer protection *in vivo* (Ferris & Donaldson, 1992).

2.3.6 Plaque assay

Prior to the assay an appropriate number of 6 well plates were seeded with $1x10^6$ wells of the appropriate cell line and incubated at 37°C until the monolayer reached 100% confluency. When the cell monolayer was 100% confluent a $\log_{10}$ dilution series (20µl virus in 200µl PBS) was prepared for each virus to be tested. The monolayers were then washed twice with PBS and 100µl of each virus dilution was overlaid onto two wells of the 6 well plate. The plates were then incubated at 37^oC for 15 minutes. In parallel, an Eagles' overlay mixture was prepared as follows: 0.6g of Indubiose (Pall corporation, USA) was added to 25ml of sterile water and melted in a microwave. To the molten Indubiose 75ml of Eagles' overlay media was then added, followed by 5ml of tryptone phosphate broth, 1ml of foetal calf serum and 1ml 100 IU/ml Penicillin and 100µg/ml Streptomycin. To keep this molten the mixture was kept at 45°C in a water bath until use. After 15 minutes incubation the 6 well plates were washed twice with PBS before 2ml of prepared Eagles' overlay mixture was added to every well. Once the overlay mixture had set the plates were incubated at 37°C for 48 hours.

After 48 hours the plates were stained by adding 2ml of 10% (w/v) trichloroacetic acid (TCA) to each well for 15 minutes at room temperature to fix the cells and dissolve the indubiose. The TCA was then washed off and 1ml of crystal violet stain (stain prepared by dissolving 1 g of crystal violet in 99 g of 20% ethanol as a stock solution. 20 ml of this stock solution was then mixed with 40 ml of 95% ethanol and 150 ml of water was added to all wells and incubated at room temperature for 30 minutes before being washed off. Plates were then photographed and the plaque sizes observed.

2.4 Sequencing methods.

2.4.1 RNA extraction of viruses.

Extraction of viral total RNA was carried out using an RNeasy mini kit (50) (Qiagen, UK) following the manufacturer's instructions. Briefly, 460µl of virus supernatant (cultured as in section 2.1.1) was added to 460µl of lysis buffer RLT, followed by an equal volume of 70% ethanol. This was vortexed for 15 seconds and 700µl was transferred to an RNeasy mini spin column and collection tube. The column was centrifuged at 13000 revolutions per minute (rpm) in a Heraeus biofuge pico centrifuge for ~ 15 seconds and the flow through was discarded. This step was repeated until the entire sample had been through the column. Following this, 700µl of wash buffer RW1 was added to the column and centrifuged as per the first step; again the flow through was discarded. 500µl of wash buffer RPE was added and this was centrifuged for 1 minute at 13000 rpm on the same centrifuge. After this step, the collection tube was discarded and the spin column placed in a fresh 1.5ml tube. 50 µl of RNase free water was added to the spin column and the column was centrifuged at 13000 rpm for 1 minute to elute the extracted RNA. Extracted RNA samples were stored at -20°C until use.

2.4.2 cDNA synthesis.

cDNA synthesis was performed on extracted RNA using a superscript III first strand synthesis system for RT-PCR (Invitrogen, Worldwide), as per the manufacturer's instructions. The following components were mixed:

Constituent	Volume (µl)
Total RNA	4
DEPC treated water	3
Random hexamers (50ng/µl)	2
10mM dNTP mix	1

The reactions were heated on a thermal cycler at 68°C for 3 minutes and placed in ice for an equivalent amount of time. Subsequently, further reagents were added as follows:

Constituent	Volume (µl)
10x RT Buffer	2
25mM $MgCl_2$	4
0.1M DDT	2
RNase out	1

Samples were placed on a thermal cycler and heated to 42°C for 2 minutes, 1µl of RT superscript III reverse transcriptase was added and samples placed back on the thermal cycler at 42°C for a further 2 hours. cDNA samples were stored at -20°C until use.

2.4.3 Polymerase chain reaction (PCR).

PCR reactions were set up using a KOD (derived from Pyrococcus kodakaraensis KOD1 polymerase (Fujiwara *et al.*, 1998)) hot start polymerase kit (Novagen, USA) according to the manufacturer's instructions. For each cDNA, a reaction mix was prepared as follows:

Constituent	Volume (µl)
10x Buffer	5
dNTPs (2mM each)	5
25 mM $MgSO_4$	4
KOD hot start DNA polymerase (1 unit/µl)	0.9
Nuclease free water	30
Forward primer (10pM)	1
Reverse primer (10pM)	1
cDNA	3

The capsid region was amplified using either the primer pair L463F (ACCTCCRACGGGTGGTACGC)/NK72R (GAAGGGCCCAGGGTTGGACTC) or L463F/EUR2B52R (GACATGTCCTCCTGCATCTGGTTGAT) resulting in an amplified product of approximately 2.5 kb, including the C-terminus of L, P1, 2A and N-terminus of 2B. For viruses that were not amplified using these universal primers, specific primers were designed and used.

The prepared reactions were placed on a thermal cycler on the following programme:

Programme

Step	Temperature (°C)	Time (seconds)
1	95	120
2	95	30
3	68	20
4	70	60
	Repeat steps 2-4 30 times	
5	70	60
6	4	hold

For some reactions, the annealing temperature was adjusted down to 64°C.

2.4.4 Gel electrophoresis of PCR product.

PCR products were analysed by electrophoresis on a 1% agarose gel. Agarose (Invitrogen, worldwide) was added to 1 x Tris Borate EDTA (TBE) to achieve a 1% agar solution; this was then melted in a microwave. After allowing this to cool to ~50^{o}C, ethidium bromide was added to a final concentration of 0.5µg/ml^{-1}. 1µl of 10x blue juice gel loading buffer (Invitrogen, worldwide) was added to 9µl of sample. This was loaded and gels run in 1 x TBE at between 100-120v. DNA fragment size was estimated by comparison of their mobility to a Track it 1kb+ ladder (Invitrogen, worldwide). A UV imager and 'Quantity One' version 4.1.0 software (Biorad, USA) were used to capture gel images.

2.4.5 Purification and quantification of DNA from PCR products.

Following PCR, the DNA was purified using a GFX^{TM} PCR and Gel band purification kit (GE Healthcare, worldwide) according to the manufacturer's protocol; briefly, 100µl of PCR product was

added to 500µl of capture buffer type 2 and vortexed for 15 seconds. This mixture was then loaded into a capillary tube (supplied with kit) and centrifuged at 13000 rpm for 30 seconds in a Heraeus biofuge pico centrifuge. The flow through was discarded and 500µl of wash buffer type 1 was added to the capillary tube. Again this was centrifuged at 13000 rpm for 30 seconds. After this the collection tube was discarded and the capillary tube was placed in a fresh, clean eppendorf. 50µl of collection buffer type 6 was added and allowed to sit at room temperature for 1 minute before being centrifuged at 13000 rpm to elute the purified DNA from the membrane.

2µl of purified DNA was run on a 1% agarose gel and run at 100-120v. DNA quantity was calculated by comparing the strength of the band given to that of a λIII molecular weight ladder. A UV imager and 'Quantity One' version 4.1.0 software (Biorad, USA) were used to capture gel images. Purified DNA was frozen at -20°C until use.

2.4.6 Capsid sequencing.

Sequencing reactions were prepared using the ABI-Seq BigDye® Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol. For every reaction to be run, a mix was set up as follows:

Reagent	Volume (µl)
5 x sequencing buffer	2
BigDye® Terminator v3.1 Ready Reaction Mix	0.25
Primer (2pM)	2
DNA*	1
Nuclease free water *	5.25

*Volumes varied depending on the concentration of DNA.

All viruses were sequenced using 24 type O Universal capsid primers (appendix two). If required, additional primers were designed and used. All viruses were sequenced on both the DNA strands

After reactions were prepared, they were placed on a thermal cycler on the following progamme:

Cycle

Step	Temperature (°C)	Time (seconds)
1	96	60
2	96	10
3	50	5
4	60	240
	Repeat steps 2-4 25 times	
5	4	Hold

The sequence reactions were then cleaned for sequencing. Briefly, 5µl of EDTA was added to each reaction along with 60µl of 100% ethanol. Reactions were left in the dark for 15 minutes before being centrifuged at 690xg for 45 minutes. The supernatant was immediately removed and 60µl of 70% ethanol added. Reactions were centrifuged at 320xg for a further 15 minutes. The supernatant was immediately removed following centrifugation. The reactions were then vacuum dried for 45 minutes in the dark before 20µl of Hi Di formamide (Applied Biosystems) was added. Samples were analysed using an applied Biosytems 3730 DNA analyser (Applied Biosystems, Worldwide).

2.4.7 Sequence assembly

Sequences were assembled using the DNAStar seqman II progamme (DNAstar, USA). Sequences were aligned and amino acid sequences translated using BioEdit software (Hall, 1999).

2.5 Cloning methods.

2.5.1 Infectious copy plasmids.

The full length O1 Kaufbeuren infectious copy PT7S3 plasmid (Zibert et el 1990, Ellard 1999) and the chimeric form of this plasmid containing the capsid coding region from O UKG 34/2001 (Bøtner et al 2011) was used for the site directed mutagenesis carried out in this study.

2.5.2 Inverse PCR site directed mutagenesis.

For each set of mutagenic primers used, an inverse PCR was set up using a KOD hot start polymerase kit (Novagen, USA) according to the manufacturer's instructions. For each primer set a reaction mix was prepared as follows:

Constituent	Volume (µl)
10x Buffer	5
dNTPs (2mM each)	7
25 mM $MgSO_4$	4
Kod hot start DNA polymerase (1 unit/µl)	1
Nuclease free water	30.5
Forward primer (30pM)	1.5
Reverse primer (30pM)	1.5
Template plasmid	2

Primer sets were prepared for each mutation to be inserted, in some cases in combination, either as a single or double nucleotide substitution. For a complete list of Mutagenic primers see appendices three and four.

The prepared reactions were placed on a thermal cycler on the following progamme:

Step	Temperature (°C)	Time (seconds)
1	95	120
2	98	10
3	68	30
4	72	480
	Repeat steps 2-4 16 times	
5	70	60
6	4	hold

2.5.3 Restriction endonuclease Dpn-1 digestion of mutagenesis products.

Inverse PCR products were digested using the restriction endonuclease Dpn-1 (New England Biolabs) to remove bacterial DNA. 1µl of Dpn-1 enzyme was added to each sample followed by 5µl of Buffer 4 (NEB). Samples were then incubated at 37°C for a minimum of 4 hours (generally overnight). Digested products were then analysed by electrophoresis as described in section 2.3.4 to determine the presence of PCR product.

2.5.4 Transformation of Dpn-1 Digested PCR products into competent DH5α cells.

DH5α cells were cultured overnight at 37°C in 2ml of Lysogeny broth (LB). The following day, the culture was diluted 1/200 in LB Broth (100µl of cells in 20ml of LB) and grown until the optical density (OD) was between 0.15-0.20 (approximately 2 hours). When the correct OD was reached the cells were pelleted by centrifugation at 4000rpm for 10 minutes, the supernatant was then discarded

and the pelleted cells resuspended in 2ml of transformation and storage solution (TSS). The cells were then left on ice for one hour. After this incubation, 5µl of the Dpn-1 digested product was added to 100µl of cells and left for a further hour on ice. The cell/plasmid mix was then heat shocked by taking from the ice and immediately placing into a water bath set at 42°C for one minute. The mix was then returned to the ice for a further 5 minutes before 900µl of Super Optimal broth with Catabolite repression (SOC) was added and then incubated for 1 hour at 37°C. Cells were then plated on LB agar plates containing 100µg/ml of Ampicllin and incubated at 37°C overnight.

2.5.5 Minipreparation of plasmid DNA (Miniprep).

All Minipreps were performed using a Qiagen Qiaprep spin miniprep kit according to the manufacturer's protocol. Briefly, individual colonies cultured overnight at 37°C on LB agar plates containing 100µg/ml of Ampicllin (see section 2.4.4) were picked and cultured overnight in 2ml of LB broth containing 100ug/ml Ampicillin. The following day, 1ml of suspension was centrifuged briefly at ~17,000xg to pellet the bacterial cells. The supernatant was then discarded and 250µl of buffer P1 was added to the pelleted cells, followed by 250µl of buffer P2 and 350µl of buffer N3. At each step the sample was mixed to ensure even dispersal of buffer. Samples were then centrifuged at 17,000xg for 10 minutes to pellet the aggregated chromosomal DNA and protein/sodium dodecyl sulphate complexes. The supernatant was then applied to a QIAprep spin column and centrifuged 17,000xg for 1 minute. The column was washed by the addition of 750µl of buffer PE and centrifuged at 17,000xg for 1 minute. The column was then transferred to a clean eppendorf tube and the DNA eluted by the addition of 50µl of buffer EB and centrifuged at 17,000xg for 1 minute. The presence of plasmid DNA was confirmed by gel electrophoresis as described in section 2.3.4. The presence of the correct mutation was determined by sequence analysis as outlined in section 2.3.6. Miniprep plasmid DNA was frozen at -20°C until use.

2.5.6 Maxipreparation of plasmid DNA (Maxiprep).

All Maxipreps were performed using a Qiagen Plasmid Maxi kit according to the manufacturer's protocol. Briefly 5µl of Miniprep DNA containing the desired mutation was transformed into DH5α cells as outlined in section 2.4.4. These cells were inoculated into a 100ml suspension of LB broth containing 1ug/ml Ampicillin incubated overnight at 37°C. The following day, the bacteria were pelleted by centrifugation at 6000xg for 15 minutes at 4°C. The supernatant was discarded and the bacterial pellet re-suspended in 10ml of buffer P1. 10ml. Buffer P2 was subsequently added and incubated at room temperature for 5 minutes. 10ml of buffer P3 was then added and the sample incubated on ice for a further 20 minutes. Samples were then centrifuged at 20,000xg for 30 minutes, the supernatant transferred to a clean 50ml falcon tube and re-centrifuged at 20,000xg for a further 15 minutes. During this centrifugation step, a Qiagen tip column was prepared by addition of 10ml of the equilibration buffer QBT and allowed to empty through gravity flow. The supernatant from the last centrifugation step was then applied to this column and allowed to empty through gravity flow. The tip column was then washed by applying 2 x 30ml of buffer QC and allowed to empty through gravity flow. To elute the DNA, 15ml of the elution buffer QF was added to the column and allowed to empty through gravity flow. 10.5ml of room temperature isopropanol was added and mixed before centrifugation at 15,000xg for 30 minutes to pellet the plasmid DNA. After centrifugation the supernatant was decanted, 5ml of 70% ethanol added and the sample re-centrifuged at 15,000xg for a further 20 minutes. Supernatant was then decanted and the DNA pellet allowed to air dry for 10 minutes before being re-dissolved in 100µl of 1M Tris EDTA buffer. The presence of plasmid DNA was confirmed by gel electrophoresis as described in section 2.3.4. Maxiprep plasmid DNA was frozen at -20°C until use.

2.5.7 Plasmid linearization using restriction endonuclease Hpa-1.

Maxiprep plasmid DNA was linearised using the restriction endonuclease Hpa-1 (New England Biolabs). For each digestion a reaction mix was prepared as follows:

Constituent	Volume (µl)	Volume (µl) Negative Ctrl
NEB* Buffer 4	2	2
Hpa-1 restriction enzyme	2	-
Nuclease free water	6	8
Maxiprep DNA	10	10

*New England biolabs.

Samples were then incubated at 37°C for 4 hours or overnight. Digested products were analysed by electrophoresis, as described in section 2.3.4, and linearization confirmed by comparison with the negative control.

2.5.8 RNA synthesis using T7 polymerase.

RNA was synthesised using a Megascript T7 kit (Ambion, USA) according to the manufacturer's instructions. Briefly, a reaction mix was prepared as follows:

Constituent	Volume (µl)
10x Buffer	2
CTP's (75mM)	2
GTP's (75mM)	2
ATP's (75mM)	2
UTP's (75mM)	2
T7 polymerase	2
Hpa-1 Digested Plasmid	8

After the samples had been incubated at 37°C for 4 hours, the RNA was extracted ready for transfection using an RNA mega clear kit (Ambion, USA), according to the manufacturer's instructions. Briefly, 80µl of elution buffer was added to the sample followed by 350µl of binding solution and 250µl of 100% (v/v) ethanol. Between the additions of each chemical, the sample was mixed by gently pippetting up and down several times. The sample was then applied to a filter

cartridge supplied with the kit, centrifuged at 13000 rpm in a Heraeus biofuge pico centrifuge for ~1 minute and the flow through discarded. 500µl of wash buffer was then applied to the filter cartridge and drawn through as in the previous step. This wash step was repeated and then the RNA was eluted by addition of 50µl of elution buffer (pre-heated to 95°C) and spinning as previously described. RNA was frozen at -80°C until use.

2.5.9 Transfection into mammalian cells

To a 4mm electroporation cuvette (BTX molecular delivery systems, USA) 20µl of the cleaned RNA product from section 2.4.8 was added to 780µl of a chilled $1x10^7$ cells/ml suspension of BHK cells in electroporation buffer. The cells were then permeablised using a Biorad 165-2670 Gene Pulser MXcell Electroporation System (Biorad, USA) set to 0.75 KV and 0.25 Ω (Ohms) to allow entry of the RNA. Samples were left at room temperature for 10 minutes and then added to a $25cm^2$ tissue culture flask (Cellstar GMBH, Germany) containing Eagles minimum essential medium supplemented with 10% adult bovine sera (ABS), 100 IU/ml Penicillin and 100µg/ml Streptomycin (EMEM). Flasks were frozen at -20°C either when >90% CPE was observed or after a time period of 48 hours.

2.5.10 Passaging of electroporated viruses

All electroporated viruses were passaged a further 3 times on either BHK, IBRS2, primary bovine Thyroid (BTY) or ZZ-R 127 foetal goat cells as outlined in section 2.1. A large number of cell lines were used to ensure that any residue changes that alter virus receptor usage had the best chance of being rescued. The capsid sequence was determined as outlined in section 2.3, to confirm the presence and the stability of each mutation. Once the correct mutation was confirmed, the viruses were aliquoted into 1ml volumes and stored at -80°C until required.

2.6 *In-silico* methods

2.6.1 Determining the surface accessibility of type O residues.

The surface exposure of each capsid residue on a central protomer, surrounded at each two fold axis of symmetry by an adjacent protomer (figure 2.1) was determined using a probe the approximate size of a water molecule (1.6Å), any residue on the surface with greater than 1Å^2 exposed was included in the analysis. The interactions of residues at interfaces between protomers were determined using the EMBL PISA program (Krissinel & Henrick, 2007). Only surface exposed residues were included in the analysis.

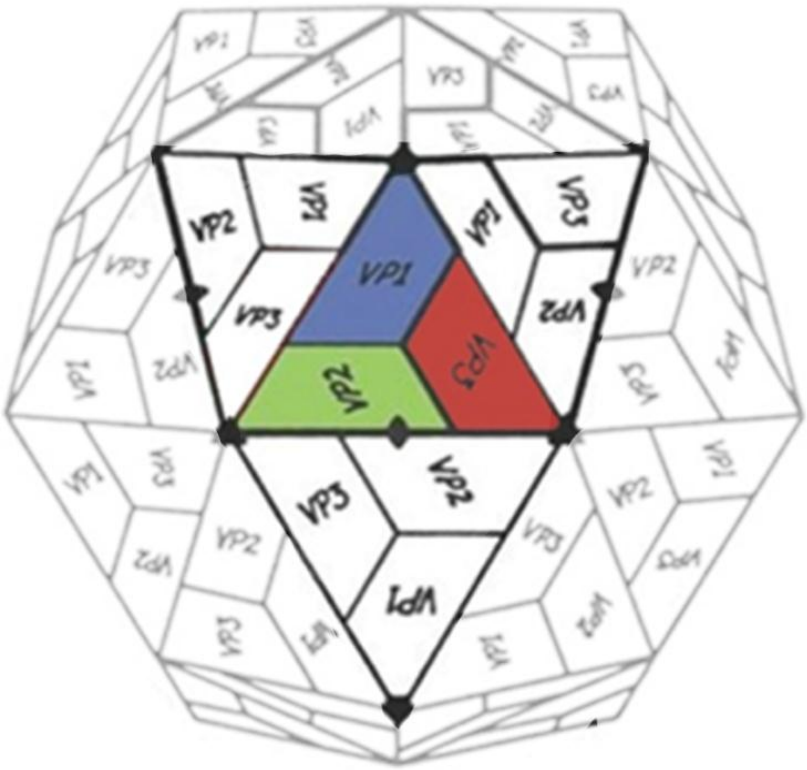


Figure 2.1: Calculating the surface exposure of an individual protomer; A central protomer with a surrounding protomer at each two fold axis of symmetry, which was used to calculate the surface exposure, is shown in black, with the remainder of the capsid shown in light grey. The capsid proteins of the central protomer are coloured in blue (VP1), green (VP2) and red (VP3).

2.6.2 Comparisons of genetic diversity

The genetic diversity of the viruses carried out in chapters 3 and 5 was determined using the Espript program (Gouet *et al.*, 1999) and a Risler similarity scoring matrix (Risler *et al.*, 1988). Firstly, the amino acid sequences (either generated in this study or from Genbank) were aligned to the

amino acid sequence of the reduced serotype O BFS structure file 1FOD.pdb using the Clustal X program (Thompson *et al.*, 1997). This alignment was subsequently divided into the individual proteins VP1-VP3 and any amino acid residue not found within the pdb file was removed. This meant that residues 212 and 213 of VP1 and residues 1-4 of VP2 were not included in the analysis. The PDB file was then divided in the individual capsid proteins. Each sequence file and the corresponding PDB file for each capsid protein, VP1-3 were uploaded into the program.

The similarity scores generated for each residue were output in the B factor column of a new PDB file, with scores given from 0 (No diversity) to 100 (the most diverse). These scores were converted to percentage diversity.

2.7 Statistical analysis

To determine the significance of each of the results an analysis of variance was performed using a general linear model with the fixed and random effects as listed in specific instances. All comparisons were performed using the Tukey method for multiple comparisons, with confidence limits set a 95%. All statistical functions performed were completed using the Minitab 16 statistical software package (Minitab Inc, USA).

Chapter 3: Analysis of sequence, virus neutralisation test (VNT) and liquid phase blocking ELISA (LPBE) results.

The O UKG and O BFS VNT work in this chapter was performed by Sasmita Upadhyaya

3.1 Summary.

This chapter describes the primary analysis of the data generated for 105 FMD type O viruses through sequencing and serological evaluation against five antisera by means of virus neutralisation tests (VNT) and liquid phase blocking ELISAs (LPBE), including an analysis of the correlations between the different findings.

The consensus sequence for the whole capsid coding region was generated for each virus after growth through three serial passages in cell culture. Phylogenetic analysis of the sequences confirmed that a broad representation of the O serotype was selected, with representatives from all nine of the currently circulating topotypes present. The serological reactivity of the viruses to 5 different bovine vaccinate sera (BVS) showed that in most cases the vaccines from which the sera were generated would provide good cross protection, with the VNT generally having a higher number of positive vaccine matches. However, the results obtained with the O KIC serum contrasted with this pattern, with most viruses not being a good match to the parent vaccine, including the closely related O KWT virus. The cause of this anomalous result was further investigated and is reported in Chapter six.

The differences between each of the five homologous viruses used to prepare antisera and the respective heterologous viruses were compared pairwise, by correlating differences in seroreactivity with differences in amino acid sequences. The latter was evaluated at the level of the whole capsid, the individual capsid proteins, at antigenic sites and with those residues identified as highly diverse. This revealed little or no correlation, suggesting that a small number of dominant changes in the capsid sequence are causative of changes in antigenicity, rather than this arising through an accumulation of sequence changes in any of the regions analysed. Further analyses to identify these dominant antigenic 'hotspots' is described in Chapter four.

3.2 Methods.

3.2.1 Generation of the serological data

Each virus (selected and grown as described in section 2.1.1) was tested twice in both the VNT and the LPBE and for each test, the r_1 value was determined as described in sections as 2.2.4 and 2.2.6 respectively. For the VNT, an r_1 value of greater than 0.3 is indicative of a vaccine match. The LPBE vaccine matching criteria for the r_1 values reported in this chapter are set at two different threshold values. As discussed in section 1.10.2, previous studies have suggested that although an r_1 value of 0.4 is generally indicative of a good level of cross protection, vaccine matches with a reciprocal value as low as 0.2 may also provide protection. The bovine vaccinate sera tested in the VNT analyses had been raised against the O UKG, O BFS and O KIC viruses. Whilst those bovine vaccinate sera tested in the LPBE had been raised against the O UKG, O BFS, O KIC, O Ken 1978 and O Uga 2002 viruses. For more details on these sera, see section 2.2.2. Different numbers of heterologous viruses were tested against each serum.

3.2.2 Generation of capsid sequences

The capsid sequences for all the viruses analysed in this chapter were generated using Sanger sequencing methods as described in section 2.3.

3.2.3 Generation of a phylogenetic tree.

To evaluate the evolutionary relationships of the viruses selected for this study a neighbour joining (NJ) tree was constructed from the nucleotide sequences of the entire capsid (P1) region using a Kimura 2-parameter model and the Mega 4 program (Tamura *et al.*, 2007).

3.2.4 Comparisons of genetic diversity and sequence variability.

Sequence diversity of the serotype O viruses generated in this chapter was determined as described in section 2.5.2. Prior to the use of the sequences to calculate amino acid diversity the sequences of the entire capsid, along with the individual capsid proteins were used to determine the

number of completely conserved amino acids. This was performed using the bioedit program which provided a summary of the number of amino acids at each location. Where only a single amino acid was present at the location (defined by n=105 under a particular amino acid within a residue number) then this amino acid was said to be completely conserved. This enabled the determination of this property both for the entire capsid and for those residues missing during the diversity analysis.

3.2.5 Correlating sequence and serology data.

The number of amino acid substitutions occurring across a given sequence was first determined by constructing files containing only the specific residues to be examined (for example the whole capsid or the antigenic sites) using the Bioedit program (Hall, 1999). These files were then uploaded into the MEGA 4 program (Tamura *et al.*, 2007) and a pairwise distance calculation was performed using the Amino acids: Number of differences model. These results were tabulated for each virus alongside the corresponding serological values. Titres were used instead of r_1 values due to the curvilinear nature of r_1 value inter-relationships.

To analyse the relationships of the changes in cross reactivity within the serology data to the number of amino acid substitutions within the selected residues, scatter plots were generated using the R statistical analysis software package (R Development Core Team. 2010). In order to measure the overall correlation between the serology and sequence data for each of the selected sequence regions, a Pearson product-moment correlation coefficient (alternatively called Pearson r) was determined. This coefficient is expressed as a number between one and minus one. If the coefficient value is 1 this implies that the relationship between the *x* and *y* variables is perfectly linear, a value of −1 implies a perfect negative correlation and a value of 0 indicates that there is no correlation between the two sets of data (http://www.statsoft.com/textbook/basic-statistics/#Correlations).

The percentage of variance in the serological data accounted for by amino acid substitutions in the selected sequence regions was then calculated by squaring the correlation coefficient value (thus

giving the R^2 value) and multiplying by 100 (see Table 3.2). This determines the percentage of the variation in the response variable (serum titre) explained by the explanatory variable (number of substitutions occurring across the sequence in question).

3.2.6 Identifying surface exposed residues of the reduced O BFS structure

The surface exposure of each residue was determined as outlined in section 2.6.1, using the reduced O BFS structure (PDB: 1FOD)

3.3 Results

3.3.1 Phylogenetic and sequence analysis.

3.3.1.1 Phylogenetic analysis

Figure 3.1 shows a phylogenetic tree, highlighting the relationships of the viruses selected for this study (n=105) and their location within the serotype O topotypes (Samuel & Knowles, 2001). All of the relationships on the phylogenetic tree were similar to those defined from previously determined VP1 sequences only (Knowles personal communication). The viruses represent a broad global distribution of viruses, with 9 of the 11 currently defined serotype O topotypes present (for the current list of topotypes see: http://www.wrlfmd.org/fmd_genotyping/prototypes.htm). The two topotypes not represented (Indonesia 1 and 2) are believed to be extinct (Knowles & Samuel, 2003).

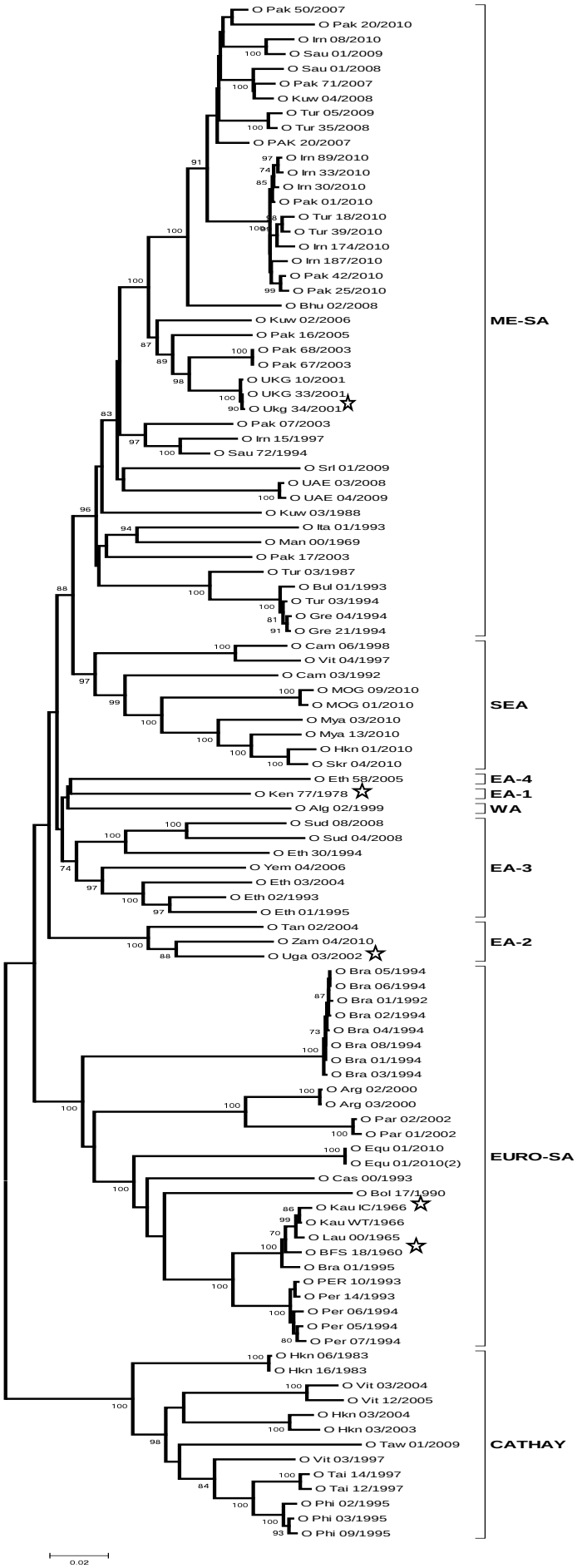


Figure 3.1: A neighbour joining tree showing the evolutionary relationships of the viruses selected for this study using the nucleotide sequence of the entire capsid. FMDV serotype O topotypes are also shown on the figure. The definitions of the abbreviations are listed below. The homologous vaccine viruses used in this study are marked with a star; The O KIC virus is named as O Kau IC/1966 on this tree, the O KWT virus is named as O Kau WT/1966.

Topotype abbreviation definitions:

Euro-SA: Europe-South America.

ME-SA: Middle East-South Asia.

SEA: South-East Asia (SEA).

Cathay: China and the east Tartary.

WA: West Africa.

EA: East Africa.

3.3.1.2 Analysis of sequence diversity

Of the 734 amino acids that make up the serotype O capsid, 25.3% show some level of variation between the 105 viruses (Figure 3.2). Of the individual capsid proteins VP1 exhibited the most variation across its 211 amino acids (35.3%- Figure 3.2), which compares to around 74% of amino acids being variable on VP1 between serotypes (Carrillo *et al.*, 2005). In addition, O Eth 58/2005 had acquired an additional amino acid at residue 45 (part of antigenic site 3). O Ecu 01/2010 and O Ecu 09/2010 had a deletion within the GH loop at position 144, O UAE 3/2008 and O UAE 4/2009 also had a deletion in the GH loop at position 140. Both of these deletions are located upstream from the RGD motif. VP2 (218 amino acids) and VP3 (220 amino acids) exhibited a similar level of variation at the amino acid level (23.4% and 20.9% respectively) with VP4 (85 amino acids) showing the lowest amount of variability, with only 15.3% of residues showing any variation (Figure 3.2). This compares to the variability observed between serotypes of 53%, 61% and 19%, respectively (Carrillo *et al.*, 2005).

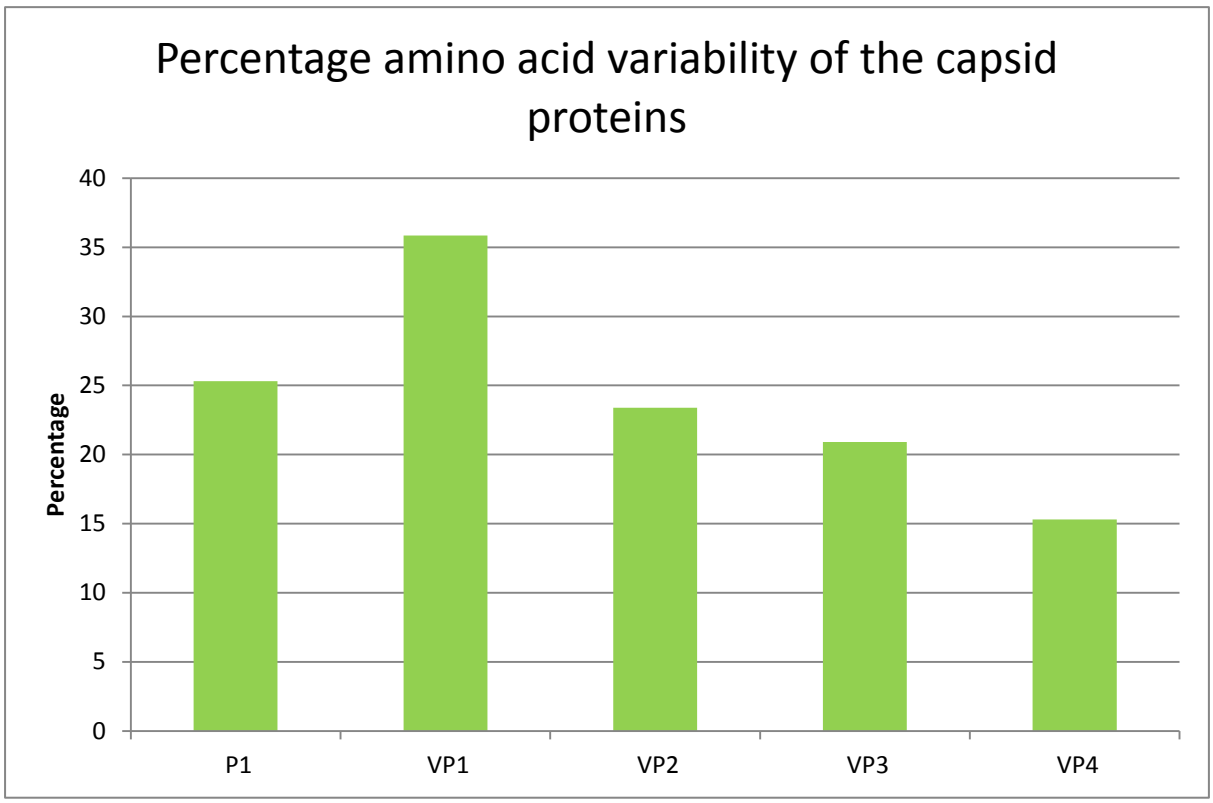


Figure 3.2: Graph showing the percentage of amino acids that have any variability in amino acid composition (percentage of residues that are variable) for the entire P1 region and the individual capsid proteins, VP1-4.

To examine whether there is a link between surface exposure and amino acid diversity (3.2.4-calculated using a risler matrix (Risler *et al.*, 1988)), the levels of amino acid diversity for the whole capsid (displayed in figure 3.3) and the surface exposed capsid proteins, VP1-3 were correlated to surface exposure. VP1, VP2 and VP3 all contribute to the capsid surface but differ in the relative proportion of their residues located there. In all cases it was observed that the majority of amino acids showing any diversity are located on the surface (figure 3.4). The proportion of surface exposed residues increases with the levels of diversity, so 62.2% of residues on the capsid exhibiting any diversity are surface exposed, this increases to 83.9% of residues with a diversity of greater than 50% on the surface and finally at 100% diversity the almost all residues (90.9%) are surface exposed (figure 3.4). Of the individual capsid proteins, the number of amino acids showing any diversity and those with greater than 50% diversity are proportionally more surface exposed on VP1, followed by VP2 with VP3 having the largest proportion of residues buried.

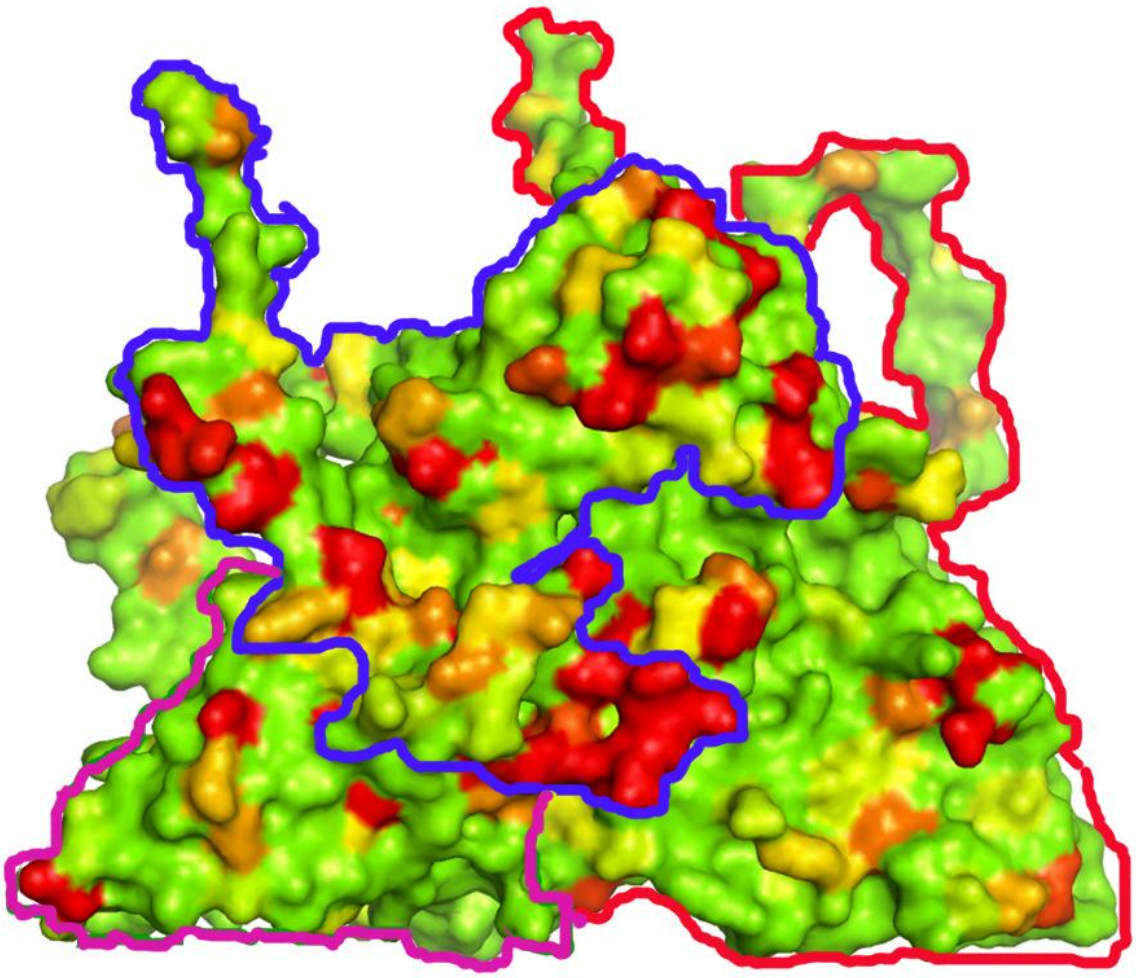


Figure 3.3: A pictorial representation of the molecular surface of the reduced O BFS (PDB ID: 1FOD) protomer made using Pymol (Schrödinger LLC) showing the sequence diversity of residues between the 105 serotype O virus capsid sequences constructed using the ESPRIPT program (Gouet et al., 1999), the colouring goes from green (most conserved) to red (least conserved). A rough outline of the capsid protiens is shown. VP1 is outlined blue (with the GH loop present in the down conformation laying over VP2), VP2 is outlined in purple and VP3 is highlighted in red.

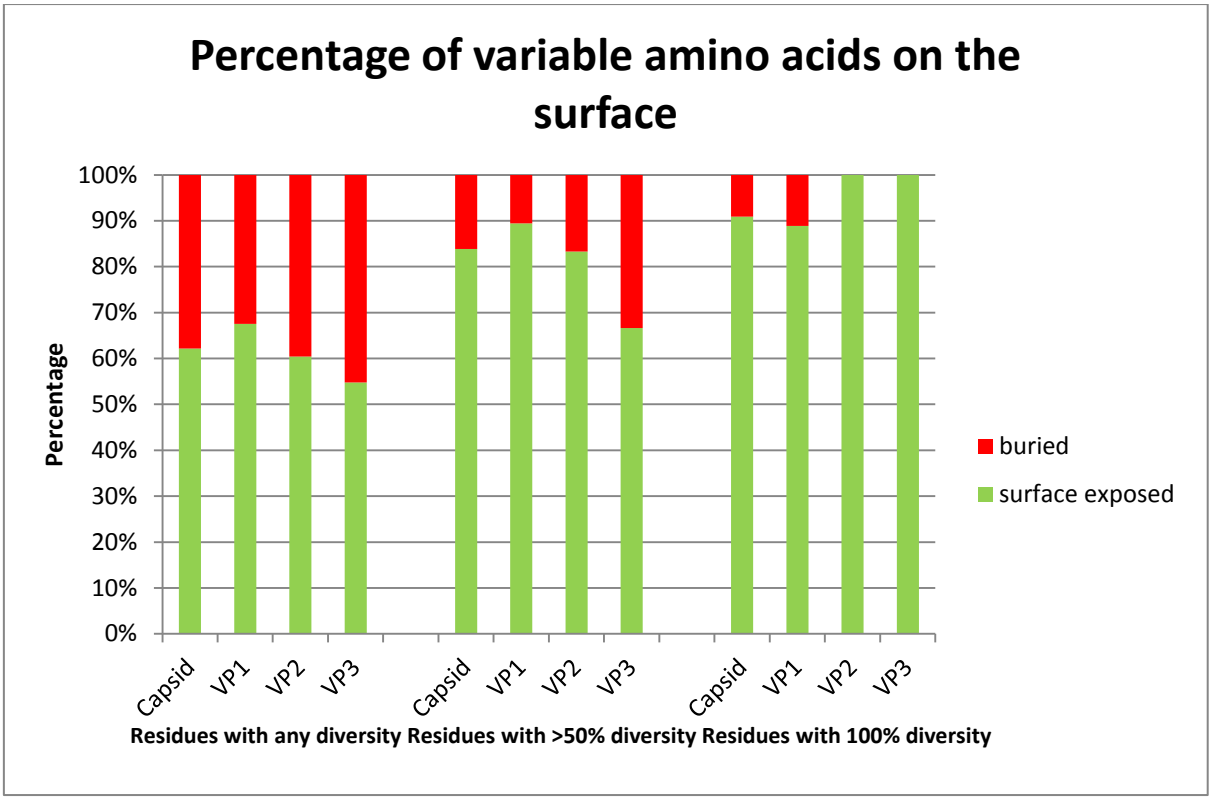


Figure 3.4: Percentage of capsid, VP1, VP2 and VP3 residues with any diversity, diversity greater than 50% and 100% diversity that are located on the surface of the capsid or buried. It should be noted here that only 33.1% of the amino acids that make up the whole capsid (VP1-4) are surface exposed so the ratio of variability to surface exposure is likely to be more significant.

The majority of residues showing 100% diversity on VP1 generally fall into the regions that encompass the previously defined antigenic sites and are surface exposed (figure 3.5). The only other residue showing 100% diversity is residue 4 of VP1 (figure 3.5), which is normally buried on the structure. The reason for such a high level of diversity at this residue is unknown, although there is a possibility that this residue may become surface exposed through capsid breathing, which has been demonstrated for residues at the N terminus of VP1 on other Picornaviruses (section 1.8). This could also explain the presence of other residues at the N terminus of VP1 that appear to be highly diverse. When those residues showing 100% diversity are examined on VP2 and VP3 it is apparent that only one residue on each exhibits this level of diversity and in each case that they are surface exposed. The residue of VP2 showing this level of heterogeneity is located within the immunodominant antigenic site two (figure 3.6), while on VP3 the residue is 60 (figure 3.7), which is associated with heparan sulphate binding *in vitro* (section 1.2.2).

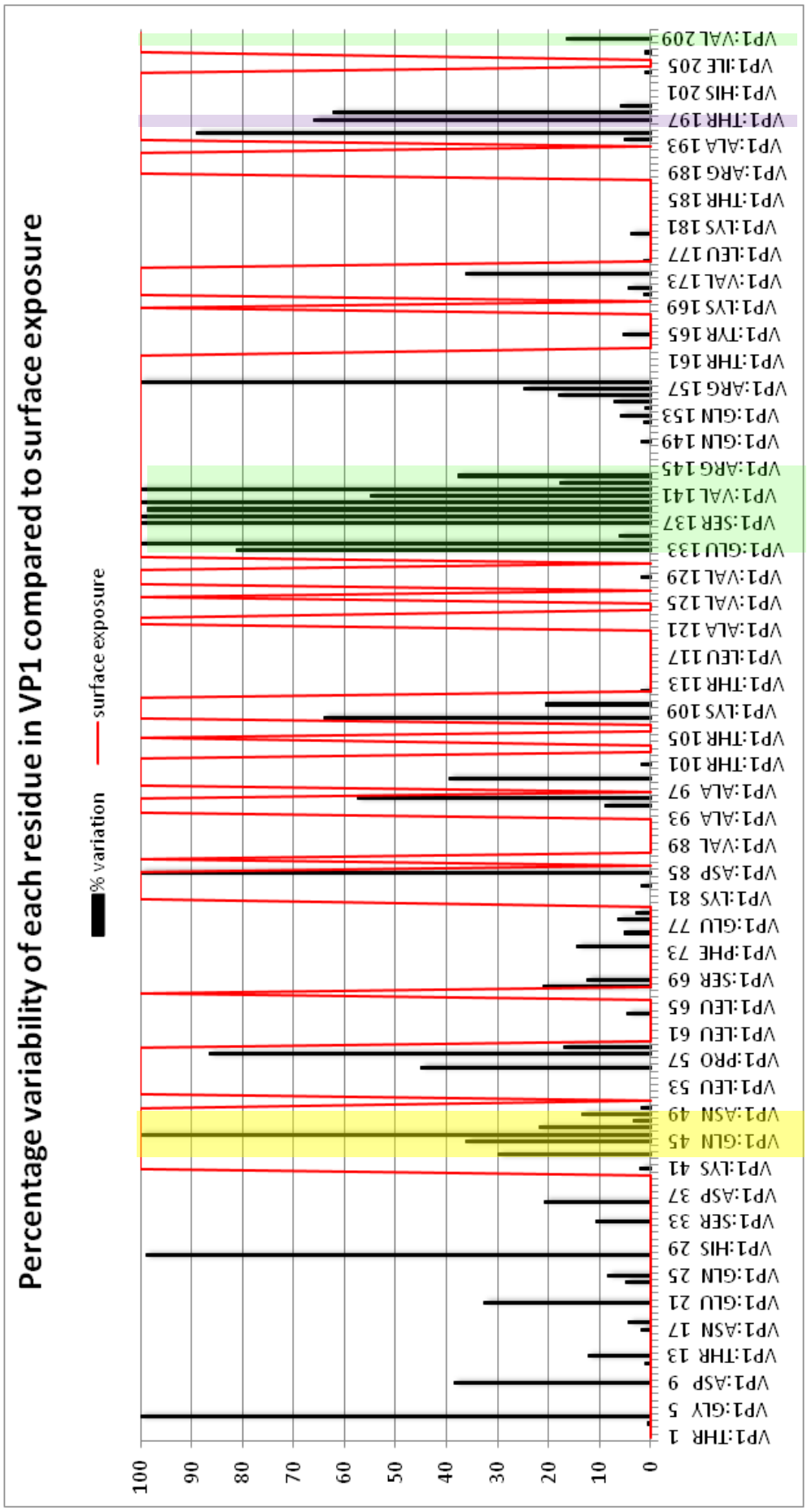


Figure 3.5: Graph comparing the percentage variation seen in VP1 to the surface accessibility of each residue. Variation calculated using the Risler scoring matrix (Risler et al., 1988). The regions where known antigenic sites are located are highlighted in yellow (site 3) and green (site 1 & site 5). The epitope identified at residue 198 is coloured purple Surface exposure at 0 on the X axis denotes that the residue is buried, while 100 represents exposure.

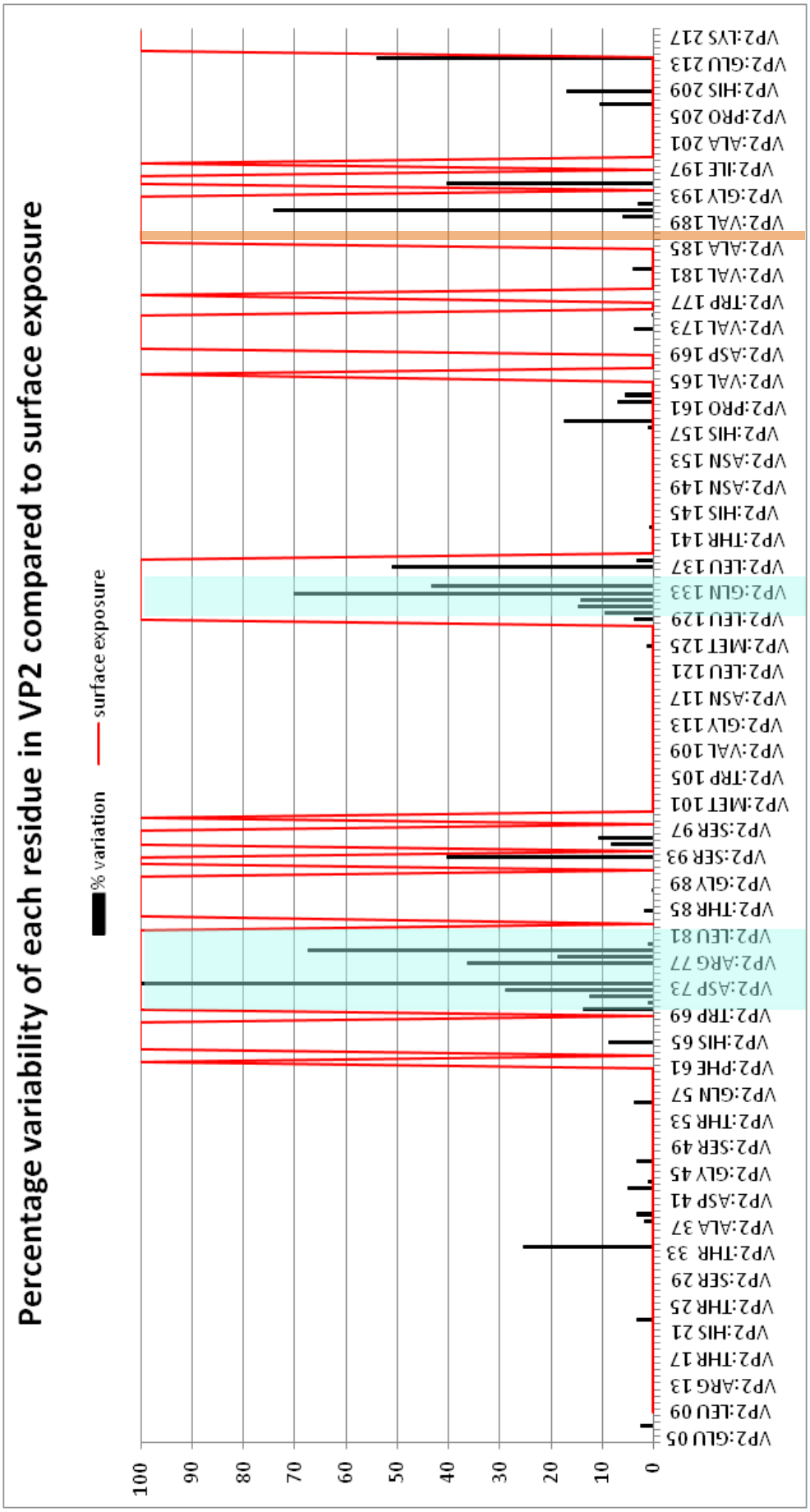


Figure 3.6: Graph comparing the percentage variation seen in VP2 to the surface accessibility of each residue. Variation calculated using the Risler scoring matrix (Risler *et al.*, 1988).The regions where antigenic sites are located are highlighted in blue (site 2) the epitope at 188 is coloured orange. Surface exposure at 0 on the X axis denotes that the residue is buried, while 100 represents exposure.

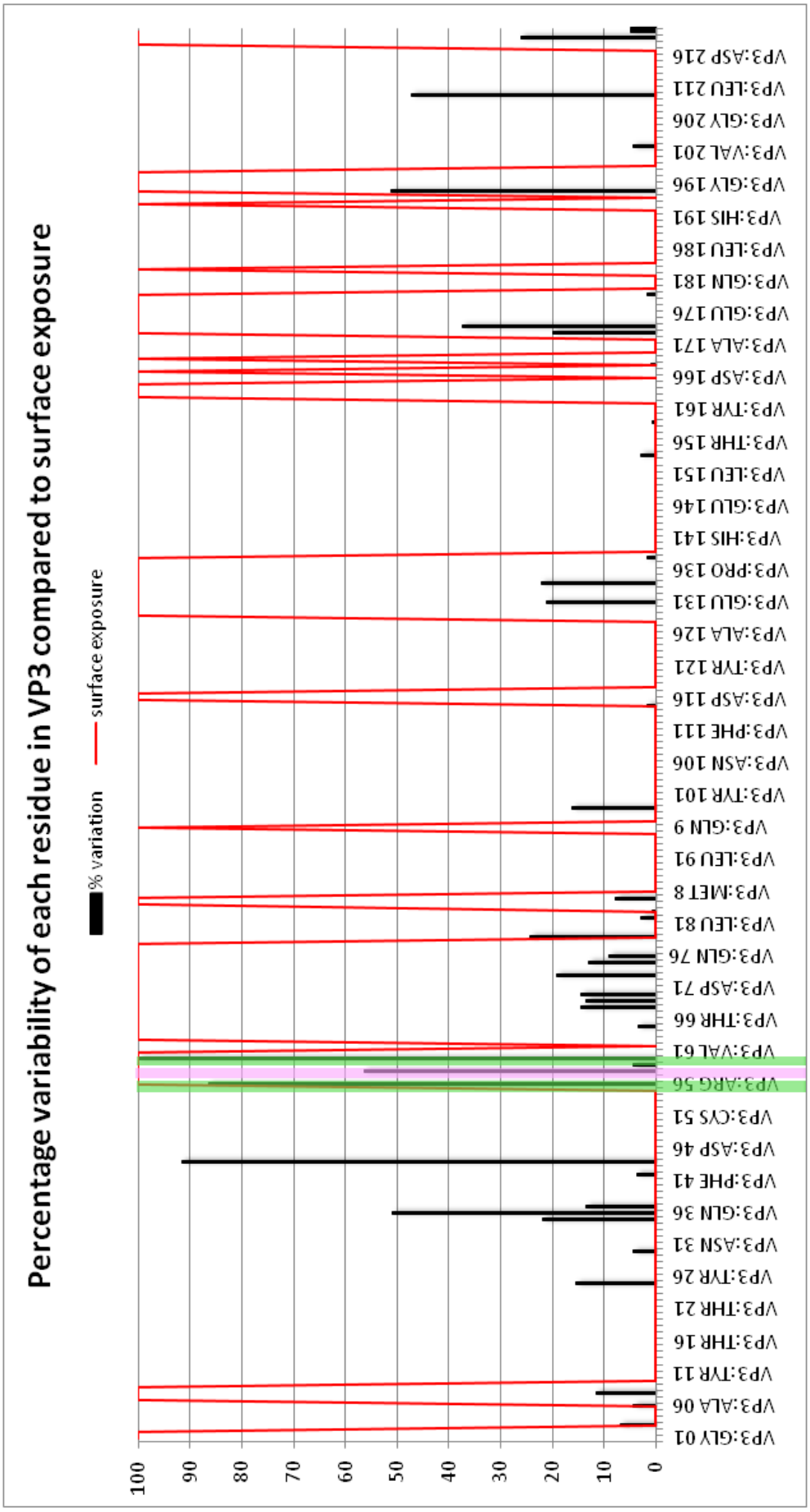


Figure 3.7: Graph comparing the percentage variation seen in VP3 to the surface accessibility of each residue. Variation calculated using the Risler scoring matrix (Risler *et al.,* 1988). The regions where antigenic sites are located are highlighted in pink (site 4). Two of the residues associated with Heparan sulphate binding are also shown in green. Surface exposure at 0 on the X axis denotes that the residue is buried, while 100 represents exposure.

The increased diversity of VP1 is due primarily to the presence of the immunogenic and highly variable GH loop, which contains a hyper-diverse region (residues 133 to 144) just prior to the RGD motif, used for receptor binding *in vivo*, and is also the location of antigenic sites 1(a) and 5 ((Crowther *et al.*, 1993b; Kitson *et al.*, 1990)-figure 3.5). Residues around VP1 43-45, 48 and 209, which form antigenic sites 3 and 1(b) respectively (Kitson *et al.*, 1990), along with the epitope at VP1 198 (Aktas & Samuel, 2000), are all located on diverse regions of the genome. The other previously defined antigenic sites on both VP2 and VP3 are also located at diverse regions of the genome (figures 3.6 and 3.7). However, the previously described epitope identified using bovine mAb's at VP2 188 ((Barnett *et al.*, 1998)-figure 3.6) appears to show no diversity.

Several other residues not previously described as antigenic, and that are not located adjacent to any antigenic sites, but which are surface exposed also show a high level (>50%) of diversity. On VP1 these are residues 58, 85, 96, 108, 158 and 195 (figure 3.5), on VP2 these are residues 138, 191 and 214 and finally VP3 residue 195 also has >50% diversity (figures 3.6 and 3.7). VP3 35 and 44 are also highly diverse; however neither of these residues are located on the surface.

In addition there are several areas of the surface that are exposed but show low levels of diversity, including VP1 52-54, 80-84, 122-128 and 190-192, VP2 64-68, 170-178 and 215-218 and VP3 163-168 and 175-178. These regions are interesting as they may provide sites to target for the future design of more cross reactive vaccines.

3.3.2 Comparison of the VNT and ELISA data.

3.3.2.1 Overview of the VNT and ELISA results.

Both the VNT and LPBE assays were performed in duplicate against the 5 different sera (as described in the Methods section). The O KIC serum was tested against 103 of a total of 105 viruses for both the VNT and LPBE. The O BFS and O UKG serum were tested against 62 viruses for the VNT[1] and 83 viruses for the LPBE. The O Uga 2002 and Ken 1978 sera were tested only in the LPBE against 49 viruses due to time constraints.

The mean serum titres against the homologous viruses from which the serum were raised were generally 0.3 $\log_{10}$/ml higher in the LPBE than the VNT (table 3.1), suggesting that the neutralising antibody component made up approximately 50% of the whole antibody response observed. However such a comparison may not be valid as the two tests are qualitatively different. The serum titres with the O UKG virus were the highest observed in the VNT while all the other serum titres were similar to each other. The O Ken 1978, O Uga 2002 and O UKG mean serum titres were similar to each other while the O KIC and O BFS titres were lower but similar to each other in the LPBE.

Homologous virus	Serum titre (VNT)	Serum titre (LPBE)
O KIC	2.06	2.55
O BFS	2.11[1]	2.44
O UKG	2.75[1]	3.19
O Ken 1978	N/A	3.37
O Uga 2002	N/A	3.35

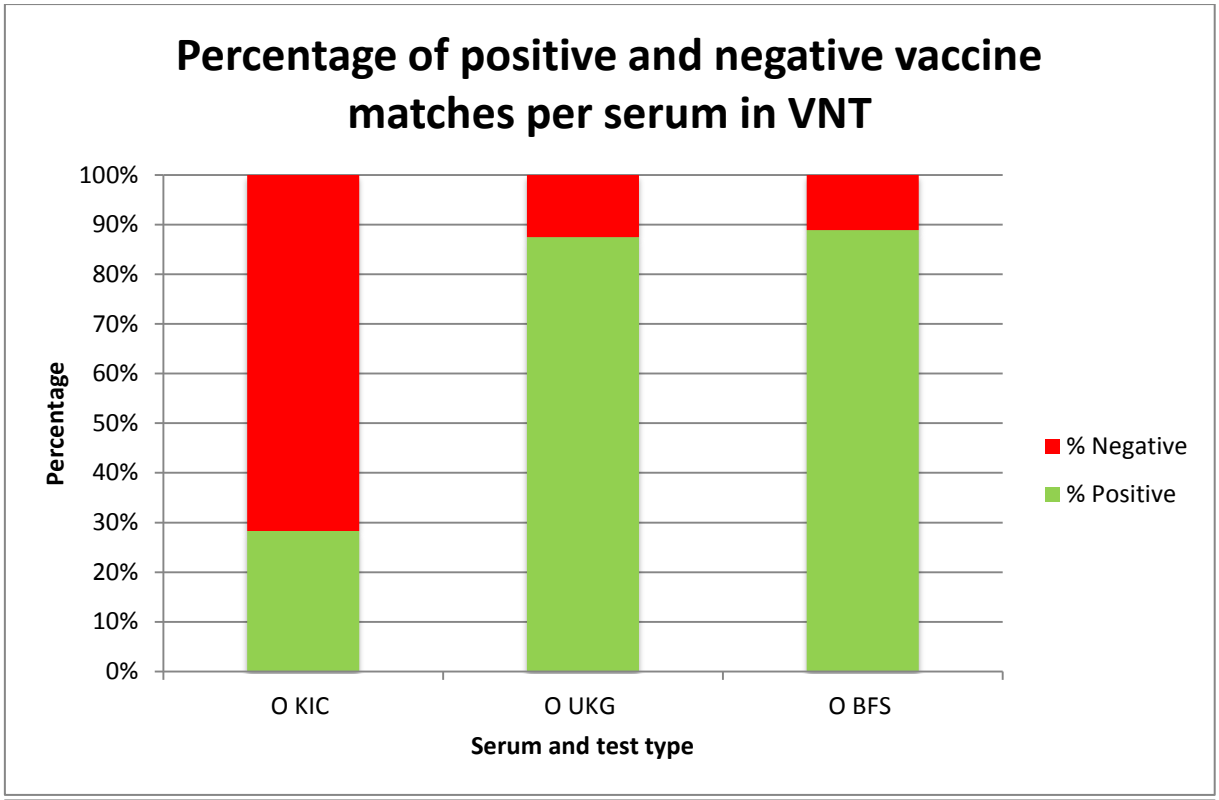
Table 3.1: Table showing the mean serum titres of the homologous viruses (from which the serum was raised) in both the VNT and LPBE assays.

The O KIC sera had the lowest number of vaccine matches (for a review of vaccine matching and FMDV see section 1.10.2) both in the VNT and LPBE, with 28.3% in the VNT and 0.94% or 18.8% at LPBE vaccine match thresholds of 0.4 and 0.2 respectively. This contrasts with 88.8% and 87.5%, for the O BFS and O UKG sera in the VNT and 58.7%, 39.1%, 45.8% and 72.9% for the O BFS, O UKG, O

[1] Work carried out by Sasmita Upadhyaya

Ken 1978 and O Uga 2002 sera in the LPBE at a threshold value for a vaccine match of 0.4 and 88.8%, 75%, 79% and 87.5% at a threshold for vaccine match of 0.2 (Figure 3.8). For those sera tested in both assays the LPBE reported far fewer vaccine matches than the VNT at an r_1 value threshold of 0.4 however when the threshold for a vaccine match was 0.2 the reported number of vaccine matches was closer to that observed in the VNT, with the percentage of viruses being a vaccine match against the O BFS serum being identical. Overall, with the exception of O KIC, each vaccine would be expected to provide a broad level of cross reactivity to other serotype O viruses, consistent with previous investigations (Doel, 2003). It should be noted here that the comparison between the percentages of vaccine matches for the VNT and LPBE do not represent agreement of the tests for the vaccine match of individual viruses. This is discussed in detail later.

Figure 3.9 shows a heatmap image representative of all the r_1 values for each of the sera tested. From the O KIC VNT and LPBE results it is evident that the r_1 values obtained for all the heterologous viruses are antigenically distant to the homologous virus (figure 3.9). In the LPBE and VNT, the homologous O KIC virus had the highest r_1 value. Even viruses that were thought to be closely related to the O KIC virus had an r_1 value that is indicative of a lack of enough cross reactivity to provide protection. This included the heterologous O Kaufbeuren (O KWT) virus from which the O KIC virus was initially derived, which had an r_1 value of 0.25 in the VNT and 0.17 in the LPBE. The results for the O KIC data contrasts not only with the data from the other serotypes but also with the general perception that serotype O viruses are not as antigenically diverse as some other serotypes (Doel, 2003).



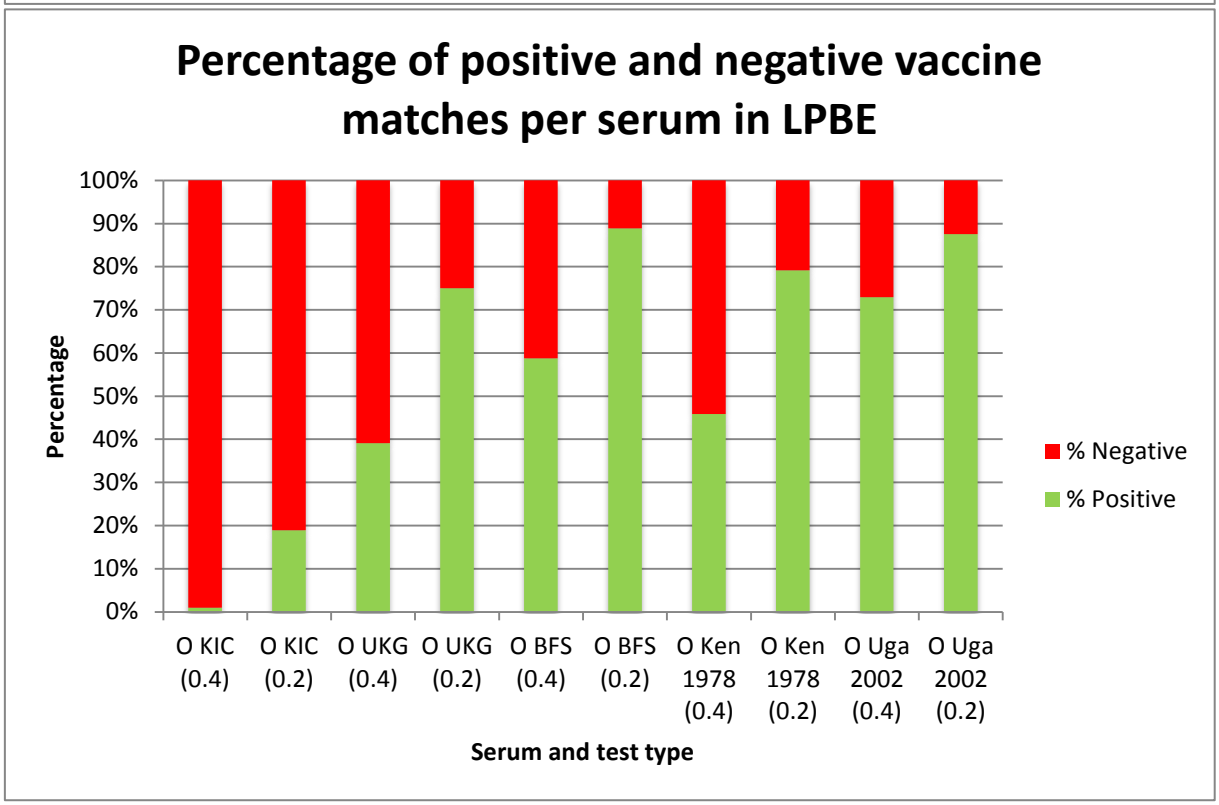


Figure 3.8: Percentage of heterologous viruses for each vaccine that are positive or negative for vaccine match by the FAO gold standard tests, the VNT (A) and LPBE (B), both when the threshold for vaccine match in the LPBE is 0.4 and 0.2. The LPBE threshold is shown in brackets.

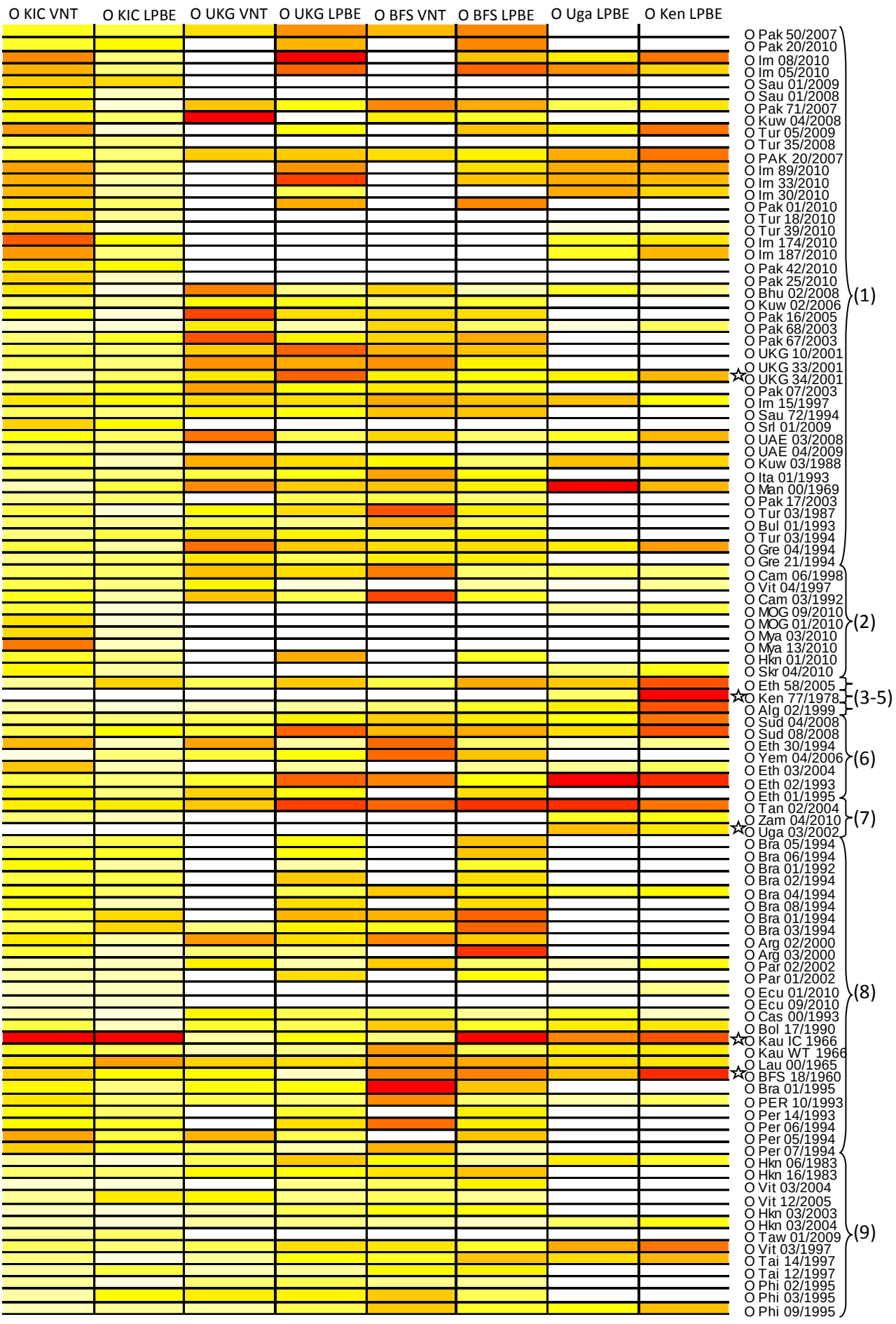

O KIC VNT
O KIC LPBE
O UKG VNT
O UKG LPBE
O BFS VNT
O BFS LPBE
O Uga LPBE
O Ken LPBE
O Pak 50/2007
O Pak 20/2010
O Irn 08/2010
O Irn 05/2010
O Sau 01/2009
O Sau 01/2008
O Pak 71/2007
O Kuw 04/2008
O Tur 05/2009
O Tur 35/2008
O PAK 20/2007
O Irn 89/2010
O Irn 33/2010
O Irn 30/2010
O Pak 01/2010
O Tur 18/2010
O Tur 39/2010
O Irn 174/2010
O Irn 187/2010
O Pak 42/2010
O Pak 25/2010
O Bhu 02/2008
O Kuw 02/2006
O Pak 16/2005
O Pak 68/2003
O Pak 67/2003
O UKG 10/2001
O UKG 33/2001
O UKG 34/2001
O Pak 07/2003
O Irn 15/1997
O Sau 72/1994
O Srl 01/2009
O UAE 03/2008
O UAE 04/2009
O Kuw 03/1988
O Ita 01/1993
O Man 00/1969
O Pak 17/2003
O Tur 03/1987
O Bul 01/1993
O Tur 03/1994
O Gre 04/1994
O Gre 21/1994
O Cam 06/1998
O Vit 04/1997
O Cam 03/1992
O MOG 09/2010
O MOG 01/2010
O Mya 03/2010
O Mya 13/2010
O Hkn 01/2010
O Skr 04/2010
O Eth 58/2005
O Ken 77/1978
O Alg 02/1999
O Sud 04/2008
O Sud 08/2008
O Eth 30/1994
O Yem 04/2006
O Eth 03/2004
O Eth 02/1993
O Eth 01/1995
O Tan 02/2004
O Zam 04/2010
O Uga 03/2002
O Bra 05/1994
O Bra 06/1994
O Bra 01/1992
O Bra 02/1994
O Bra 04/1994
O Bra 08/1994
O Bra 01/1994
O Bra 03/1994
O Arg 02/2000
O Arg 03/2000
O Par 02/2002
O Par 01/2002
O Ecu 01/2010
O Ecu 09/2010
O Cas 00/1993
O Bol 17/1990
O Kau IC 1966
O Kau WT 1966
O Lau 00/1965
O BFS 18/1960
O Bra 01/1995
O PER 10/1993
O Per 14/1993
O Per 06/1994
O Per 05/1994
O Per 07/1994
O Hkn 06/1983
O Hkn 16/1983
O Vit 03/2004
O Vit 12/2005
O Hkn 03/2003
O Hkn 03/2004
O Taw 01/2009
O Vit 03/1997
O Tai 14/1997
O Tai 12/1997
O Phi 02/1995
O Phi 03/1995
O Phi 09/1995
(1)
(2)
(3-5)
(6)
(7)
(8)
(9)

Figure 3.9: Heatmap image showing the r_1 values of each virus, ordered by topotype (as figure 3.1) to the various different serum and tests (columns). r_1 values are coloured from highest (red) to lowest (light yellow). Boxes coloured white indicates that virus was not tested against a specific serum. The topotypes are indicated by the braces on the right of the virus names. (1) is Middle East–South Asia (2) is South-East Asia, (3) is East Africa 4, (4) is East Africa 1, (5) is West Africa, (6) is East Africa 3, (7) is East Africa 2, (8) is Europe–South America and (9) is Cathay: China and the east Tartary. Viruses marked with a star are the homologous viruses. The O KIC virus is named as O Kau IC/1966 on this heatmap.

The O Ken 1978 virus had the highest recorded r_1 value against its homologous serum. However, for the remaining sera, in either the VNT or the LPBE, the homologous virus did not have the highest r_1 value against the respective serum (the highest titre is shown in red on the heatmap). There are many heterologous viruses that, after two repeats appear to have a level of cross reactivity with the serum higher than the homologous virus. The O UKG VNT serum titres had the highest number, with 35% of viruses having an r_1 value of greater than 1 (the homologous value), the O Uga 2002 LPBE results reported the same with 20% of viruses tested. Overall 17% and 6% of the O BFS VNT and LPBE r_1 values were also above one. The virus with the highest r_1 value against the O BFS serum in the LPBE was the O KIC virus, which contrasts with the reactivity of the serum generated against this virus. The least number of viruses with an r_1 value greater than 1 (excluding the O KIC serum) were reported in the O UKG LPBE (6%).

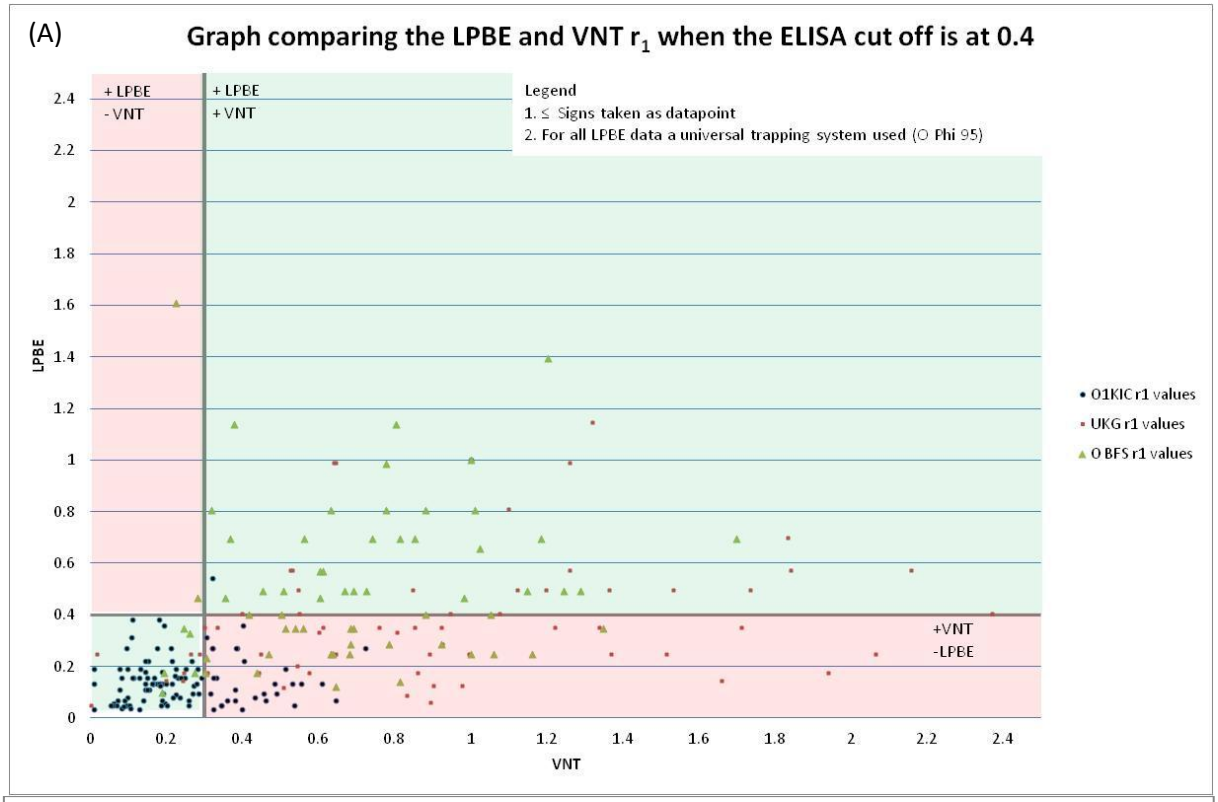
The heatmap (figure 3.9) also shows the cross-reactivity of all the viruses to each serum, with the viruses grouped according to the topotypes defined in the phyogenetic tree. This shows that in several instances viruses that are genetically divergent can be closely related antigenically. For example, the O BFS VNT serum results are strongly cross reactive to viruses both within the European-South American topotype but also viruses of the East Africa-3 topotype and a couple of viruses of the South East Asia topotype. This can be observed for many of the sera tested. Generally, all topotypes showed some cross reactivity of viruses to serum from other topotypes. The viruses that appear to have the least cross reactivity to others are the Cathay topotype viruses. These results, and the large changes in the level of serological cross reactivity observed even between

viruses that are genetically very similar appears to show that there is not a strong correlation between the genetic and antigenic relationship of serotype O viruses. This is consistent with previous investigations, discussed in section 1.6.2.

3.3.2.2 Comparison of the VNT and ELISA r_1 values for each virus.

For each heterologous virus the agreement of the VNT and LPBE vaccine matching was compared using the r_1 values generated for the three sera for which both the VNT and LPBE results had been collected using two different threshold values for the LPBE (0.4 and 0.2- section 1.10.2). Previous studies have suggested that although a vaccine match threshold value of 0.4 is generally indicative of a good level of cross protection, vaccine matches with a reciprocal value as low as 0.2 may also provide some level of protection (Ferris & Donaldson, 1992). The aim of these comparisons was to evaluate which of these thresholds correlate better with the results of the VNT.

When the threshold for a vaccine match in the LPBE is 0.4 then the agreement between the VNT and LPBE is highest for the O KIC sera, with 72.9% agreement on vaccine match for each heterologous virus between both tests. For the O BFS and the O UKG serum the agreement was poorer, with only 63.5% of and 46.9% of the tests in agreement for the vaccine match for each heterologous virus (Figure 3.10(B)). However when the threshold for a vaccine match is 0.2 the agreement between the VNT and LPBE for both the O BFS and O UKG sera increases greatly with 87.3% and 75% of tests respectively now in agreement on vaccine match. The level of agreement for the O KIC vaccine matches in contrast decreases slightly to 70% (Figure 3.11(B)).



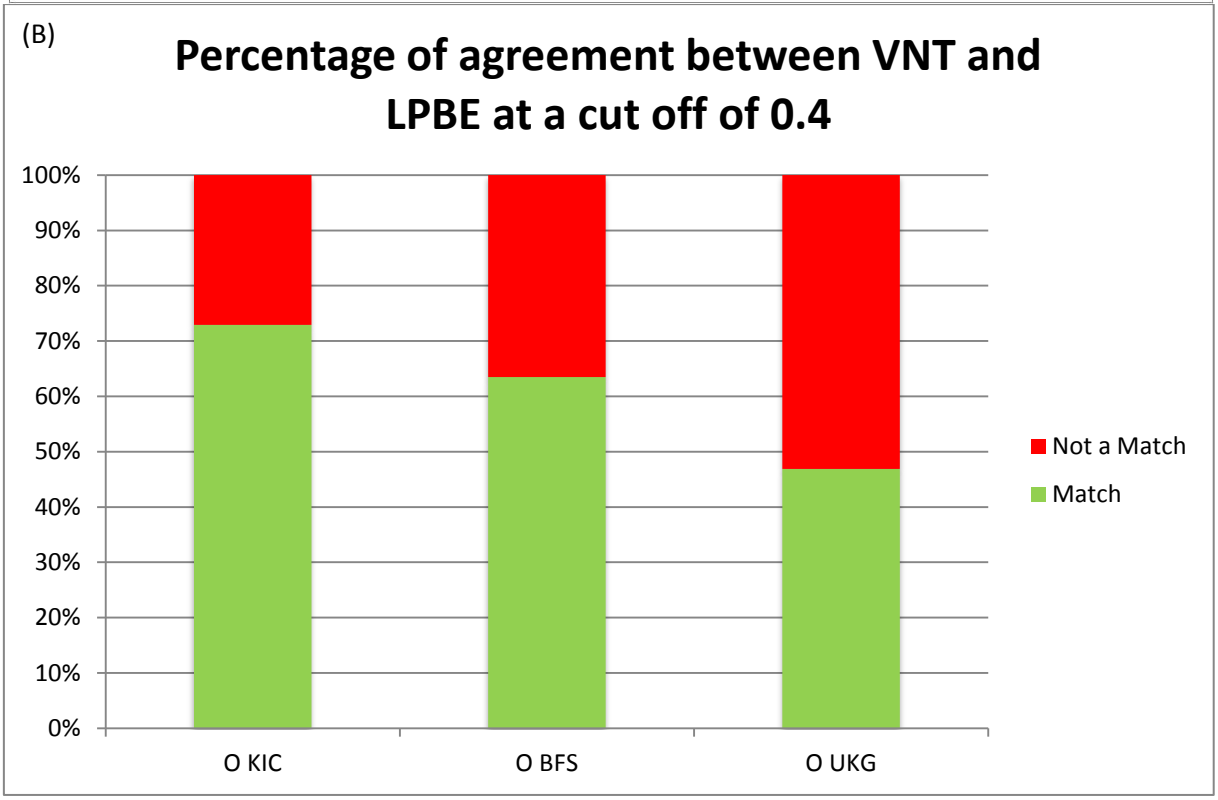


Figure 3.10: Graphs showing the level of agreement over vaccine match between the VNT and LPBE tests with antisera against three viruses (O KIC, OBFS, O UKG) when the threshold for the LPBE is set at 0.4. (A) shows the r_1 values for each virus in both the VNT (x axis) and LPBE (y axis). Those in the green boxes highlight agreement in between the VNT and LPBE, those in the red boxes do not agree. (B) shows the percentage agreement of the VNT and LPBE for each serum.

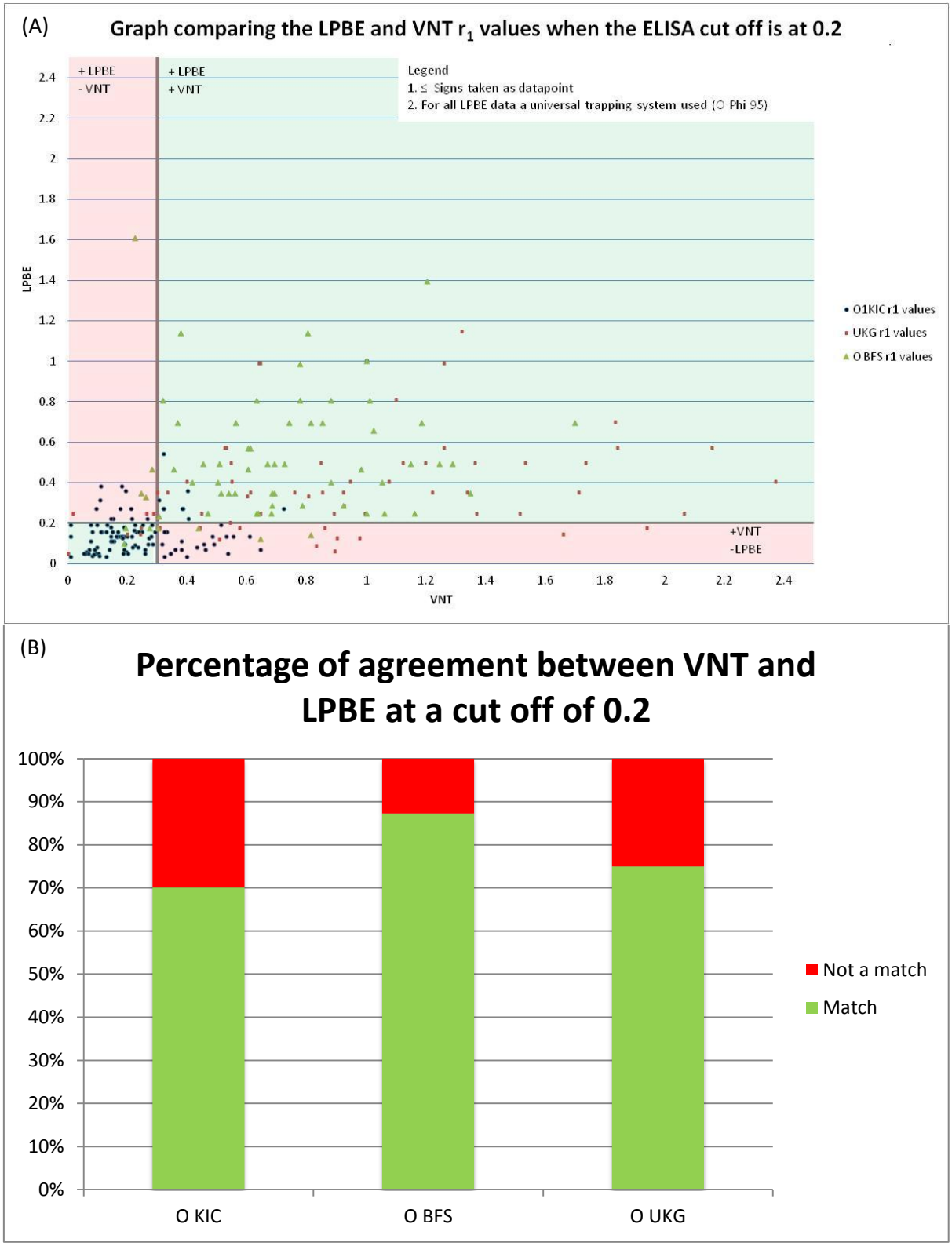


Figure 3.11: Graphs showing the level of agreement over vaccine match between the VNT and LPBE test when the threshold for the LPBE is set 0.2. (A) shows the r_1 values for each virus in both the VNT (x axis) and LPBE (y axis). Those in the green boxes highlight agreement in between the VNT and LPBE, those in the red boxes do not agree. (B) shows the percentage agreement of the VNT and LPBE for each serum.

When the individual r_1 values for each heterologous virus, generated in both the VNT and LPBE, are compared it can be seen that in many cases the r_1 value can vary greatly between the tests (figures 3.10(A) and 3.11(A)). For all the sera tested, there was a large percentage of heterologous viruses that were positive for vaccine match in the VNT but negative in the vaccine match in the LPBE, when the vaccine match threshold of 0.4 is applied to the LPBE data (table 3.2), with the highest reported for the O BFS serum of 49.2%. Conversely at this threshold for LPBE vaccine match there are essentially no heterologous viruses that are vaccine match positive in the LPBE, but not a vaccine match in the VNT (table 3.2). When the LPBE threshold for vaccine match is set to 0.2 then the numbers vaccine match positive in one test but negative in the other become more comparable, but there is still a higher percentage VNT vaccine match positive and LPBE vaccine match negative than vice versa against the O KIC and O UKG sera, although for the O BFS sera the number positive in either test but negative in the other reach parity (table 3.2).

Serum	% VNT positive, LPBE negative @ 0.4	% VNT positive, LPBE negative @ 0.2	% LPBE positive (@0.4), VNT negative	% LPBE positive (@0.2), VNT negative
O KIC	27.4	19.3	0	10.3
O UKG	49.2	18.8	0	6.25
O BFS	33.3	6.34	3.17	6.34

Table 3.2: Table showing the percentage of heterologous viruses that were vaccine match positive in the VNT assay, but negative for vaccine match in the LPBE assay at the two LPBE vaccine match r_1 value thresholds of 0.2 and 0.4. The percentage of viruses that were LPBE vaccine match positive but negative for vaccine match in the VNT are also shown.

An r_1 value of greater than 1 (higher than that of the homologous virus the serum was raised against) was recorded for a heterologous virus in the VNT when a r_1 value lower than 0.4 was reported in the LPBE (vaccine match threshold) on nine out of 64 comparisons (14%) for the O UKG data and for 3 out of 63 comparisons (4.76%) for the O BFS data (Figure 3.10(A)). When the vaccine match threshold is lowered to 0.2 for the LPBE this incidence decreases to only 3 comparisons (4.68%) in the O UKG data and none the BFS data (Figure 3.11(A)). There is only a single occasion when an LPBE r_1 value of greater than 1 was recorded where the VNT r_1 value was less than 0.3 (the

vaccine match threshold for the VNT), against the O BFS sera (figure 3.10 and 3.11 (A)). There was no occasion where an r_1 value of greater than one was reported against the O KIC sera in either test.

The above results demonstrate that the VNT is more sensitive at determining vaccine match than the LPBE at both thresholds, although the results are much more consistent when an r_1 value threshold of 0.2 is applied. However the comparisons of the two tests conducted here are relative to each other and not to *in vivo* results so it is difficult to draw any definitive conclusions.

3.3.3 Evaluation of the correlation between the number of amino acid sequence changes and the antigenic relationships of viruses in both VNT and ELISA.

To determine whether or not the accumulation of capsid amino acid changes occurring on the heterologous viruses used in this study has a link to the changes in antigenicity, the level of correlation was examined between the number of amino acid substitutions occurring and the level of antigenic variation recorded in the VNT and LPBE for each of the sera tested.

3.3.3.1 The level of correlation between sequence changes on the whole capsid and serum titre variation

For all of the sera tested, there was no significant correlation observed between the number of capsid amino acid substitutions and the changes in serum cross reactivity seen. This was the case for both the VNT (figure 3.12) and the LPBE (figure 3.13). The percentage of variation in the serum titre explained by the accumulation of sequence changes was in all cases below 20% (Table 3.3). Generally, the LPBE data had a lower correlation than the VNT data. The highest level of correlation was observed for the O UKG VNT (18.32%) with the lowest observed being the O Uga 2002 LPBE results (6.71%).

3.3.3.2 The level of correlation between sequence changes on the individual capsid proteins, antigenic sites and residues with greater that 50% diversity and serum titre variation

For all of the comparisons made no significant correlations were observed (Table 3.3). The correlation of the serological data to the variability of the amino acids across the individual capsid proteins was, in most cases less than that observed for the whole capsid. The number of substitutions occurring at the known antigenic sites and at those residues with a diversity of greater than 50% (calculated as described in section 3.2.4) also had a correlation worse than the changes at the whole capsid level, with just three exceptions. Generally the percentage variability accounted for by the variation in the number of residue changes occurring across the sequence was not higher than 20% (with just three exceptions across all the comparisons made- on VP2 and VP4 for the O KIC VNT results and on VP4 for the O BFS data) and in the majority of cases a percentage of less than 10% was recorded. This suggests that the number of changes occurring on any region of the capsid investigated here does not explain the variability observed and that a simple accumulation of amino acid changes does not drive the antigenic changes observed across this dataset.

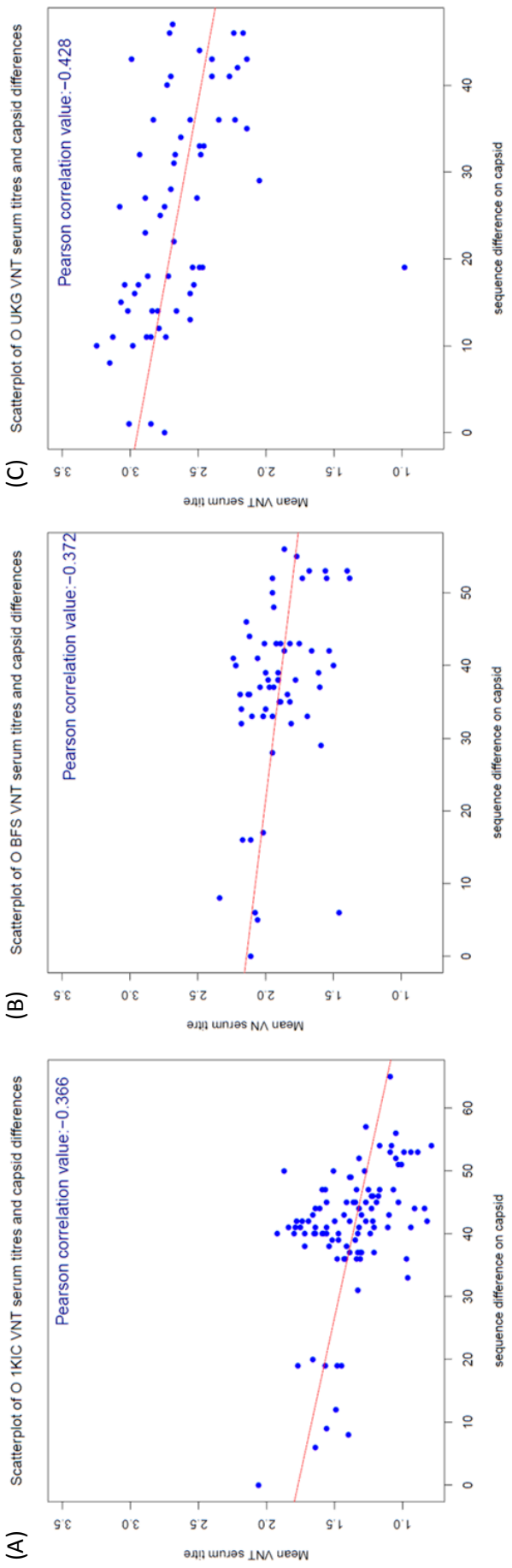
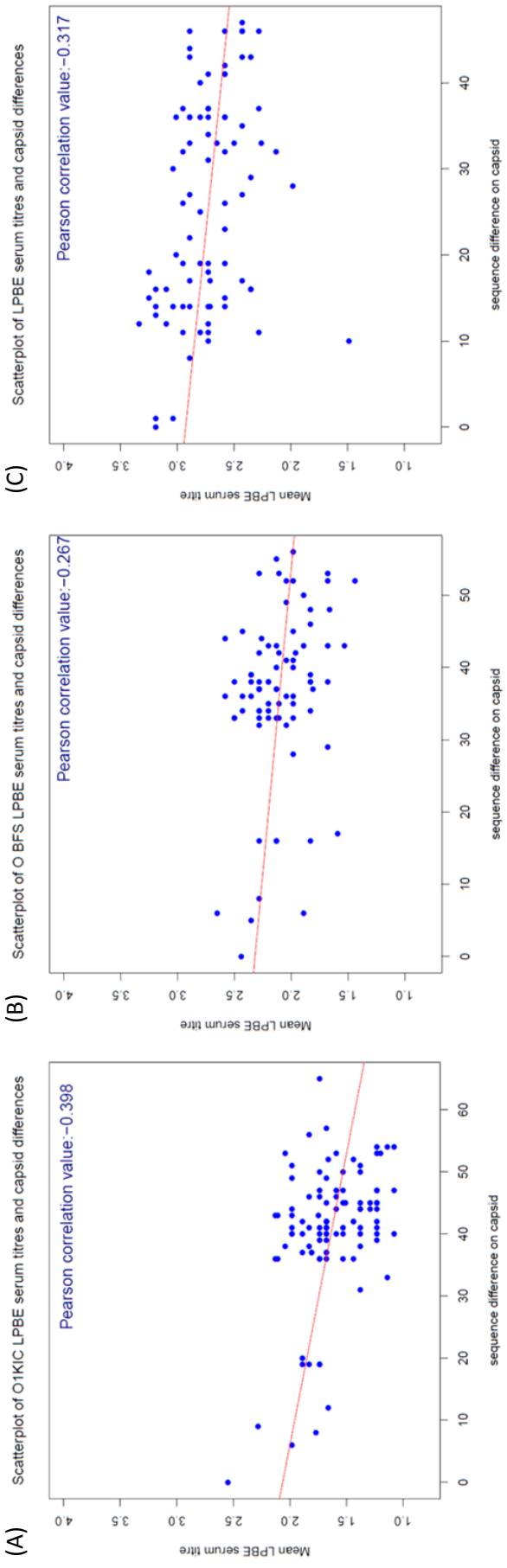


Figure 3.12: Scatterplot showing the relationship between changes in VNT seroreactivity within the O KIC (A), O BFS (B) and O UKG (C) serum and the number of amino acid changes on the whole capsid sequence of each virus tested. Individual solid blue dots represent the data for one virus. A best fit line is represented in red and the Pearson correlation value is given in the top right of the graph in dark blue



3.13: Scatterplot showing the relationship between changes in LPBE seroreactivity within the O KIC (A), O BFS (B), O UKG (C), O Ken 1978 (D) and O Uga 2002 (E) sera in the LPBE and the number of amino acid changes on the whole capsid sequence of each virus tested. Individual solid blue dots represent the data for one virus. A best fit line is represented in red and the Pearson correlation value is given in the top right of the graph in dark blue.

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	OKIC VNT	OKIC LPBE	O UKG VNT	O UKG LPBE	O BFS VNT	O BFS LPBE	O Uga 2002 LPBE	O Ken 1978 LPBE
Capsid	16.32%	15.84%	18.32%	10.05%	13.84%	7.13%	6.71%	12.46%
VP1	6.00%	12.25%	17.14%	11.63%	12.18%	7.67%	3.50%	11.09%
VP2	23.43%	8.47%	5.76%	4.08%	9.06%	5.81%	14.82%	12.46%
VP3	2.59%	4.16%	9.92%	2.19%	0.76%	0.16%	4.54%	15.13%
VP4	31.92%	2.62%	11.36%	2.40%	20.61%	9.42%	1.02%	0.85%
Ag sites	11.63%	12.3%	16.73%	12.67%	12.96%	6.40%	6.45%	7.78%
50%+ Div.	9.61%	11.42%	19.89%	9.86%	11.49%	3.28%	5.38%	13.03%

Table 3.3: Table showing the percentage of variation in the serum titres in each assay accounted for by the number of amino acid changes occurring across whole capsid, VP1-VP4, the previously described antigenic sites and those residues which have greater than 50% diversity

3.4 Discussion

The phylogenetic relationships demonstrate that the selected viruses are representative of the topotypes of serotype O and cover a wide geographic and temporal distribution. Additionally, these viruses have provided us with a broad spectrum of antigenicity, with a range of serum titres recorded. Some of the titres against heterologous viruses are higher than those for the virus homologous to the antiserum in question, consistent with previous studies for both the VNT (Barnett *et al.*, 2001; Rai, 1983; Rweyemamu & Ouldridge, 1982) and LPBE (Samuel, 2000). This kind of cross reactivity has also been reported in flaviviruses, where flavivirus serum in some instances reacts more strongly with heterologous viruses than the homologous ones (Calisher *et al.*, 1989; De Madrid & Porterfield, 1974). However, these reports, along with the examples previously reported for FMDV, offer no explanation for these findings.

One possible explanation for the phenomenon of "high avidity" (r_1 values greater than 1) viruses in the VNT is that the substitution of some amino acids on the surface could result in a loss of intermolecular antigenic competition between binding sites. If an epitope elicits a strong antibody response that is not very effective at neutralising or clearing the virus in question, it may out-compete a less immunodominant epitope for binding to antibodies that would be more effective at virus neutralisation or clearance. Substitutions that inhibit the binding of the dominant epitope, could remove competition and thereby enhance the binding to and neutralisation/clearance of virus. The potential presence of heteroclitic antibodies in the polyclonal serum response may also play a role. In addition, the ratio of live to dead virus particles may also have an effect in the VNT, with the numbers of antibodies available to neutralise virus reduced by binding to defective interfering (DI) virus particles; if the ratio of DI to live particles was 1:1, then half the available antibodies may bind to DI particles, it would therefore take double the volume of serum to neutralise the same number of live virus particles than if no DI particles were present. This could effectively increase the titre of serum needed to neutralise a heterologous virus to greater than that needed to neutralise the homologous virus. A similar scenario might be possible for the LPBE if antibodies bind to incomplete

capsid subunits within the viral suspension. However if either of these final explanations were the case then a virus would more than likely be of a higher avidity universally regardless of the serum tested, which is not the case here. All of this is speculative and in order to determine the underlying cause of these reactions further study is needed.

The genetic diversity of the capsid sequences determined here demonstrated that most of the diversity occurs on the surface of the virus. This is to be expected as these regions are most likely to come under selective pressures from the immune response. The loop regions that form part of the β barrel structures of the capsid proteins are the most exposed regions of the capsid and also seem the most able to tolerate amino acid substitutions without disrupting the structure. As expected, VP1 was the most genetically diverse of the capsid proteins, with the most diversity reported at the hypervariable region preceding the integrin binding RGD motif on the GH loop (antigenic site 1).

The other previously defined antigenic sites showed a high level of diversity, including around the immunodominant antigenic site 2 (Mahapatra *et al.*, 2012) located on VP2 and antigenic site 4 on VP3. The residues VP3 56 and 60, associated with adaptations that enable the binding of serotype O virus to heparan sulphate *in vitro* (section 1.2.4) are located on regions of high diversity. This could be explained by the varying levels of *in vitro* adaptation of the viruses studied, through culture in IBRS-2 cells. That all of the viruses did not change receptor affinity over the course of three passages seems to reflect the fact that, although the selective pressure is there for switching to heparan sulphate *in vitro,* viruses are still able to use integrin αvβ8 to infect IBRS-2 cells (Johns *et al.*, 2009), slowing the rate of adaptation. A change at residue VP3 56 from histidine to arginine is the key substitution involved in acquiring heparan sulphate binding capacity for enhanced cell culture infectivity. VP3 60 is also involved in the interaction (although no change associated with this binding has been attributed to VP3 60- section 1.2.2) and therefore the diversity seen at these two residues is unlikely to be associated with any naturally occurring selective pressures. However, at VP3 56

there is a change from histidine to tyrosine seen on the Cathay topotype viruses that has not been associated with receptor alteration *in vitro* and is therefore likely to be a natural substitution.

There are also several other highly diverse residues outside of the known serotype O antigenic sites. This could be an indicator that these sites are antigenic, as the high levels of diversity may be caused by selective pressure brought about by interaction of the capsid with antibodies. In addition some of these residues are known to be antigenic on other serotypes of FMDV. Both of these inferences suggest that some of these residues may well be epitopes on serotype O that are yet to be identified. Another interesting finding is that several regions on the surface are highly conserved between the O serotype viruses studied here. This is potentially of importance because these areas may provide a target for the development of more cross-reactive vaccines, if it was found that antibodies were raised to them. This could be achieved by removing some of the dominant antigenic sites driving the antibody response to target these more conserved epitopes.

Like VP1, VP3 appears to have several highly diverse residues outside of those areas that are surface exposed although VP3, out of all the surface exposed proteins, seems to have the least diversity in terms of percentage variability over all of its residues. For VP2 all of the highly diverse residues are located within surface exposed areas of the protein. One interesting residue that has a high diversity and was not located on the surface is VP1 4, which is normally buried internally. A recent study looking at mAb escape mutants for serotype A FMDV found a substitution on an escape mutant located at residue 3 of VP1 (Mahapatra *et al.*, 2011) and poliovirus antibodies binding to residues at the N terminus of VP1 that are normally buried have been identified (Li *et al.*, 1994). This suggests that this variability may be driven by immune selection via exposure of these residues during capsid breathing in a similar process to that observed for other Picornaviruses (section 1.8).

As expected for serotype O viruses there was generally a broad range of antigenic coverage provided by the vaccine strains (Doel, 2003). However, in contrast, the O KIC bovine vaccinate serum appeared to be antigenically distinct from all the viruses that were evaluated and in the case

of the LPBE only one virus was close enough to score positive as a vaccine match at a threshold of 0.4. This included not only viruses from different topotypes but also several viruses that were closely related phylogenetically. These viruses were anticipated to be closely cross reactive to this serum and included the wild type O Kaufbeuren (O KWT) virus. Upon comparison of the two sequences only 7 residues were variable between the two viruses. The causative amino acids behind this distinct antigenic phenotype will be investigated further in a later chapter.

Historical comparisons of vaccine matching by VNT and LPBE have found a good correlation; however, these have compared the serum titres generated in both tests without correlating the relationships with the homologous virus through the use of r_1 values (Hamblin *et al.*, 1986a; Hamblin *et al.*, 1987; Van Maanen & Terpstra, 1989). Significantly, when the generated r_1 values for each virus and vaccine were compared in this analysis, it was observed that in many cases the r_1 values for viruses in the LPBE and VNT can differ substantially. Nevertheless, in terms of predicting whether a vaccine is a match then the two tests appear to have a better correlation to each other when the threshold for declaring a positive vaccine match for the LPBE is 0.2, rather than 0.4. This is consistent with recent findings correlating *in vivo* protection and vaccine matching conducted with serotype A viruses that also suggest a r_1 value of 0.2 should be used for the LPBE (Mattion *et al.*, 2009). However, again this is not the case for the O KIC serum where a threshold of 0.4 had a marginally better level of agreement between the two tests. Overall, it would seem that the VNT is a more sensitive assay in terms of detecting numbers of positive vaccine matches and that based on these data the threshold for declaring a vaccine match in the LPBE should be set at 0.2, at least for serotype O FMDV's.

When the accumulation of sequence changes across the capsid, individual capsid proteins, the antigenic sites and those residues that showed > 50% diversity was compared to variation in the serum titres of the viruses it was apparent that there was very little or no correlation. However, this does not necessarily mean that correlations do not exist, just that they do not appear in the areas tested in this chapter and more work will be done in later chapters to try and identify these regions.

Additionally viruses that are genetically closely related can also be distantly related antigenically and vice versa. The above points suggest that rather than the changes in the antigenicity of type O FMDV being caused by the accumulation of sequence changes across the capsid, instead, a smaller number of dominant changes in the capsid sequence cause the phenotype changes, again consistent with previous investigations (see section 1.5.2). This demonstrates the need for more sophisticated methods to try and identify the dominant changes that are causative of the changes in antigenicity of serotype O FMDV, for which the data presented in this study provides a good, representative set for analysis.

Chapter 4: Generation of linear mixed effects model to predict antigenic relationships from Capsid sequence data and identify novel epitopes of serotype O FMDV.

Part of the work conducted within this chapter will be submitted for publication along with the results from chapter 7:

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The statistical modelling within this chapter was performed by Dr. Richard Reeve.

4.1 Summary.

FMDV vaccine selection is currently based on serological assays using bovine polyclonal vaccine antisera (BVS) , but inherent variability in both the assays and the antisera themselves can make the results poorly reproducible and difficult to interpret (as shown in the results from chapter three). This, along with the time constraints and the expense of using these techniques means that the development of other, additional tools for vaccine matching free from some of these constraints would be of great benefit. Recent work has shown that data similar to those generated in chapter three can be used to build a sequence based vaccine match model, that predicts vaccine match by quantifying the importance of amino acid changes at specific regions of the capsid that correlated with changes in cross reactivity for the SAT serotypes (Reeve et al., 2010- discussed in section 1.10.2). In addition this model was adapted to identify individual residues that are causative of the changes in sero-reactivity observed. In this chapter a similar model for vaccine matching and epitope prediction is developed for serotype O FMDV using the LPBE (but not the VNT due to confidentiality issues) and sequence data generated in chapter three.

The sequence based vaccine matching model, developed here for the O serotype, identified three surface exposed regions of the virus capsid where the number of changes to the amino acid sequence between viruses correlated with changes in serological cross-reactivity occurring between viruses across the LPBE data. However, using only the number of changes in amino acid sequence at these three regions of the capsid resulted in a model that predicted vaccine match significantly poorer than using data derived from a single replicate of the LPBE assay, when compared to the more statistically valid "best estimate" r_1 values developed in this chapter as part of the model development.

Adaptation of the above vaccine match model that uses the phylogenetic relationships between viruses to provide a structure relating each virus to one another through the sequence allowed the prediction of four antigenic residues for serotype O FMDV, one of these residues is a previously

defined epitope while another is located on the GH loop of VP1, which contains two known antigenic sites. The remaining two residues that were predicted as antigenic by this model are at sites not previously described as antigenic for serotype O. These predictions can now be validated using reverse genetics techniques. In addition a vaccine match model based just on amino acid changes occurring across the four residues predicted to be antigenic (above) performed better than the model developed using the three surface exposed regions of the virus capsid, resulting in a final model that performed as well at predicting r_1 values as the those from a single replicate of the LPBE assay. These results suggest that, with further refinement, this may make a suitable model for vaccine matching based on sequence data alone.

4.2 Methods

4.2.1. Serological data

The LPBE data used for the model development was generated in chapter three.

4.2.2 Sequence data

The sequence data used for the model development was generated in chapter three.

4.2.3 Determining the candidate regions used for model development

Firstly the surface exposure of each residue within the capsid was determined as described in section 2.6.1. Once the surface exposure of the residues was determined this data was applied to the sequences of the viruses selected for this study. The capsid sequences of the viruses to be examined were aligned using the Bioedit program (Hall, T. A. 1999) and the sequences of the three surface exposed capsid proteins (VP1-3) were further divided into linear sub-sequences based on surface exposure, starting from the first surface exposed residue and proceeding through the sequence in a linear fashion, stopping at a residue just prior to the first buried residue, forming sub sequence one and so on.

In applying this method the capsid protein sequences were divided into 47 linear contiguous surface exposed regions, made up of between 1-31 residues (see table 4.1). The VP1 GH loop is the longest of these (and contains antigenic site 1a and 5 of Serotype O- (Crowther *et al.*, 1993b; Kitson *et al.*, 1990) with most linear regions being between 4-10 amino acids long. It is these linear regions (and the changes occurring within them) that form the candidate regions assessed within the predictive vaccine matching model, with their antigenic significance and their correlation to r_1 values assessed.

VP1	VP2	VP3
Residues 41-50*	Residues 62-68	Residues 7-9
Residues 52-59	Residues 70-82*	Residues 56-61*
Residue 67	Residues 84-90	Residues 63-78
Residues 81-85	Residues 92-93	Residues 84-85
Residue 87	Residues 95-97	Residue 96
Residues 94-96	Residue 99	Residues 116-117
Residues 98-102	Residues 129-138*	Residues 129-138
Residue 105	Residue 166	Residues 163-165
Residues 108-111	Residues 170-175	Residue 167
Residues 122-123	Residue 178	Residue 169
Residue 126	Residues 186-193*	Residues 173-179
Residues 128-130	Residues 195-196	Residue 183
Residues 132-162*	Residue 198	Residue 193
Residue 169	Residues 215-220	Residues 195-198
Residues 171-175		Residues 218-220
Residues 189-192		
Residues 194-204*		
Residues 207-210*		

Table 4.1: The linear surface exposed regions from each of the surface exposed capsid proteins, VP1-3, that make up the candidate regions used in the model. * Denotes a region containing a known antigenic site/epitope

4.2.4 Statistical analysis.

All analysis carried out in this chapter was performed using the R statistical software package (R Development Core Team, 2009)) and the lme4 mixed effects modelling package (Bates & Maechler, 2010).

4.2.5 Phylogenetic analysis.

A phylogenetic tree was generated using the BEAST and tracer programs (Drummond & Rambaut, 2007). A relaxed uncorrelated exponential clock and a GTR+CP_{112}+ Γ_{112}+ I nucleotide model (Shapiro *et al.*, 2006) was used to construct the trees from the available nucleotide sequence data from chapter three. This model was identified as the best model previously in the SAT work (Reeve *et al.*, 2010).

4.3 Results

4.3.1 Generating 'best estimate' r_1 values.

Currently vaccine match is evaluated by determining the relationships of heterologous viruses to homologous strains by VNT and LPBE. The r_1 value of an heterologous virus is determined by comparing the serum titre of this virus to the serum titre of the homologous virus from which the serum was derived, generated as a control on the same day the heterologous titre was obtained, referred to as the results from a single 'day of test' within this chapter (see section 1.10.2 for a review of current methods for vaccine selection and section 2.2.6 for r_1 value calculation method). There are inherently a large number of variables within the LPBE (the test evaluated here) and in order to give confidence in the results the test is repeated at least twice for each heterologous virus by the same user. Using the homologous virus serum titre from the day the test was performed to generate the r_1 value of the heterologous virus should help control for any variation within a given test. The individual r_1 values from each day of test are then averaged to give a final result.

The LPBE work carried out between the five homologous viruses (and serum thereof) and heterologous viruses in chapter three resulted in generation of a final dataset comprising 865 replicates of individual titres (and r_1 values). This data was used to generate all the following models.

For this analysis, as with the work conducted on the SAT viruses (Reeve *et al.*, 2010), it was decided that a more statistical approach to generating r_1 values should be developed using a linear mixed effects model. This enables the comparison of data generated on different days by different users, taking into account both the fixed and random effects in order to give a set of "best estimates" for the r_1 values of an heterologous virus to a given homologous virus. This was performed by building a model to estimate the true underlying titres of these viruses using the mean LPBE serum titres of both the heterologous and homologous viruses throughout the tests as the response variable (data normally distributed-Lilliefors normality test $P>0.05$). The homologous virus, the

heterologous virus and the interactions between them were included as fixed effects. The inclusion of each of these parameters had a significant effect (P<0.05) on the model fit. One other parameter, the date the test was conducted, was found to have significant effects (P<0.05) on the titres obtained and so was included in the model as random effect. Once the true titres had been determined then the best estimate r_1 values could be calculated. These best estimate r_1 values represent a more precise way of estimating the true relationships between viruses and are taken forward as the gold standard dataset for the subsequent data analysis.

The final model used for the best estimate (true) titres (and hence the r_1 values) is as follows (equation 1):

$$\log(t_{p,c}) = \mu_{p,c} + \varepsilon_D + \varepsilon_R$$

Where $\varepsilon_i \sim N(0, \sigma_i^2)$

Where $\mu_{p,c}$ is the fixed effects of the mean titre of the homologous (p) and heterologous virus (c), ε_D is the best linear unbiased predictor (BLUP) of the random effect of the date the test was conducted and ε_R is the residual error not accounted for by the model. For each of these effects there is also an associated variance (σ_i^2). The ε_i part of the equation is assuming that each random factor is approximately normally distributed, which was reaffirmed by testing for normality of the residuals (data not shown). The results from this analysis identified that the sum of the variance that is normally encountered when using the standard procedure of comparing two r_1 values generated on different days (that is the random effects of date the test is conducted and the residual variation – the variation between sera is not a consideration as only one pooled serum is used for each vaccine strain) is 0.31 $\log_e$ which gives rise to confidence intervals (CI) for the estimation of an individual r_1 values of +/- 0.86 $\log_e$ titres (for a result from an individual test). Using the $\mu_{p,c}$ or the mean titres derived from all the repeat tests between a homologous and heterologous viruses resulted in smaller confidence intervals ranging from +/- 0.31 $\log_e$ for viruses with four repeats to a maximum of +/-0.66

$\log_e$ titres when a virus was only tested once. This indicates that the method developed here, like the model developed for the SAT data results in an improved method for estimated log titres.

4.3.2 The generation of linear mixed effects model for vaccine matching of serotype O viruses using the LPBE data.

In order to develop a predictive model of r_1 values for the LPBE data the equation used to generate best estimate r_1 values was adapted. The fixed effect of the interaction between the mean serum titres of both the heterologous and homologous viruses that was used to calculate the r_1 value ($\mu_{p,c}$) was removed and replaced with a predictive term based on the non-synonymous changes in capsid-coding amino acid sequence between the homologous and heterologous viruses used in the LPBE within the candidate regions identified in section 4.2.3.

Essentially each region was assessed within the model to determine the significance of the effect of the number of amino acid changes within the region on improving the fit of the predictive model to the best estimate titres of viruses (determined in section 4.3). The region that most improved the predictive model was added first, followed by other regions in a standard stepwise regression that sequentially added all regions deemed to have a significant impact in improving the predictive model.

The probability that the inclusion of a candidate region generated a prediction model that was significantly better than if it was not included was assessed by a likelihood ratio test, since the models were nested. For multiple tests, each with p-values p_i the statistic $-2 \Sigma \log p_i$ is expected to be χ^2 distributed with twice as many degrees of freedom as tests under the null hypotheses (Fisher, 1970). When this was not the case ($p<0.05$), then the best predictor that was individually significant (after a Holm-Bonferroni correction for the number of terms (Holm, 1979)) were used as the basis for the next step of the regression. Essentially the most significant candidate region was included in the model as a fixed effect and the model run again to determine the next most important region. This regression was repeated until no more candidate regions could be added (as none of the remaining

regions were significant), thereby forming a predictive model with only a small number of the candidate regions.

This model differed from the model that was developed for the SAT-1 data (Reeve 2010), where the model had a better fit for the dataset when a random effect for the heterologous virus titres was included, which was not the case for this dataset. In this model the fixed effect of the interaction between the homologous and heterologous titres from equation one ($\mu_{p,c}$) was replaced by fixed effects for the reactivity of both the homologous and heterologous viruses and a predictive term based on the number of amino acid differences between the homologous and heterologous viruses at each of candidate regions.

The final model is (equation 2):

$$\log(t_{p,c}) = k_0 + \sum_{i=1}^{N} k_i \times d_i(p,c) + \mu_p + v_c + \varepsilon_D + \varepsilon_R$$

Where k_0 is the average titre, k_i is the regression coefficient of the count of changes occurring for the i^{th} region, d_i is the number of changes occurring between the homologous (p) and the heterologous (c) viruses, μ_p and v_c are fixed effects for each of the homologous serum and the heterologous viruses used. The random effects and variances thereof are the same as those listed In the equation for best estimate r_1 values.

The vaccine match LPBE model with the best fit for the data contained just 3 candidate regions, amino acid changes within which significantly correlated with the changes in cross reactivity observed within the serological data. The most significant region in the model was VP1 132-162 (represented as GH in the below equation), which is part of antigenic site one and antigenic site five (Crowther *et al.*, 1993b; Kitson *et al.*, 1990), this was followed by VP3 84-85, represented as DE in the below equation (believed to be the effect of the OKIC serum, which had an unusually low level of cross reactivity- chapter 3), and then VP1 194-204 (represented as CT in the below equation),

residues within which have been described as epitopes in the O, A and SAT-1 serotypes (Aktas & Samuel, 2000; Baxt *et al.*, 1989; Grazioli, 2006). The locations of the regions on the molecular surface of the virus are shown in figure 4.1.

The final equation for the LPBE r_1 value predictor is:

Equation 3:

$$r_1(p,c) = 0.964^{d}GH^{(p,c)} \times 0.864^{d}CT^{(p,c)} \times 0.637^{d}DE^{(p,c)} \times e^{v_p - v_c}$$

Where v_p and v_c were the fixed effects of the homologous virus (p) and the heterologous virus respectively (c). To determine the performance of the model a set of predictions were made using changes within those regions listed above to try and predict r_1 values, these were then compared with the r_1 values generated on the day of test against the "best estimate" r_1 values generated in section 4.3. Of the r_1 values predictions made by the model, using just the number of amino acid changes that occurred in the three regions (listed above), just 65% r_1 values fell within 95% Confidence limits of the best estimate r_1 values, which was significantly lower ($P<0.001$) than the r_1 values generated on the day of test, of which 74% fell within the confidence intervals (figure 4.2).

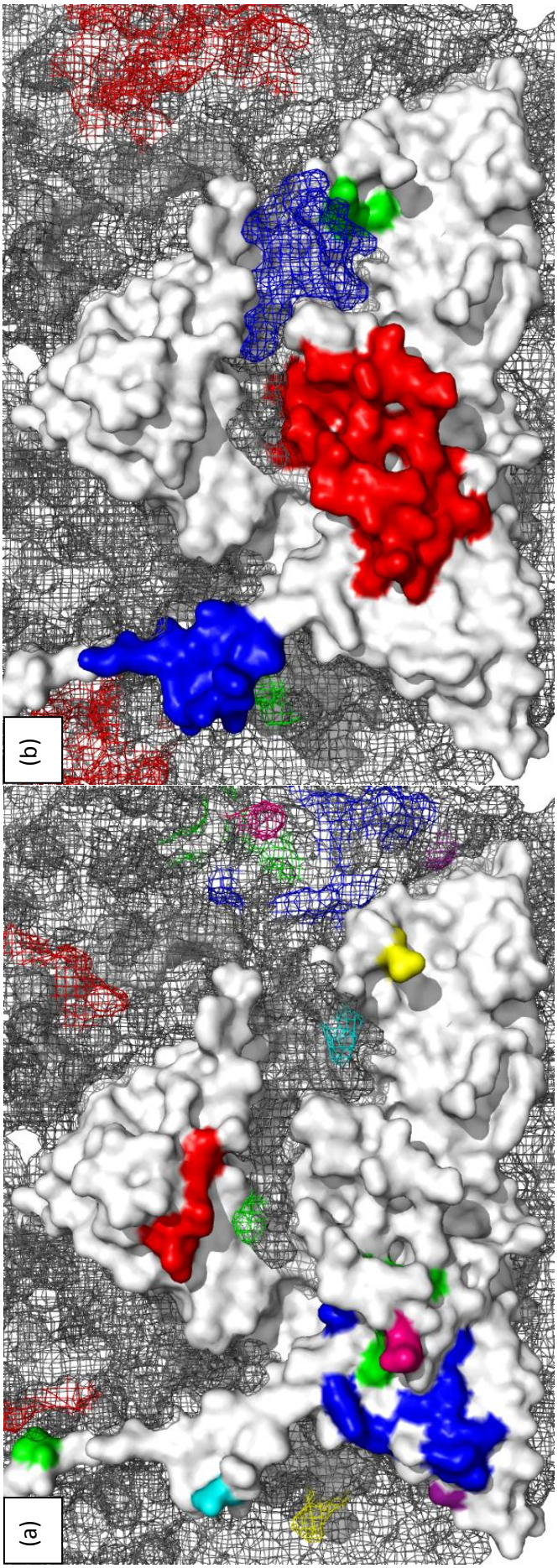

Figure 4.1: A side by side comparison of O BFS (PDB ID 1FOD) showing the molecular surface of the capsid, with a central protomer (in a reduced conformation with the GH loop present) represented in solid white and surrounding protomers as a grey mesh highlighting the known antigenic sites (a) Antigenic site one is coloured green, site two is blue, site three is red, site four is yellow and site five is pink. The additional residues identified from other studies are coloured purple (VP2 188) and teal (VP1 198). The location of each of the candidate regions selected for the LPBE vaccine match model are shown in (b); Blue is VP1 194-204, green is VP3 84-85 and red is the VP1 132-162.

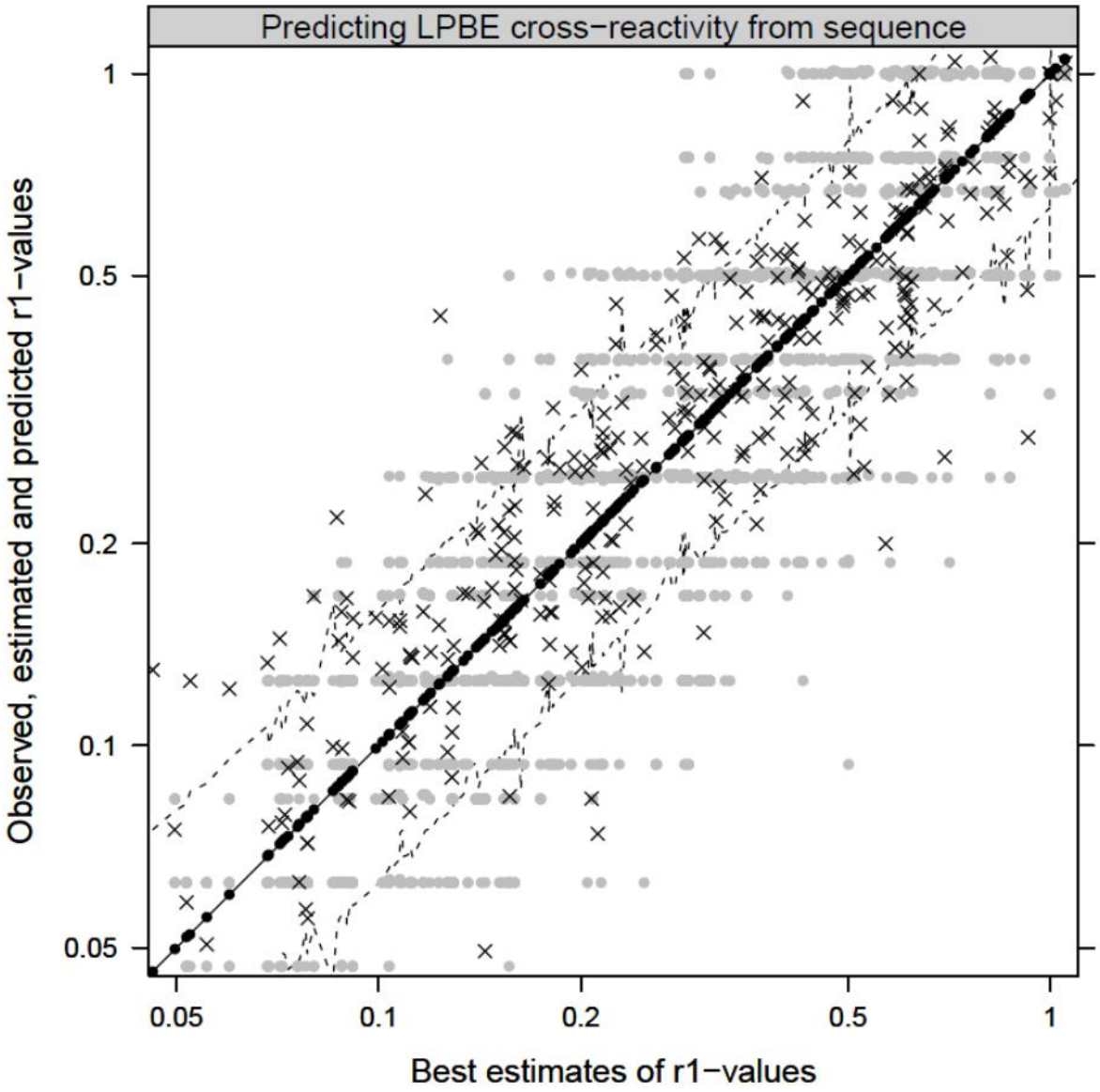

Figure 4.2: Predicted and actual r_1 values for the LPBE compared to the best estimate r_1 values. The predicted r_1 values used here were generated using changes in the three candidate regions that correlated significantly with changes in antigenicity observed. Bootstrap samples of individual serological r_1-values (Grey dots), predictions (black crosses) and matching best estimates (black dots) with the confidence intervals of the best estimates for type O r_1-values shown as the grey dashed line. Because of the log-normally distributed variance structure of the r1-values, data are plotted on a log scale.

4.3.3 Results of the model development for determining further epitopes using the LPBE and sequence data.

In order to determine epitopes, the first step is to control for the phylogenetic relationships between viruses, enabling the identification of the changes in amino acid sequence relating them. The previous model identified regions of the capsid, the number of amino acid changes within which correlate significantly to the changes in antigenicity observed across the LPBE data. However there is no way within this model to determine which (if any) of these correlative changes are directly responsible (causative) for any of the changes in cross reactivity observed. By inserting a phylogenetic control that uses the branches from a constructed phylogenetic tree as control terms the relationships between viruses becomes apparent, so that now the antigenic relationships of viruses to each other can only be caused by amino acid substitutions that occur across the branches of the phylogenetic tree that connect these viruses. This allows the identification of changes in cross reactivity between different viruses that have a common cause (that is the same mutation occurring in a specific branch of the tree); this also removes any repeated measures as each residue within a branch can only be significant once. A similar model was therefore generated to include a fixed effect for each branch on the phylogenetic tree (figure 4.3), which is a zero if the branch is not travelled between the homologous and heterologous viruses in question and a one if the branch is traversed. In order to do this a model similar to that developed for the vaccine matching can be constructed as follows (Equation 4):

$$\log(t_{p,c}) = k_0 + \sum_{i=1}^{N} m_i \times \delta_i(p,c) + \mu_p + v_c + \varepsilon_D + \varepsilon_R$$

Where m_i is the regression coefficient of the effect of substitutions occurring on each branch of the tree, δ is a delta function which is one if the homologous (p) and the heterologous (c) viruses are separated by branch i and zero in all other circumstances. The fixed effect, k_0, the other effects and variances thereof are as those listed in the best estimate r_1 value model (equation 2).

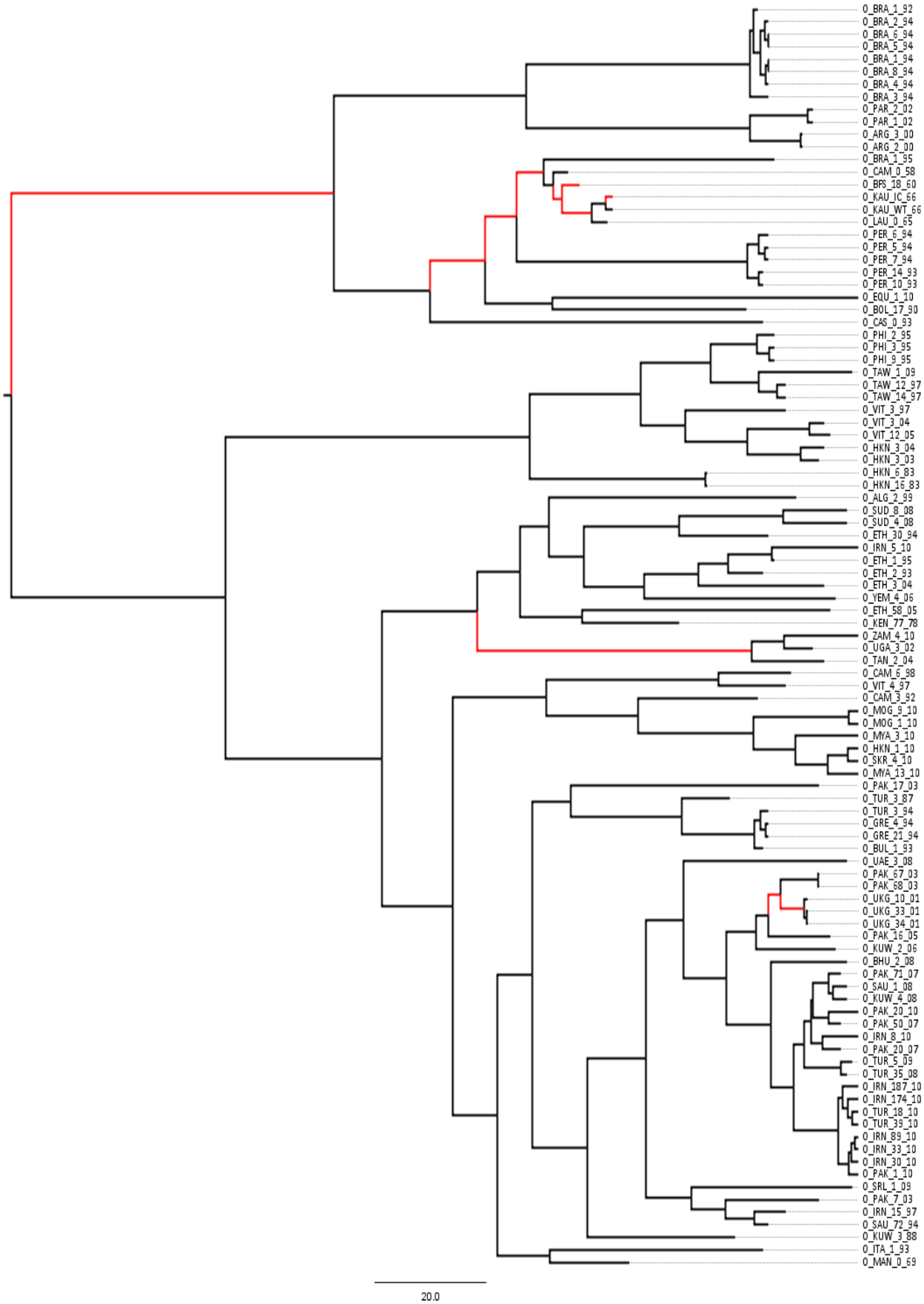

Figure 4.3: Phylogenetic tree with the branches that had a significant effect on the serum titres observed in the LPBE highlighted in red

The phylogenetic tree generated for use in this study used a more sophisticated model when compared to the tree developed in chapter three, however all of the viruses are still located within the same topotypes and the relationships between the topotypes is essentially the same. As with the SAT models there are only a limited number of homologous viruses so the number of branches explored is limited only to those that link the five homologous viruses to the heterologous viruses tested. In order to determine which branches (and hence the substitutions within them – all substitutions, at this point you do not know which individual residues are significant) are significantly associated with changes in cross reactivity and those that are not, all branches were added to the model and then removed one by one to finally include only those branches that were significant.

The final model for the serotype O LPBE data identified 11 branches of the phylogenetic tree that were associated with significant changes ($P<0.05$) in cross reactivity that occur between viruses (figure 4.3). Two of these significant branches lead directly to a single virus at terminal points on the phylogenetic tree, which equates to there being a significant difference between the virus on this branch and all other viruses caused by the sequence changes located on this branch. The first of these is the branching between the O KIC and O KWT (named O Kau IC_66 and O Kau WT_66 on Figure 4.3) respectively. The second terminal branch is located very close to the first and is again a branch that leads to one of the homologous viruses, O BFS. As discussed in chapter three there is indeed a substantial difference in serum titre between the O KIC virus and all other viruses tested, although this is not the case for the O BFS virus. However there is also a large amount of data for both of these viruses so branches associated with smaller changes in cross reactivity may be significant.

The remaining significant branches are located at internal points on the tree and represent significant antigenic differences between different clades of viruses. Two of these branches are at points where the branches divide into topotypes, the first of which is the branch that separates the European-South American topotype and the second is at the branch that separates the three viruses

belonging to the East Africa 2 topotype. This indicates that these topotypes differ significantly in antigenicity individually from all the other topotypes. Five other branches are located in the European-South American topotype, with the remaining two branches located within the Middle East-South Asia topotype just back from the terminal branch for the O UKG viruses, one of which (O UKG 34/01) was a homologous virus.

As mentioned above, that several of the significant branches are located at points leading to the homologous viruses does not necessarily mean that each of branches is associated with a large effect on cross reactivity, in fact there may be branches with larger effect sizes that are missed due to the lack of available data, however the large amount of data associated with each homologous virus means that even small changes in cross reactivity can be measured and observed as significant. It is also important to note here that the locations of these branches are an approximation, as using differing parameters to generate the phylogenetic tree may lead to different placement of branches on the tree.

Once the phylogenetic relationships between viruses has been controlled for then each of the 103 surface exposed residues varying across the 11 branches identified as significant can be examined in a new model to try and identify any that are significantly affecting the serum titres. In order to identify which residues are causative of significant losses in cross reactivity a model similar to the model for vaccine matching, modified to include a term to look at individual residues instead of candidate regions, can be combined with the model controlling for the phylogeny to give the final model (equation 5):

$$\log(t_{p,c}) = k_0 + k_1 \times s_1(p,c) + \sum_{i=1}^{N} m_i \times \delta_i(p,c) + \mu_p + v_c + \varepsilon_D + \varepsilon_R$$

Where s_1 represents a change occurring at an individual residue between the homologous (p) and heterologous (c) viruses and k_1 is the associated regression coefficient: all the other factors are

explained in the previous models. As discussed previously the phylogenetic control terms that are included in this model account for the repeated measurement of the shared phylogenetic history that is significant for this dataset. These terms also mean that any directly significant effects of substitutions that occur only on an individual branch of the tree will not be identified (as repeated measures are lost at the level of the phylogenetic control), however significant interactions involving convergent and/or multiple substitutions that happen at the same residues of the capsid on multiple branches of the phylogenetic tree will still be identified and will be strong indicators of a causative effect, the significance of which can therefore be determined. Therefore in order for an epitope to be predicted it must have had a significant effect on more than one branch, otherwise it cannot be demonstrated that it is causative of any drops in cross reactivity. This also makes this a very conservative approach to identifying epitopes.

When observed collectively the changes occurring across the 103 residues are significant ($P<0.005$). However of all the substitutions that had occurred only 4 residues were individually significant as causative of the changes in cross reactivity observed. The most significant was a substitution at VP3 219 (the C terminus of VP3 – see figure 4.4 for the location of the residues on the surface) where a substitution from a threonine to an alanine was found to cause a drop of 0.27 $\log_e$ (CI +- 0.18). VP3 218 has previously been identified as an epitope of the Asia-1 serotype (Grazioli *et al.*, 2004). The second residue is VP1 138 located on the GH loop, residues of which form antigenic site one and five of serotype O (Crowther *et al.*, 1993b; Kitson *et al.*, 1990). A substitution at this residue from arginine to glutamic acid was identified as causative of a drop in serum titre of 0.19 $\log_e$ (CI +/- 0.15), with a similar drop reported for a change to aspartic acid. The third residue is VP2 191, located on the HI loop, where a change of residue from threonine to asparagine is predicted to cause a drop in LPBE titre of 0.15 $\log_e$ (CI +/- 0.11). This residue has not previously been identified as an epitope on any serotype of FMDV, however this residue is only slightly downstream from VP2 188, which has been previously identified as an epitope on serotype O (Barnett *et al.*, 1998). The final

residue is VP1 198 where a change from aspartic acid to glutamic acid is predicted to cause a drop in LPBE titre of 0.17 $\log_e$ (CI +/- 0.16). This residue is a known epitope for the O serotype (Aktas & Samuel, 2000).

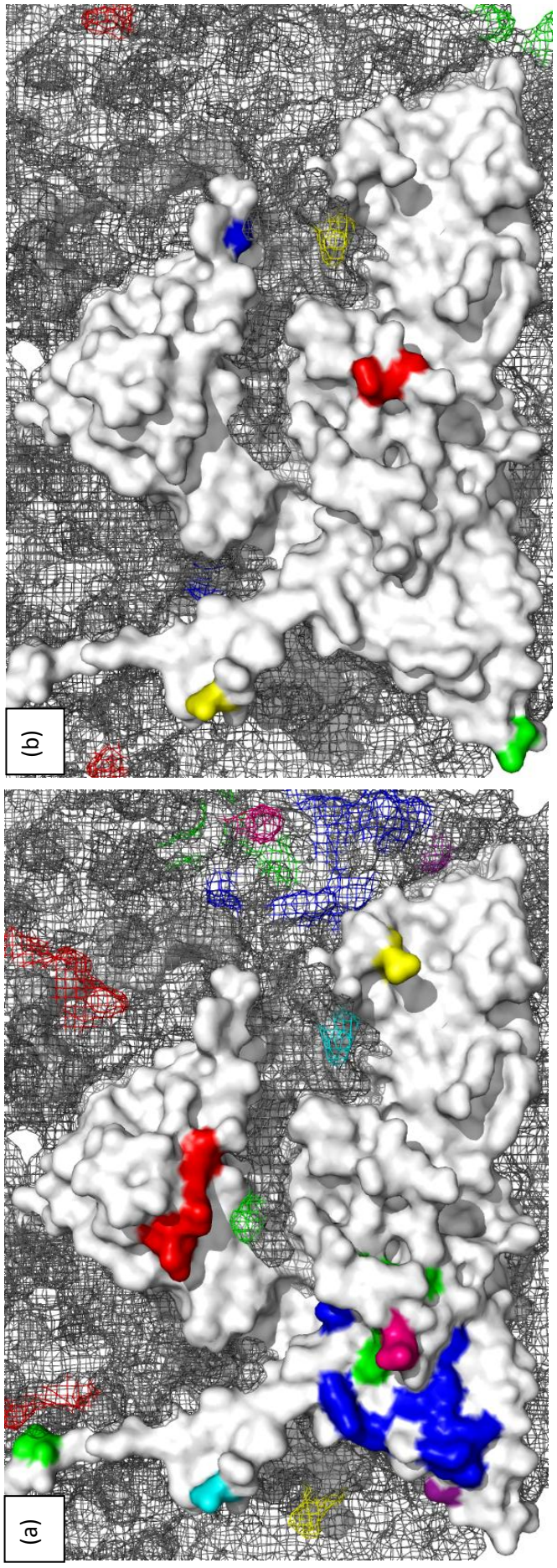

Figure 4.4: A side by side comparison of O BFS (PDB ID 1FOD) showing the molecular surface of the capsid, with a central protomer (in a reduced conformation with the GH loop present) represented in solid white and surrounding protomers as a grey mesh highlighting the known antigenic sites (a) Antigenic site one is coloured green, site two is blue, site three is red, site four is yellow and site five is pink. The additional residues identified from other studies are coloured purple (VP2 188) and teal (VP1 198). The location of each of predicted epitopes is shown in (b); Red is VP1 138, green is VP2 191 red is the VP3 219 and yellow is VP1 198.

4.3.4 Refining the vaccine matching model using just the changes that have occurred on the four residues

As discussed in section 4.4 the best model that could be developed for vaccine matching using amino acid changes in the candidate regions performed significantly poorer at predicting vaccine match than the results of the serological data generated on a single day of test, when compared to the gold standard best estimate r_1 values. In order to determine if this model could be improved, by just looking at the variation in those four residues identified as epitopes (but not the specific substitutions identified at these residues), the final model for vaccine match was adapted to remove the terms for the candidate regions and add in the terms for changes at just those four residues. In this case the model was improved by inserting an additional control that distinguished between the homologous and heterologous titres to account for further differences in cross reactivity not explained by these four residues alone.

The final equation for the LPBE r_1 value predictor using just these four residues is (equation 6):

$$r_1(p,c) = 0.904^{d}VP1\ 138^{(p,c)} \times 0.842VP2\ 191^{(p,c)} \times 0.808^{d}VP3\ 219^{(p,c)} \times\ 0.801^{d}VP1\ 198^{(p,c)} \times\ 0.524^{p \neq c} \times\ e^{v_p - v_c}$$

Where the v_p and $v_c$were the fixed effects of either to homologous (p) or the the heterologous virus (c) listed in the above equation and $p \neq c$ is the control for distinguishing between homologous and heterologous titres. To determine the performance of the model a set of predictions were made using changes within those residues listed above to try and predict r_1 values, these were then compared with the r_1 values generated on the day of test against the "best estimate" r_1 values generated in section 4.3. Of the predictions, 71% of r_1 values fell within 95% Confidence limits of the best estimate r_1 values, which is not significantly different (P=0.3) than the r_1 values generated on the day of test, of which 74% fell within the confidence intervals (figure 4.5).

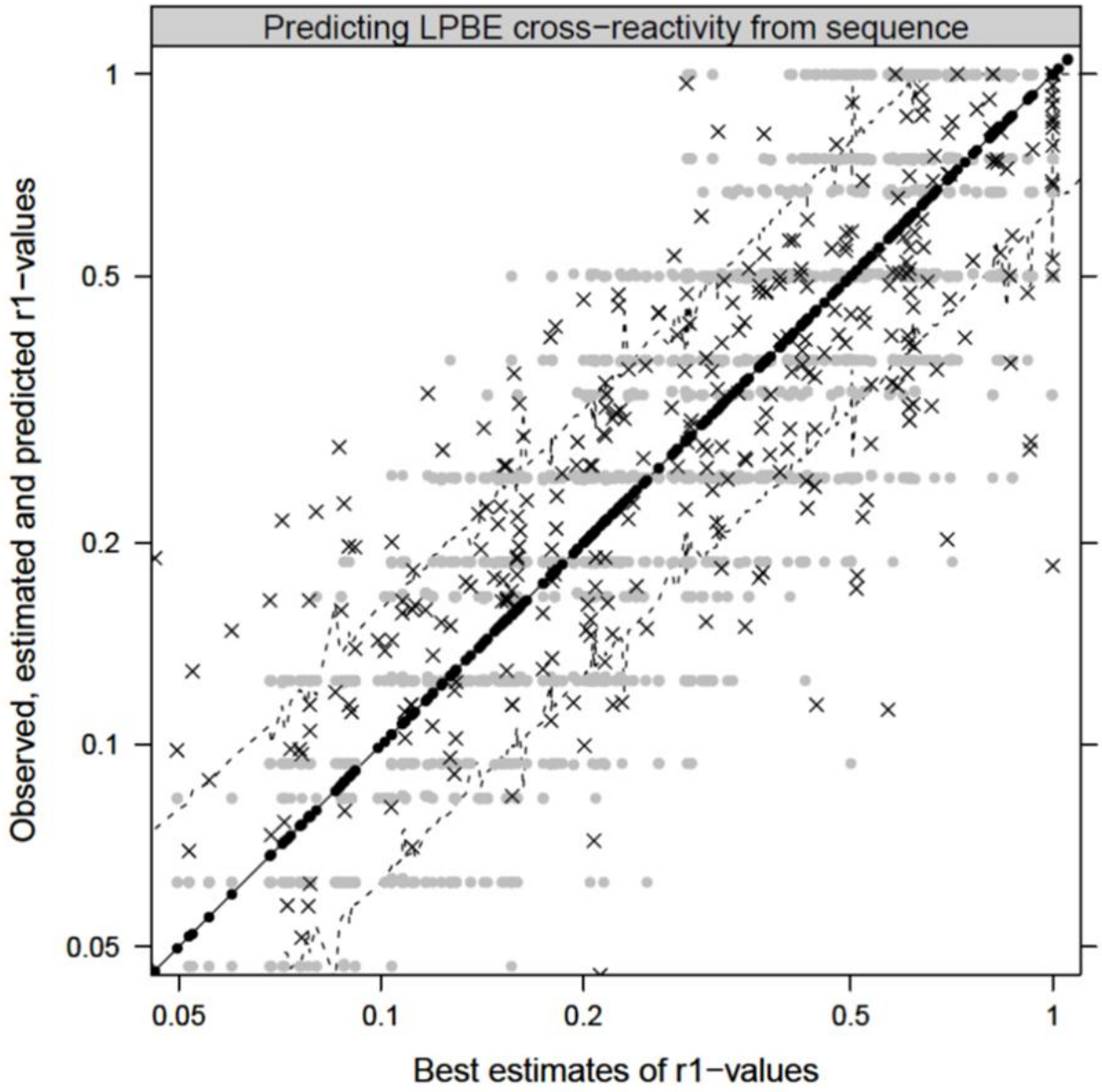

Figure 4.5: Predicted and actual r_1 values for the LPBE compared to the best estimate r_1 values. The predicted r_1 values used here were generated using changes occurring in the four residues that have been predicted as epitopes. Bootstrap samples of individual serological r_1-values (Grey dots), predictions (black crosses) and matching best estimates (black dots) with the confidence intervals of the best estimates for type O r_1-values shown as the grey dashed line. Because of the log-normally distributed variance structure of the r1-values, data are plotted on a log scale.

4.4 Discussion

Traditional methods of identifying the most appropriate vaccines for use in an outbreak situation (described in section 1.10.2) can be expensive and time consuming, which is not ideal in a situation where a rapid response is vital to contain an outbreak. As such an approach for vaccine matching that does not rely on serological methods, but can identify vaccine matches based on sequence data alone would be advantageous for both its speed and cost. As mentioned previously (section 1.10.2) a model for vaccine matching using a set of sequence and serology data, relying on sequence data alone, has been developed for serotypes SAT-1 and SAT-2 (Reeve *et al.*, 2010). This study also developed a model to identify potential epitopes, by controlling for the phylogenetic relationships between viruses. The aim of this chapter was to see if similar models could be developed for the O serotype using the LPBE and sequence dataset generated in chapter three.

The vaccine match model developed using the candidate regions was significantly poorer at predicting vaccine match than the data generated from the day of test, when compared to the gold standard "best estimate" r_1 values (a more statistically valid method for generating r_1 values). Therefore this model does not perform well enough to be considered as an alternative to the current serological methods for vaccine matching (unfortunately due to confidentiality issues, the VNT data from chapter three was not available for the analysis performed in this chapter). However the model was able to identify three sub-sequences on the capsid where the number of amino acid substitutions correlated with the observed changes in serological titre. The most significant of the three regions identified was VP1 132 to 162 (the GH loop and beyond) which is known to contain a hypervariable region, just prior to the integrin binding RGD motif. This region also contains two known antigenic sites of serotype O FMDV (Crowther *et al.*, 1993b; Kitson *et al.*, 1990) and was previously found significantly to correlate to the changes observed in the SAT-1 VNT data (Reeve *et al.*, 2010). This correlation is perhaps not surprising given that the hypervariable region of the GH loop is highly flexible and free from many of the structural constraints of the capsid, enabling almost

all of the amino acids on this 12 residue long stretch to substitute to different amino acids across the viruses tested, with the majority of the other residues in this 31 amino acid long stretch conserved (data not shown). This region may therefore act as a 'molecular clock' accumulating residue changes at a given rate and therefore as viruses diversify this area will accumulate changes. This is unlike other parts of the capsid, where constraints may limit the frequency and the type of changes that occur, which may mean that in order to evade the immune response those residues that can vary may be dominant and switch back and forward between certain amino acids to evade the host immune response

The second region was VP3 84-85, where the only substitution occurs on the O KIC virus from a histidine (on all the other viruses) to a glutamine. Given that all of the viruses tested in the LPBE were antigenically distinct from the OKIC virus, then it is not surprising that this residue would provide a strong signal. The final region was VP1 residues 194-204, within this region a single residue has been identified as an epitope on O Manisa (Aktas & Samuel, 2000). This region is on an area that protrudes from the surface of the capsid and therefore may come under a large amount of selective pressure from antibodies. These results demonstrate that there are small sub-sequences of the capsid that do accumulate changes that correlate with drifting antigenicity of FMDV, contrasting with some other reports. Although the sites identified are not necessarily obvious and are not based on the specific residues of previously identified antigenic sites or any other regions investigated in chapter three. However it is worth noting that, although these regions correlate to the changes in antigenicity observed, the model does not identify if these regions are causative of the changes in antigenicity seen.

Further expansion of this model to control for the phylogenetic structure of the viruses predicted four residues that are causative of the changes in antigenicity of serotype O FMDV seen. One residue (VP1 198) is an already known epitope of serotype O FMDV and another residue (VP1 138) is located within the GH loop, residues of which do form two antigenic sites (Aktas & Samuel, 2000; Crowther

et al., 1993b; Kitson *et al.*, 1990). The remaining two residues (VP3 219 and VP2 191) are also located near residues that have been previously described as part of an epitope, on at least one FMDV serotype. Development of a vaccine matching model which included just these four residues and not the candidate regions (although interestingly two of these residues are predicted to be antigenic and are located within the candidate regions selected as correlates in the initial vaccine matching model) gave a better predictor for vaccine match, resulting in a model that was not significantly worse at predicting r_1 values than those generated from a single day of test, when compared to the gold standard "best estimate" r_1 values. This model could potentially be useful for predicting vaccine match, particularly in instances when a rapid vaccine match selection is required.

Only one of the four residues predicted to be antigenic has been previously identified in the literature. It could be that the other residues are simply ones that have yet to be identified, due to the limited amount of work performed using monoclonal antibody techniques, or it may be because the work conducted here used LPBE data. The LPBE would identify regions important in the whole antibody response (i.e. including antibodies that do not neutralise) and not just those regions involved in generating the neutralising component of the antibody response, from which the mAb's used to identify the known antigenic sites, were derived.

Approximately $^3/_4$ of the branches identified as significant from the phylogenetic modelling are found within the European-South American topotype, leading directly to two of the homologous viruses (O BFS and OKIC), with all the other branches (excluding one) in a region of the tree that leads to another of the homologous viruses, O UKG. This is not surprising given the relatively large amount of data collected for the homologous viruses. However this technique, if correct, would identify residues that may be more representative of epitopes that are important in antigenicity across a serotype than those identified using monoclonal antibodies, where often the binding of a mAb is only tested using the virus against which the mAb was raised or other closely related strains and then assumed to be representative of the serotype.

In summary the work performed in this chapter, the first to look at the potential application of these models for use both within serotype O FMDV, using data generated in the LPBE failed to generate a vaccine matching model that performs significantly better than current serological methods. However the second vaccine matching model based on changes occurring at just the four predicted epitopes may be of use in some instances. The modelling has led to identification of three potentially novel serotype O epitopes, the validity of which will now be tested using reverse genetics approaches.

Chapter 5: Evaluation and use of in-silico structure-based epitope prediction with foot-and-mouth disease virus.

The work conducted within this chapter has been submitted for publication to PLOS One:

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5.1 Summary

As discussed in section 1.9 several structure based B cell epitope prediction tools have been developed recently. The aim of this chapter is to examine the potential power of these structure-based approaches to predict unidentified Picornavirus epitopes. In particular this could provide a much more rapid, cheap and easy means of predicting viral epitopes conserved across several serotypes when compared to the expensive and time consuming serological methods like those used in chapters three and four.

This chapter has analysed, for the first time, how reliably several freely available structure-based B cell epitope prediction programs can identify already known viral epitopes of FMDV. To do this a simple objective metric was constructed to measure the sensitivity and discrimination of such algorithms. After optimising the parameters for five methods, using an independent training set consisting of other picornavirus structures, this measure was then used to evaluate these methods. Individually any one algorithm performed rather poorly (three performing better than the other two), suggesting that there may be value in developing virus-specific software. However using a consensus of any two of the three best methods for each serotype marginally improved the predictive power of the programs.

Taking a very conservative (but accurate) approach requiring a consensus between all three top methods predicts a number of previously described antigenic residues as potential epitopes on more than one serotype of FMDV, consistent with experimental results. The consensus results also identified novel residues as potential epitopes on more than one serotype. These include residues 190-192 of VP2 (not previously determined to be antigenic), residues 69-71 and 193-197 of VP3 spanning the pentamer-pentamer interface, and another region incorporating residues 83, 84 and 169-174 of VP1 (all only previously experimentally defined on serotype A). Two of the residues identified here were also predicted in the analysis carried out in chapter five. These results can now be taken forward for experimental validation.

5.2 Methods

5.2.1 Program selection

The programs selected for evaluation, along with a brief description of the algorithms used to determine the likelihood of a residue on a structure forming part of a B cell epitope, are listed below:

A. BEPro (Sweredoski & Baldi, 2008): uses an amino acid propensity scale generated by the developers of discotope combined with the solvent accessibility, side chain position and exposure of each amino acid within the structure.
B. Discotope (Haste Andersen *et al.*, 2006): combines surface exposure with amino acid statistics and spatial information from the analysis of 76 X ray structures of epitope/paratope interactions.
C. Ellipro (Ponomarenko *et al.*, 2008): approximates the structure of the input protein as an ellipsoid and then evaluates each residue based on how many atoms protrude from the surface of the ellipsoid (the Protrusion Index), the program then considers the arrangement of residues with a protrusion index greater than the set threshold and assesses the likelihood that any cluster to form a discontinuous epitope, assigning a score to each residue based on this likelihood.
D. Epitopia (Rubinstein *et al.*, 2009): uses a naïve Bayes classifier trained to identify epitopes using a training set of 66 X ray structures of epitope/paratope interactions. This trained Classifier then covers all regions of the surface the size of an average epitope. The score of each region is assigned to the residue at the centre, giving each individual residue a score by this method.
E. Seppa (Sun *et al.*, 2009): identifies patches on the surface within a 4 Å distance that contain two epitope residues, based on the training dataset of 82 X ray structures of epitope/paratope interactions and assigns a propensity score to each. For each residue the number of patches within a 15 Å radius are identified and the sum of the propensity scores is

calculated. This is then combined with a clustering coefficient based on the neighbouring residues to output an immunogenicity score.

These programs are believed to represent the most recent programs available, with the exclusion of the EPCES and EPSVR servers (Liang *et al.*, 2010), for which the size of the Protein Data Bank (PDB) files for the multimeric structures being used was prohibitive.

Generally, for each program, a single value is assigned to each residue predicting its likelihood of being an epitope. However, for Epitopia two values are included in the output for each residue, one being an immunogenicity score and the second being a probability score. For the purposes of this analysis the probability score was used to determine the optimal threshold for identification of Picornavirus epitopes. Since the two values are strongly correlated this should not impact on the results.

5.2.2 Structure selection

Two Picornavirus structures were selected for program optimisation; Poliovirus P1 Mahoney strain (PDB code 1HXS- (Miller *et al.*, 2001)) and Rhinovirus 14 (4HRV- (Arnold & Rossmann, 1988)). These were chosen as they are the most well characterised Picornaviruses in terms of identified epitope residues. For FMDV, a structure was selected for each serotype (where available) for program evaluation. The list of structures used was as follows: Serotype C-S8c1 (1FMD- (Lea *et al.*, 1994)), A1061 (1ZBE: (Fry *et al.*, 2005b)), O (O_1 Kaufbeuren- (Lea *et al.*, 1995)), SAT-1 (2WZR- (Reeve *et al.*, 2010)). An additional structure for serotype O (O_1 Kaufbeuren- data not published) with the G-H loop present in a reduced conformation (referred to in this chapter as reduced O1K) was also included in order to evaluate the impact of the presence of the G-H loop not normally present on the structure due to its highly disorganised nature: see section 1.3 for more detail.

5.2.3 Sequence and conformational comparison of structures

Poliovirus and Rhinovirus are both members of the enterovirus genera of the Picornaviruses. The sequences of the two virus structures examined here share a 47% sequence identity to each other while the structures have an average route mean square distance (RMSD) calculated using the structural homology program [32]of equivalenced Cα of 0.96 Å. The sequences from both of these structures have approximately 22-24% identity (calculated in MEGA 5 (Tamura *et al.*, 2007) using a p-dist matrix and multiplying by 100- used for all Identity calculations) with the sequences obtained from the four FMDVs, whose structures were used in this evaluation. The RMSD between Rhinovirus and serotype O FMDV Cα (excluding the G-H Loop) has previously been reported as 2.3Å for VP1, with the divergence between both VP2 and VP3 of 1.8Å [34] (Acharya *et al.*, 1989).

Serotypes O, A and C of FMDV share an approximate 80-82% sequence identity across the specific sequences of each structure selected while the SAT-1 virus sequence identity is approximately 65% that of the the other serotypes. VP1 exhibits the most variation (~30% for O, A and C and 50% for SAT-1) and VP2 exhibits the least variation of the surface exposed proteins (~17% between O, A and C and 30% for SAT-1). On average the RMSD (calculated using the structural homology program [32]) of each of the surface exposed virus proteins was 0.85Å for VP1 (excluding the G-H loop), 0.68 Å for VP2 and 0.66 Å for VP3.

5.2.4 Structure preparation

In the virus, the protein subunits are arranged with icosahedral symmetry, so the first stage was to add the appropriate context to the selected icosahedral protomeric unit. To do this, a complete icosahedral capsid for each structure was first generated from a protomeric subunit using the 60 non-crystallographic symmetry matrices included in each coordinate file and the General Averaging Program (GAP, Stuart D.I and Grimes J.S. unpublished). From this complete structure, an icosahedral (triangular) protomeric subunit comprising VP1-4 was selected together with the similar protomer bounding each of the sides of the first, forming a tetrameric multimer (figure 5.1(b)).

The individual polypeptide chains (VP1, VP2, VP3 and VP4) that make up each protomer were relabeled using the Moleman software (Kleywegt, 2001) so each protomer was represented as a single chain, with the central protomer labeled as chain 1 and the outer protomers labeled as chains 2, 3 and 4 respectively (Figure 5.1(b)). This enabled the central protomer to be selected as a single chain for analysis, with all interfacing protomers being taken into account as non-selected chains where possible. Where this was not possible the entire multimer was uploaded into the program and only the data for the central protomer taken forward for further analysis.

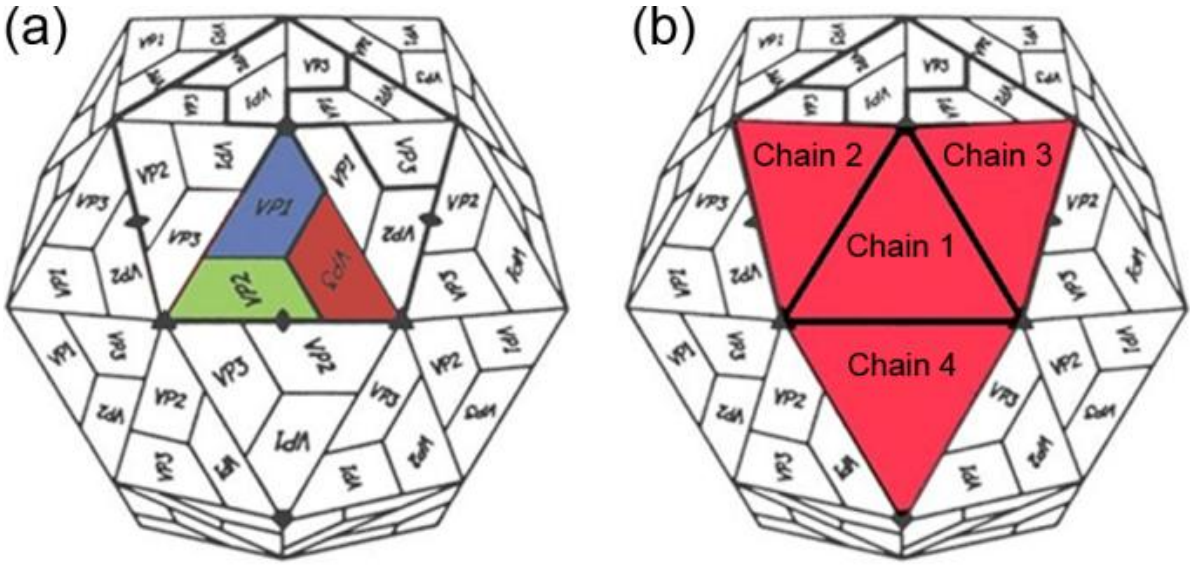

Figure 5.1: Building the multimer. (a) Schematic depiction of the Foot-and-Mouth disease icosahedral capsid with the individual virion peptides labelled. A viral triangular subunit is coloured as per the standard colouring sytem; VP1 is blue, VP2 is in green and VP3 is red. (b) Schematic depiction of the of the same structure as (a) with the four subunits selected to make the multimer labelled as chains 1-4 (highlighted in red). These four protomers form the multimeric structure that was used in the analysis.

5.2.5 Identifying surface exposed residues of the reduced O Kaufbeuren structure

The surface exposure of each residue was determined as outlined in section 2.5.1, using the reduced O BFS structure (PDB: 1FOD). Only the surface exposed residues were used in this analysis.

5.2.6 Identification of Picornavirus epitopes

A literature search was conducted to collate all identified epitopes for each of the structures selected. A total of 53 and 22 neutralising epitopes were identified for Poliovirus serotype 1 (Diamond *et al.*, 1985; Minor, 1986, 1990; Minor *et al.*, 1986; Minor *et al.*, 1983; Page *et al.*, 1988; Wiegers *et al.*, 1989) and Rhinovirus serotype 14 (Sherry & Rueckert, 1985; Sherry *et al.*, 1986) respectively. For each serotype of FMDV the following numbers of epitopes were identified; 25 for serotype A (Baxt *et al.*, 1989; Fry *et al.*, 2005b; Mahapatra *et al.*, 2011; Saiz *et al.*, 1991; Saiz *et al.*, 1989; Thomas *et al.*, 1988), 20 for serotype O (Aktas & Samuel, 2000; Barnett *et al.*, 1998; Crowther *et al.*, 1993b, c; Kitson *et al.*, 1990; Mahapatra *et al.*, 2008), 16 for serotype SAT-1 (Grazioli, 2006) and 10 for C (Diez *et al.*, 1990; Lea *et al.*, 1994; Mateu *et al.*, 1990; Mateu *et al.*, 1994). Asia-1 (Grazioli *et al.*, 2004) and SAT-2 epitopes (Crowther *et al.*, 1993a) were also identified, although they were not included in the analysis as there was no available capsid structure for either serotype. All of the epitopes described here, with the exception of residue 188 of VP2 which was identified using bovine mAbs, (Barnett *et al.*, 1998) are derived from murine mAbs. Only the exact residues identified were examined.

For all of the FMD structures, with the exception of the reduced O_1 Kaufbeuren (O_1K) structure the immunogenic and mobile G-H loop was not represented and therefore any antigenic residues previously described on this loop were not included in the program evaluation. Additionally, as the analysis utilises structural data, only epitopes identified on the native capsid were included (i.e. monoclonal antibody escape mutants).

5.2.7 Calculating genetic diversity.

Sequence diversity within serotype O and between serotypes was conducted using 150 and 255 available capsid sequences from the genbank (http://www.ncbi.nlm.nih.gov/genbank) database respectively. The methods were as described in section 2.5.2.

5.2.8 Determination of program performance

In order to evaluate the performance of each program the following parameters were determined as follows:

Sensitivity = number of true positives/(number of true positives + number of false negatives)

Specificity = number of true negatives/(number of true negatives + number of false positives)

Accuracy = (true positives+ true negatives)/(true positives+number of false positives+false negatives+true negatives)

Precision = true positives/(true positives+false positives) where 'truth' is defined as agreement with the experimental data described above.

Probability excess = (sensitivity+specificity) – 1

5.2.9 Statistical analysis

Statistical analysis was performed as outlined in section 2.6, with the virus name and the program as fixed effects.

5.3 Results

5.3.1 Program optimisation

For each program in the trial, the program authors had previously established an optimal value for the threshold at which a residue is predicted to be part of an epitope by optimisation against a broad set of epitopes in a database of test structures. The metric used to set the threshold was the so-called receiver operating characteristic area under the curve (ROC AUC) value: (this is simply the area under the curve obtained by plotting true positives against false positives, both as fractions) a value greater than 0.5 represents a robust predictor performing better than chance with a value of 1 indicative of a perfect predictor. However, as this threshold is set against a broad range of epitopes it

may not be appropriate for a particular system, such as virus capsids, where neotopes are likely to arise due to the juxtaposition of repeated protomer subunits.

To evaluate the utility of the suggested threshold values the performance of each of the programs was tested at 6 different threshold values against both Poliovirus and Rhinovirus. The results demonstrate that for each program a single threshold value yielded the optimal performance against both of the viruses; however, the performance between Polio and Rhinovirus is in several cases markedly different at this value (Table 5.1). For example, the Discotope server performed better at identifying Polio epitopes than at identifying Rhinovirus epitopes. The threshold values determined by our enterovirus based analyses were in many cases different to the default threshold suggested by the program developers, for example the Discotope server suggests a default threshold of -7.7, whereas our evaluation determined the optimal threshold to be -10.7. Additionally, the results demonstrated the generally poor sensitivity and specificity of each program tested. The picornavirus optimised threshold values were then used in the analysis of the FMDV outputs.

	Default server	Optimal Threshold	Poliovirus		Rhinovirus	
Program	setting	value	Sensitivity	Specificity	Sensitivity	Specificity
Discotope	-7.7	-10.7	0.868	0.746	0.591	0.686
Seppa	1.8	1.75	0.66	0.585	0.545	0.654
Epitopia	Not given	0.174	0.528	0.788	0.727	0.766
BEPro	1.3	0.8	0.717	0.767	0.682	0.612
Ellipro	0.5	0.3	0.811	0.648	0.864	0.553

Table 5.1: Sensitivity and specificity results for polio and rhinovirus at the optimum threshold value for scoring a residue an epitope (the default given by the developers is also shown for comparison).

5.3.2 Performance of each program at identifying known FMDV epitopes

The best performing programs in terms of sensitivity, specificity and average probability excess were Epitopia and Ellipro, followed by Discotope (see Figures 5.4 and 5.5). These programs also performed well in terms of overall number of FMDV epitope residues identified (Figure 5.2), with Discotope correctly identifying 60.60% of the total of the previously described epitopes for all serotypes of FMDV, followed by Ellipro (57.57%) and Epitopia (56.06%). Seppa and BEPro performed

poorly in terms of sensitivity and specificity where in some cases the programs performed worse than if the residues had been selected by chance, and average probability excess, with epitopia performing significantly ($P<0.05$) better than both of these programs (Figures 5.3 and 5.4), In addition BEPro and Seppa also identified the least number of epitopes (Figure 5.2), with Seppa identifying 34% and BEPro 22% of the total number respectively.

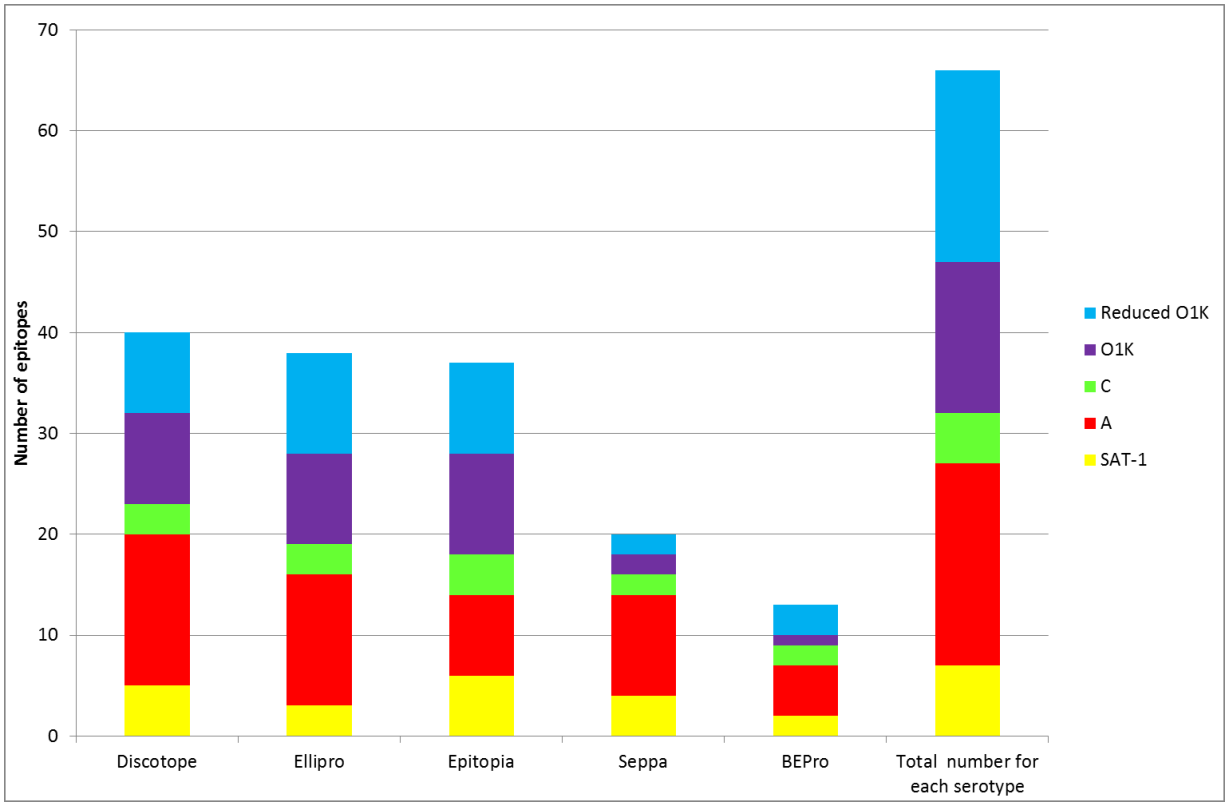

Figure 5.2: Graph showing the number of previously described epitopes for each serotype identified by each program compared to the total number of previously described epitopes.

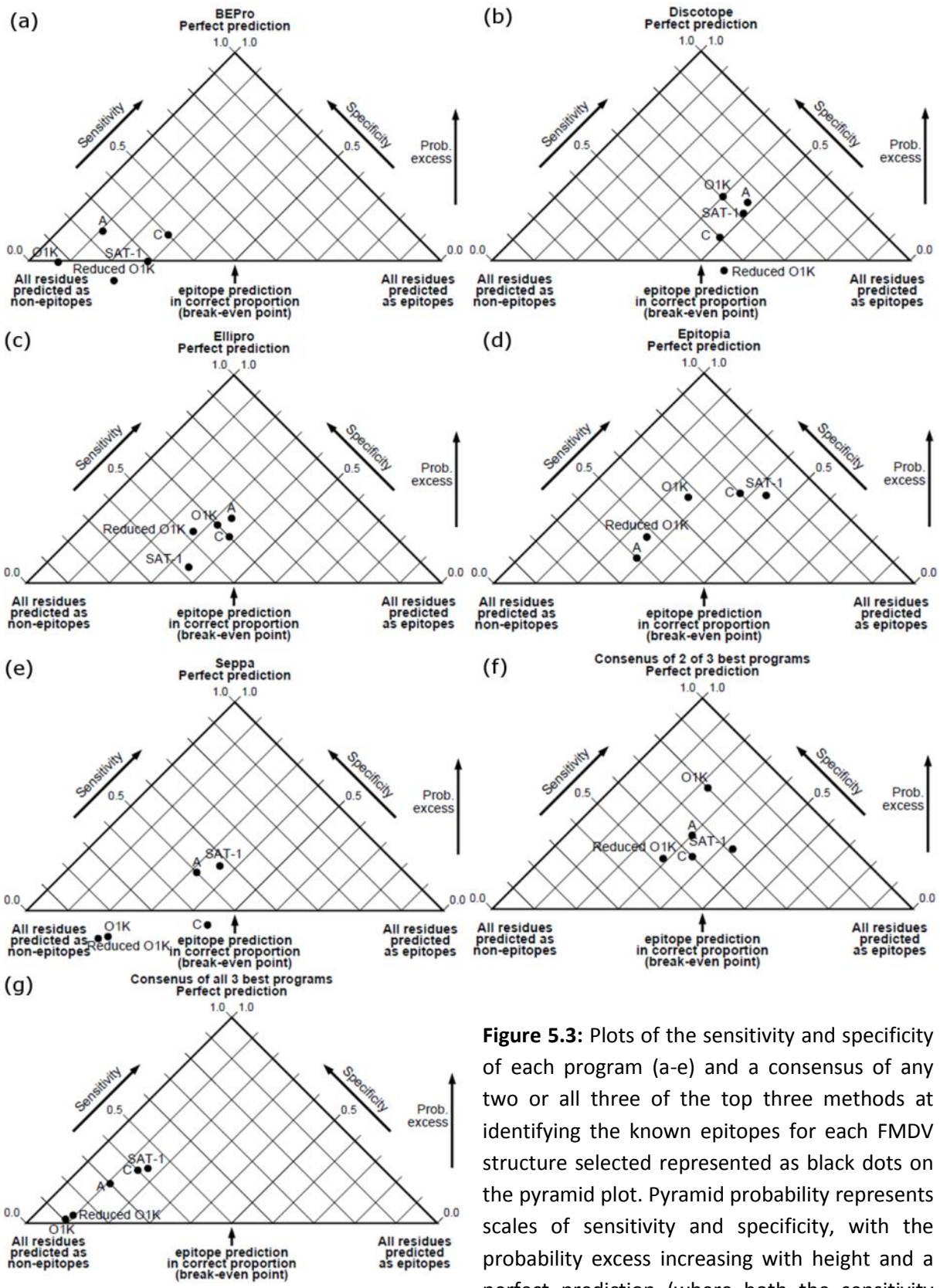

Figure 5.3: Plots of the sensitivity and specificity of each program (a-e) and a consensus of any two or all three of the top three methods at identifying the known epitopes for each FMDV structure selected represented as black dots on the pyramid plot. Pyramid probability represents scales of sensitivity and specificity, with the probability excess increasing with height and a perfect prediction (where both the sensitivity and specificity score a value of 1) coming at the tip of the pyramid. The bottom line of the plot represents the point at which results would be expected to be generated by chance.

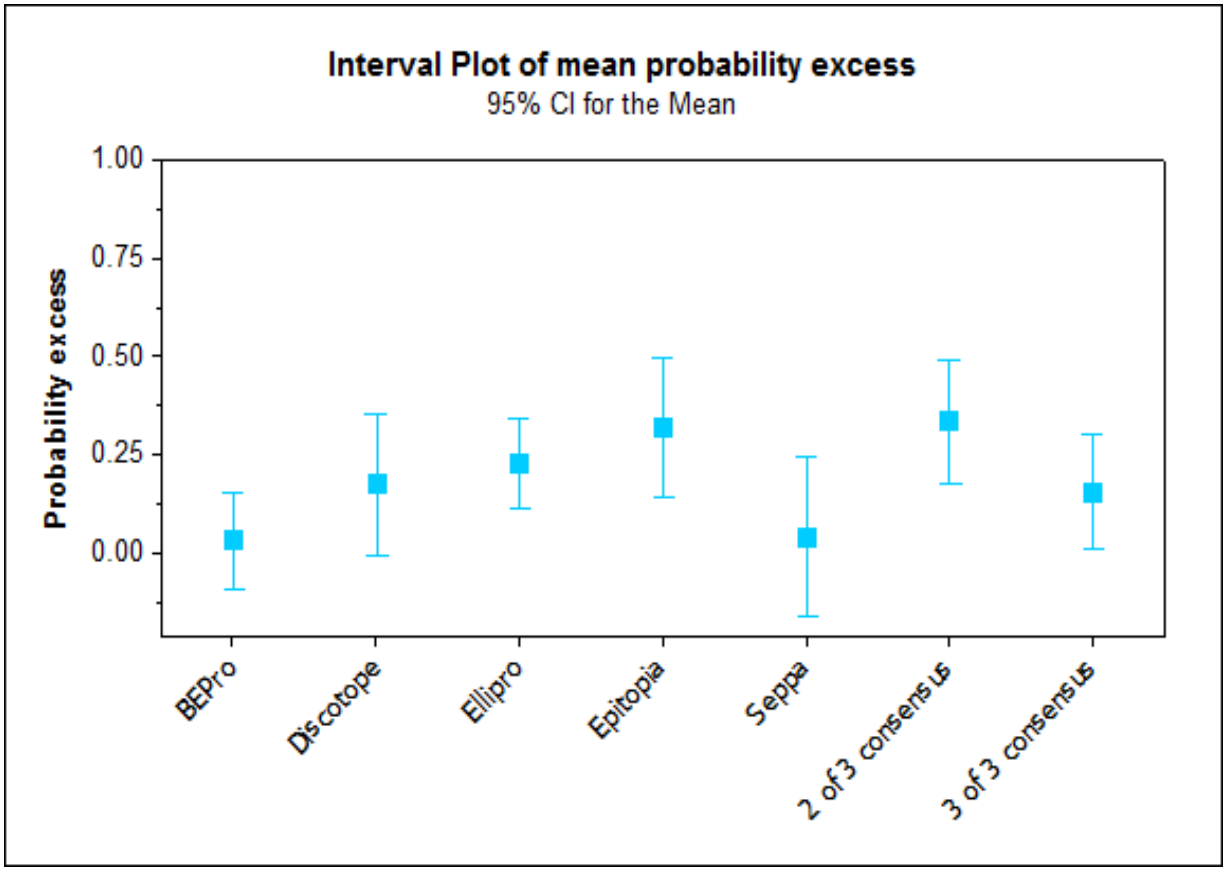

Figure 5.4: Interval plot showing the mean probability excess (and confidence intervals) of each program and a consensus of any two or all three of the top three methods.

All of the programs performed poorly in terms of precision (Figure 5.5), with most results below 0.2. However all performed better in terms of accuracy of the results obtained, with BEPro performing the best (average 0.761), followed by Ellipro, Epitopia, Seppa and Discotope (Figure 5.5).

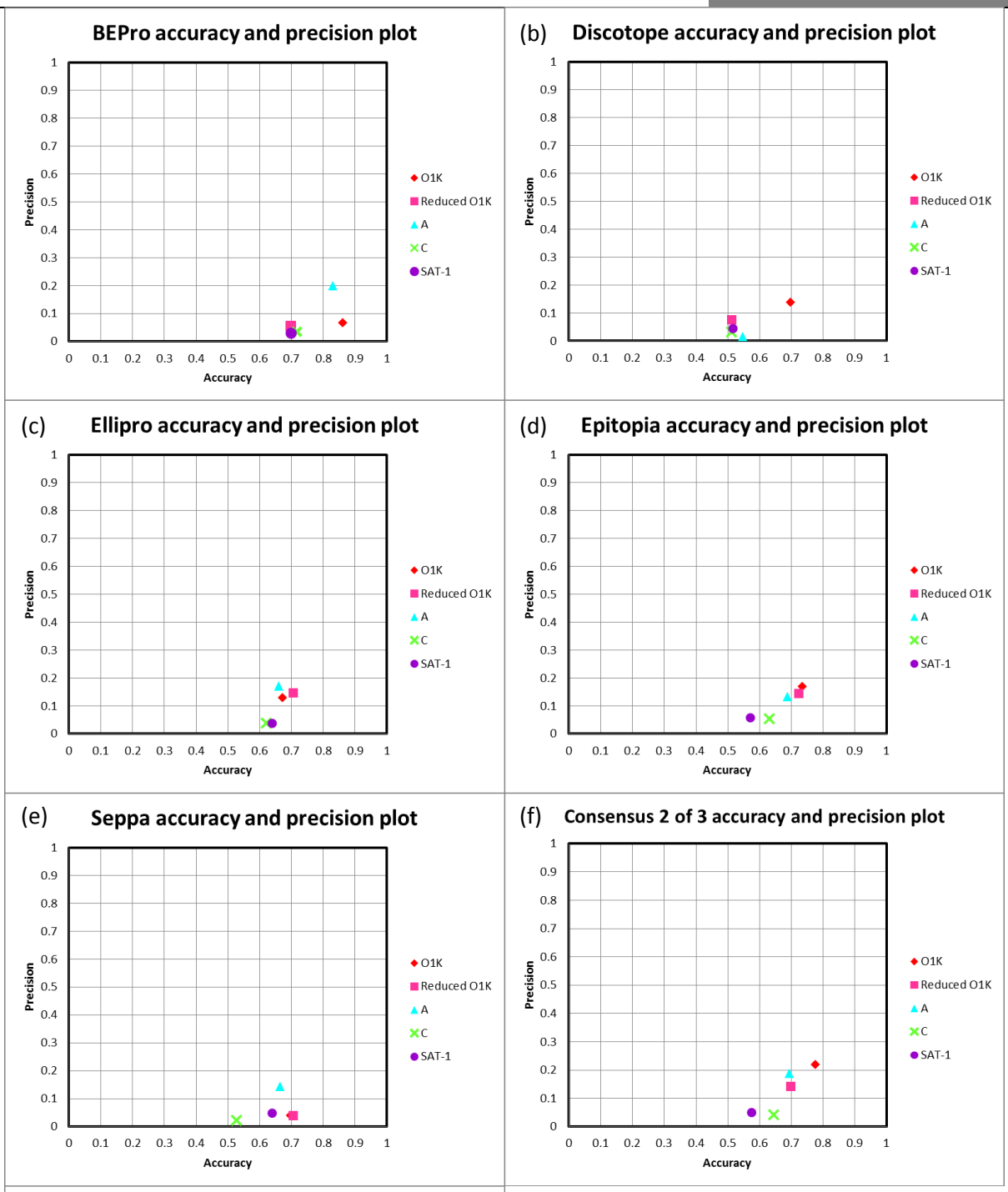

Figure 5.5: Plots of the precision and accuracy of each program (a-e) and a consensus of any two or all three of the top three methods at identifying the known epitopes for each serotype with a structure available.

Although the programs utilise different algorithms there was considerable overlap in the residues identified, with all of the epitopes on serotype A, C and SAT-1 identified either by more than one program or no program, with just two residues out of the 16 on the O_1 Kaufbeuren structure only predicted by one program (figure 5.6). This contrasts with the results for the reduced O_1 Kaufbeuren where a quarter of the residues were only identified by a single program, all of which were at regions of interaction between the GH loop, where it is laying across and VP2 (data not shown). Of those residues not identified by any program VP2 79 is of particular interest as it is an epitope on four of the structures used but is not identified by any program (data not shown). These results suggest that although the programs utilise different methods for identifying epitopes they are not fully independent.

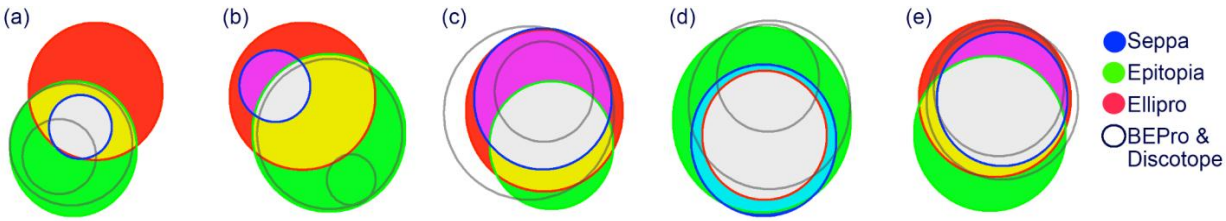

Figure 5.6: Venn diagrams showing the regognition of residues by each program for FMDV serotype O1K-Reduced (a), O1K (b) , A1061 (c), SAT-1 (d) and Cs8 c1 (e). The key on the far right hand side indicates the colour of each program as represented on the Venn Diagram. For clarity the two worst perfoming algorithms are not coloured. In regions of overalap the colour is represented as the sum of the RGB colour channels of the overlapping mathods Diagrams made using the Venn master program (Kestler et al., 2008). Note that formally there need be no perfect projection of the multi-dimensional overlap information into the Venn diagram, so these represent best approximations.

5.3.3 Using a consensus approach to identify novel epitopes

To investigate the potential of using a consensus approach to predict novel epitopes the results for the three best performing programs in terms of average probability excess were selected (Epitopia, Ellipro and Discotope - see section above). The residues that were predicted to be antigenic by a consensus of all three programs on the selected structures were tabulated and compared to the known antigenic residues described previously (see table 5.2). Figure 5.3(g) indicates, as expected, that the sensitivity of such an approach is limited, however the significantly ($P<0.01$) increased accuracy obtained over any individual program (except the poorly performing program, BEPro $P=0.12$) or using a consensus of 2 of the 3 programs (Figure 5.5(g)) validates the use of this approach. Additionally for the purpose of predicting novel epitopes using such a conservative approach is appropriate as the number of false positives need to be as small as possible. It is also apparent that selecting the consensus results from any two of the best three methods gives an overall improvement in consistency, with all the sensitivity and specificity results in the right proportions (figure 5.3(f)). Significant ($P<0.01$) improvements in the average probability excess were obtained over both Seppa and BEPro using a consensus of two of three best performing programs, and marginal (but not significant- average probability excess 0.338 compared to 0.319 for the best performing program, Epitopia) gains in probability excess compared to the remaining programs (Figure 5.3 and Figure 5.4) but not precision or accuracy over any single program. It is possible that this may be useful for some applications.

Chain ID	O1K	Reduced O1K	A	C	SAT-1	Known Antigenic site?	
VP1	VP1:ASN 46 VP1:GLN 47	VP1:ASN 46			VP1:ASN 46 VP1:ASP 47	45 &48 Antigenic site on type O, 48 on SAT-1	
				VP1:THR 83		Antigenic Site on type A	A
	VP1:GLY 84		VP1:GLY 84				
			VP1:GLN 169			Antigenic Site on type A	B
	VP1:THR 171	VP1:THR 171		VP1:GLU 171			
	VP1:ARG 172	VP1:ARG 172		VP1:THR 172		173 Antigenic site on type A	
	VP1:THR 174	VP1:THR 174					
					VP1:TYR 195		
					VP1:ASP 196		
					VP1:HIS 197		
					VP1:ASN 198		
					VP1:GLY 199		
					VP1:ARG 200	Antigenic Site on type C (193)	
		VP1:ILE 194	VP1:ILE 192	VP1:ILE 194	VP1:ASP 201		C
	VP1:HIS 195	VP1:HIS 195	VP1:LYS 193	VP1:GLN 195	VP1:ARG 202		
	VP1:PRO 196	VP1:PRO 196	VP1:VAL 194	VP1:PRO 196	VP1:TYR 203		
	VP1:THR 197	VP1:THR 197	VP1:THR 195	VP1:THR 197	VP1:LYS 204		
	VP1:GLU 198	VP1:GLU 198	VP1:SER 196	VP1:GLY 198		Antigenic site on type O	
			VP1:GLN 197	VP1:ASP 199			
			VP1:ASP 198			Antigenic site on type A	
	VP1:ALA 199	VP1:ALA 199	VP1:ARG 199				
				VP1:PRO 204		Antigenic site on type A	
			VP1:ILE 205				
VP2			VP2:ASP 68	VP2:ASP 68		67 Antigenic site on type Asia-1	
				VP2:VAL 70		Antigenic site on type O	
				VP2:PRO 71		Antigenic site on type O	
	VP2:SER 72	VP2:SER 72	VP2:ASP 72	VP2:SER 72	VP2:ASP 72	Antigenic on all tested serotypes	D
				VP2:GLN 73			
				VP2:ASN 74		Antigenic site on type C and Asia-1	
				VP2:ASP 134	VP2:ASP 134	Potential Antigenic site on type O	
				VP2:PRO 186			
				VP2:THR 188		Antigenic site on type O	
				VP2:THR 189			
	VP2:ASN 190	VP2:ASN 190	VP2:SER 190	VP2:ASN 190	VP2:ASP 190		E
	VP2:THR 191	VP2:THR 191	VP2:ASN 191	VP2:THR 191	VP2:GLN 191		
	VP2:GLU 192	VP2:GLU 192		VP2:ALA 192	VP2:THR 192		
				VP2:GLY 193	VP2:ILE 193		
					VP2:GLY 194		
				VP2:GLN 195			
				VP2:GLN 196		Antigenic site on type A	
VP3		VP3:GLU 58			VP3:ASN 58	Antigenic site on type O, A, C and Asia-1	
		VP3:GLY 59			VP3:GLY 59	Antigenic site on type A and Asia-1	
		VP3:GLY 60			VP3:VAL 60		
		VP3:VAL 61				Antigenic site on type A	
					VP3:GLN 66		
					VP3:SER 67		
			VP3:ALA 68		VP3:ASN 68		
	VP3:ASP 69	VP3:ASP 69	VP3:ASP 69		VP3:SER 69	Antigenic site on type A	F
			VP3:ASP 70		VP3:GLY 70		
	VP3:ASP 71	VP3:ASP 71	VP3:THR 71		VP3:SER 71		
					VP3:THR 112		
					VP3:GLY 113		
			VP3:SER 114				
			VP3:ASP 116				
					VP3:GLU 135	Antigenic site on SAT-1	
					VP3:THR 191		
			VP3:HIS 192				
			VP3:GLY 193		VP3:THR 193		G
			VP3:LYS 194		VP3:HIS 194		
				VP3:ASP 195		Antigenic site on type A	
			VP3:GLU 196		VP3:LYS 196		
			VP3:ASN 197		VP3:ASP 197		
					VP3:ALA 199		
				VP3:GLN 219		218 antigenic site on Asia-1	
				VP3:GLN 220	VP3:ARG 220		
					VP3:GLN 221		

Table 5.2: List of residues selected by a consensus of the three best performing programs (Discotope, Ellipro and Epitopia) for each selected FMDV structure compared to locations of known antigenic sites of all serotypes. Those residues coloured red are an already known epitope on at least one serotype of FMDV, those in blue are adjacent to a known epitope of FMDV. Regions A-G are predicted to be antigenic on the majority of the serotypes tested and are coloured the same on Figures 5.8 and 5.9. The remaining residues are coloured grey, as figures 5.8 and 5.9. Note that the SAT-1 virus VP1 has a incorporated several additional residues into the G-H loop and the A serotype also aligns slightly differently to the O and C structures therefore the numbering is different in region C as they are aligned according to position on structure. All other residues are in approximately the same location relative to each other.

The majority of residues identified using this consensus approach (consensus of all top three programs) are located at regions previously described as antigenic on at least one serotype. Additionally, several of these regions were also selected for the majority (or all) of the serotypes tested (table 5.2). This suggests that some of these residues may comprise presently unidentified epitopes on certain serotypes. The regions predicted as antigenic on the majority of serotypes are mapped onto the surface of a serotype O structure in Figures 5.8 and 5.9.

One region that is predicted for all serotypes but not previously identified as an epitope in the literature is residues 190 to 192 of VP2. This region is around the 3-fold axis and is 16.9Å (from the Cα of the central amino acid VP2 191 to the Cα of VP2) away from residue 72 of VP2, which has previously been described as an epitope on all serotypes tested and is also predicted across all serotypes tested. This region also lies close to two other regions predicted on multiple serotypes, VP3 69-71 and 193-197 (~25Å and ~14Å from the Cα of the central amino acid VP2 191 to the Cα of VP3 70 and 195 respectively), potentially forming an antigenic site spanning the 3-fold axis across pentamers. A further area of interest lies towards the 5-fold axis of symmetry, with two regions (VP1 residues 83-84 and 169-174) identified as potentially forming a conformational epitope, again on multiple serotypes. The location of these regions is shown in Figure 5.8, coloured as described in table 5.2.

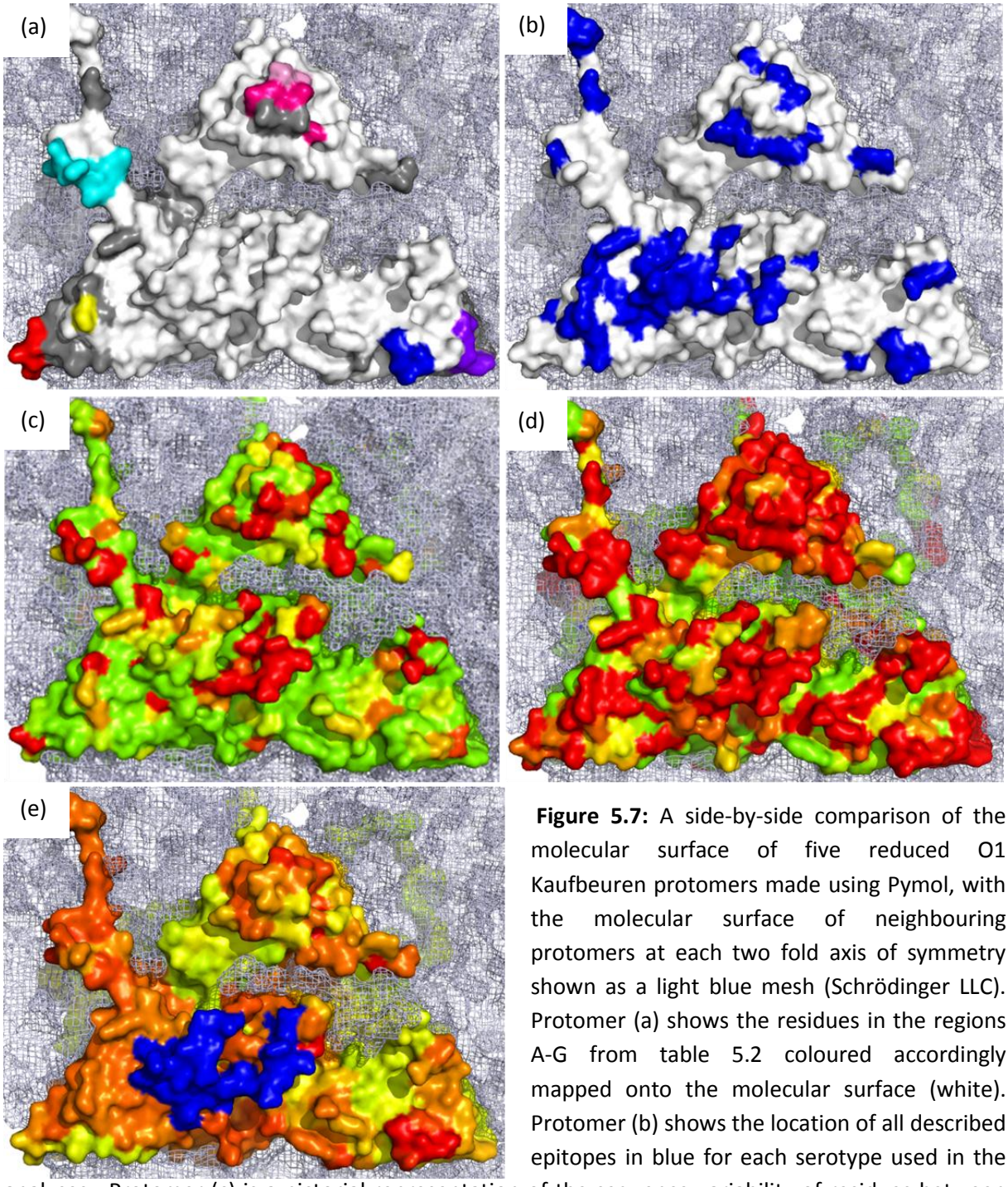

Figure 5.7: A side-by-side comparison of the molecular surface of five reduced O1 Kaufbeuren protomers made using Pymol, with the molecular surface of neighbouring protomers at each two fold axis of symmetry shown as a light blue mesh (Schrödinger LLC). Protomer (a) shows the residues in the regions A-G from table 5.2 coloured accordingly mapped onto the molecular surface (white). Protomer (b) shows the location of all described epitopes in blue for each serotype used in the analyses. Protomer (c) is a pictorial representation of the sequence variability of residues between 105 serotype O virus capsid sequences and Protomer (d) is a representation of the sequence variability of residues between 255 capsid sequences from all 7 serotypes. Both (c) and (d) were constructed using the ESPRIPT program [63] as described in the methods, the coloring goes from green (most conserved) to red (least conserved). Those sites predicted by a consensus of all programs on three or more structures tend to be located at areas of variability both inter and intra-serotypically, the two regions circled in black are particularly variable within serotype O. Region VP3 69-71 (highlighted in red on (a) and (c)) is completely conserved within serotype O, however it appears to be highly divergent between serotypes. (e) is the average RMSD across the serotypes coloured from green (smallest) to red (largest). Blue indicates the GH loop, missing on all but one structure.

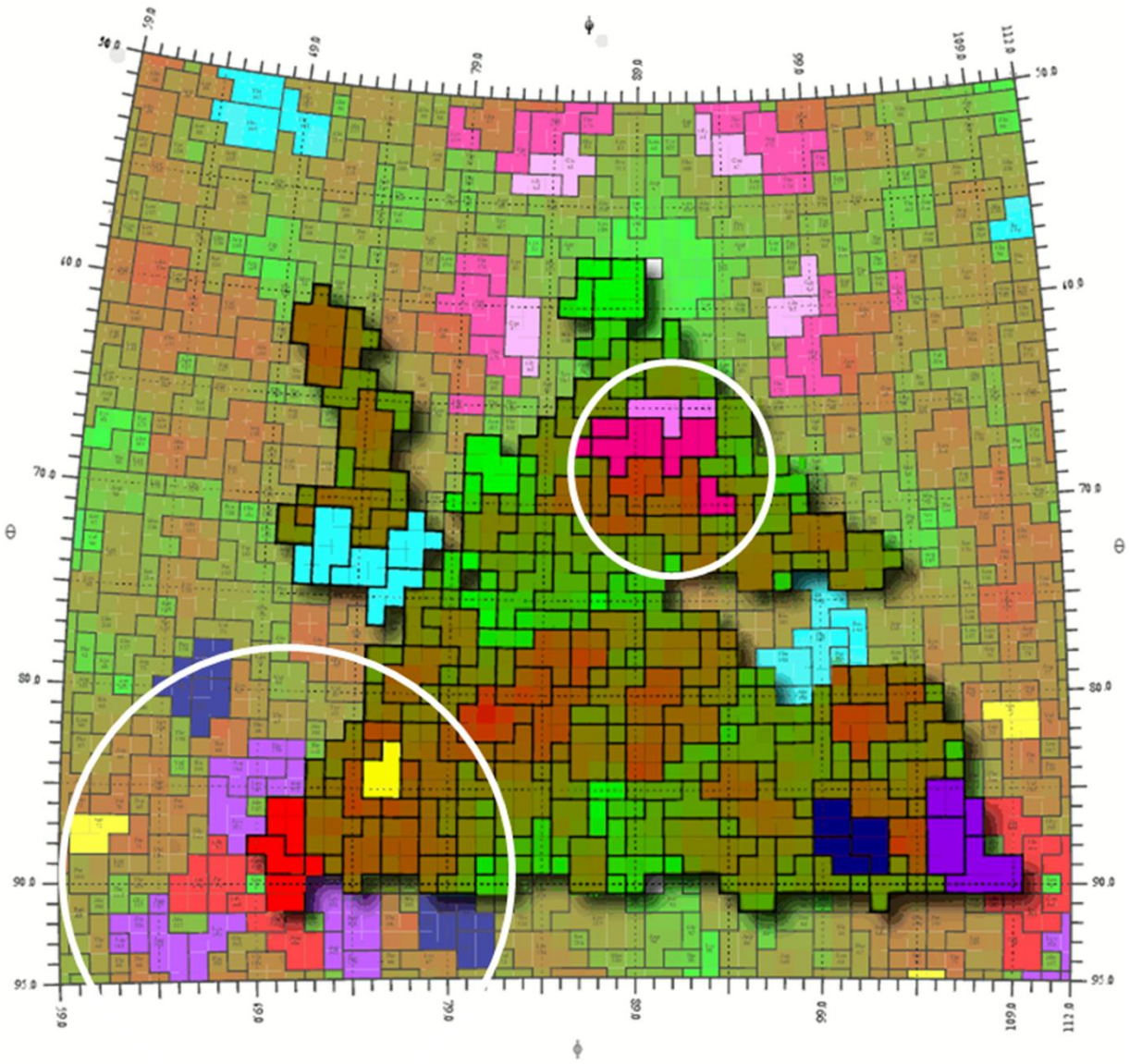

Figure 5.8: Roadmap illustration of a zoomed in area of the reduced O1K capsid generated using the RIVEM program (Xiao & Rossmann, 2007). The location of a single protomer is outlined. The occupancy of each residue on the surface is shown as an area corresponding to the surface accessibility with a black outline, with the protomer coloured according to the radius of each residue from the centre of the icosahedral capsid, coloured from dark green (least exposed) to brown (most exposed). The residues predicted by the best performing programs on the majority of the structures evaluated are coloured as in Table 5.2. When the individual areas are examined it can be seen that several regions are in very close proximity across the 3-fold axis, potentially forming a conformational epitope. Additionally two of the regions are adjacent to each other near the 5-fold axis, again potentially forming a conformational epitope. These two regions are circled in white.

When the sequences of these putative epitopes are compared between different serotype O viruses (Figure 5.7) it can be seen that VP2 190-192 and VP1 194-199 in particular are highly variable, and so could contribute to the antigenic variation observed within this serotype. The average amino acid diversity of all surface exposed residues across the serotypes (when aligned to the reduced O1K structure) is 53.9%, this compares to an amino acid diversity of 69.5% within the predicted sites (Figure 5.7), which is slightly greater than the diversity observed across the known antigenic sites (67.8%). In contrast one region (VP3 69-71) is completely conserved in sequence within serotype O and so antibodies against this putative epitope might be cross-reactive against all serotype O viruses. Nevertheless even this region is not conserved across all serotypes and appears to be located on the most significant region for structural variation (Figure 5.7(e)). It would appear from the variability observed between the serotypes that the prospect of finding an inter-serotypically conserved epitope is poor, at least on those parts of the capsid that are most exposed (Figure 5.7(d)).

The average RMSD values (when aligned to the O1 Kaufbeuren structure) for the predicted sites is also greater than that the average of the surface exposed residues at 2.32Å, which, although slightly higher than the average (2.20Å) is rather lower than the RMSD observed at the antigenic sites (2.49Å).

5.3.4 The impact of G-H loop conformation

The presence or absence of the G-H loop (representing the “up” and “down” conformations- see section 1.3 for more on the GH loop structure) did not appear to greatly influence the results, with a similar number of epitopes identified by each program for both structures (figure 5.2). The same regions were also predicted as being antigenic on both structures. However there were some epitopes that were recognised by the programs on one structure and not another (Figure 5.6) especially around antigenic site two, where the GH loop in a reduced “down” conformation interacts with VP2. A further difference observed was in the decreased sensitivity and specificity of the programs (in particular Discotope) at identifying already known epitopes on the reduced (ordered

loop) form of the O_1Kaufbeuren (Figure 5.3). This could be due to the inclusion of four additional epitopes located on the G-H loop that are not present on the non-reduced structure. As the G-H loop is considered to be a linear epitope these programs may not be well suited to identifying these residues. The G-H loop in the down conformation is also probably poorly representative of of the native antigenic state of the virus

5.4 Discussion.

A number of methods have been developed to identify B cell epitopes using structural approaches, generally refined by training against globular protein/antibody complexes. Each of these methods tends to analyse surface accessibility in conjunction with patch location or clustering and may include residue propensity information. The methods are therefore not entirely independent but the nature of their correlations is not fully characterised.

The evaluation performed in this chapter has demonstrated limitations in the sensitivity and specificity of each program at detecting known Picornavirus epitopes. However, it is quite likely that there are additional residues that form part of the antigenic composition of the viruses used in this evaluation. If this is true then some of the residues considered as false positives might be genuine, so that the programs might be performing better in this respect than our metrics indicate (although this would be likely offset by the fact that some of the negatives classified currently as correct would be reclassified as false negatives).

Additionally, the optimal threshold values of the programs for identifying both Polio and Rhinovirus epitopes differed in some cases from those identified as optimal for performance by the program developers. This difference in threshold may be explained by the way the programs are optimised using a wide range of epitope structures instead of being focused as we are here on viral epitopes. This suggests that the best method for utilising these servers to predict viral epitopes is to identify the optimal threshold value for each program using a virus related closely to that being examined.

The model predictions are only rendered marginally more reliable by using a consensus of any two of the best three performing programs (Figure 5.3(f) and figure 5.4). When a consensus of all three best performing programs is taken the sensitivity of the results decreases but the specificity and the accuracy increase, indicating that this provides a very conservative approach to identify epitopes, resulting in less false positives and giving more confidence in the results. This is of particular importance when using the program outputs as predictions to base time consuming and expensive experiments on. Based on this performance we examined the conservative consensus results to see if there are residues which may represent undiscovered FMDV epitopes on each serotype evaluated. Several such putative epitopes were discovered and there are circumstantial reasons to believe that these predictions contain some truth. The majority of the residues predicted are located on or adjacent to a known epitope identified by monoclonal antibody techniques on at least one serotype of FMDV. Potentially these residues are epitopes on all serotypes given the strong structural similarity of the FMDV capsids but due to the random nature of mAb selection and the discrepancy in the depth of investigation using these techniques between serotypes they have yet to be described. Furthermore several regions are also predicted to be epitopes across serotypes. Finally the putative epitopes are on highly variable and mobile regions of the capsid which would be consistent with these regions coming under some kind of selective pressure, potentially from antibodies.

The consensus results predicted one completely novel epitope at residues 190-192 of VP2, residues which are highly variable in serotype O. Although this region is novel it should be noted that it is only slightly downstream of an epitope identified on serotype O (Barnett *et al*., 1998). Mapping this region to the capsid suggests that there might be a conformational epitope including both VP2 and VP3 residues spanning the 3 fold axis (and hence the pentamer-pentamer interfaces). A second conformational epitope is also suggested on VP1 towards the 5-fold axis at the centre of the pentamer.

Of the four predicted epitopes identified using the sequence and serology data in chapter four two are identified here on serotype O. VP2 191 is part of the region VP2 190-192 and VP1 198 is in region 194-198. Both of these regions are predicted as epitopes across the different serotypes of FMDV. The two other residues identified from the modelling carried out in chapter four were not identified as epitopes in this structural analysis, at least for serotype O. However the VP3 C terminus was identified using this approach for serotypes SAT-1 and C. The other residue, VP1 138 was not identified on any serotype. This is not surprising as it is located on the highly mobile GH loop which is missing on most structures. For the reduced serotype O structure this residue was not identified, but this could be because this residue lies on a structure that is unusual when compared to the other structures used to train these programs.

The evaluation performed here, the first to specifically examine the outputs of these programs with a virus, has yielded valuable insight into how these programs perform in identifying already known viral epitopes. The results highlighted the very limited predictive power of an individual program at identifying established epitopes, suggesting that it may be prudent to develop a program more specifically designed for the task of identifying viral epitopes, for example in using exclusively only epitope/paratope structures derived from viruses. However using a simple consensus of the results from the best performing programs goes some way to overcoming these limitations, and suggests that, suitably tuned and used in concert, these programs could already provide a quick, cheap, and reasonably reliable method for predicting epitopes on several virus serotypes simultaneously. In order to determine the accuracy of this analysis a small subset of predicted epitopes will be taken forward for analysis using reverse genetics techniques.

Chapter 6: Determination of the cause of the distant antigenic relationship between the O Kaufbeuren wild type and infectious copy viruses.

Mutant virus M1 was rescued by Amin Asfor.

6.1 Summary.

One of the sera used for the analysis in Chapter three was against the O KIC virus that was expected to be very similar to the field strain, O KWT. In fact it was found that the O KIC virus was antigenically distinct from the O KWT virus and indeed from all other type O viruses tested in chapter three. The reason is likely to be the extensive (64) passages in cell culture to derive the O KIC virus. This was investigated by sequencing and comparing both the O KIC and O KWT virus. This chapter presents those results and, via the construction of a series of 20 mutant viruses, a dissection of the correlation between sequence changes and antigenic effect.

On sequencing the capsid encoding genes, eight amino acid differences were identified on the capsid between the O KIC and the O KWT viruses. Using the O KIC PT7S3 full length plasmid, mutant viruses were made with single and multiple amino acid reversions towards the O KWT virus. In addition, two mutants were constructed in which the existing amino acids at sites of particular interest (at VP2 130 and at VP3 56) were replaced by the neutral amino acid alanine. The mutant viruses selected were passaged 3 times, to confirm the stability of the mutation, and then tested using the VNT and LPBE assay against both the O KIC and O BFS sera. The O BFS serum was used to determine if any substitution was more broadly affecting serotype O antigenicity.

Reversion of residue VP2 130 from tyrosine back to cysteine (which usually forms a disulphide bridge with VP1, modulating the VP1 GH loop structure), initially hypothesised as the cause of the unique sero-reactivity, had little or no effect on virus antigenicity when this substitution was made individually. However, a change at this same residue from a tyrosine to an alanine resulted in significant reductions in both the VNT and LPBE titres. Although VP2 130 is not an alanine on any of the heterologous viruses investigated here, this result highlights the effect that modulating this residue can have on the antigenicity of serotype O viruses, both in the LPBE and VNT.

VP3 173 reversion had the biggest impact on cross reactivity in both the VNT and LPBE. The O KIC virus is unique among the O serotype viruses tested in having a glycine at this site, so it appears

highly likely that this residue, interacting with others, is the cause of the narrow sero-reactivity of the O KIC serum, both in the VNT and LPBE. VP3 68 and 85, individually, were significant only in lowering the LPBE titres, but against both sera, and so may be epitopes that although not important for *in vitro* virus neutralisation, are important in the overall antibody response to serotype O FMDV. Residues surrounding the heparan sulphate binding site were of limited antigenic importance; however, the plaque assay results demonstrated that additional residues may be important in determining receptor affinity. Other substitutions were more serum specific and individually not likely to be epitopes across the O serotype. The results here demonstrate the variation in importance of different residues between different sera of the same serotype, which again demonstrates the importance of using as broad a set of serological samples from across a serotype as possible when evaluating what epitopes might be cross reactive both within and between serotypes.

6.2 Methods.

6.2.1 Generation of mutant viruses

All mutant viruses were generated as described in section 2.5 using the O KIC PT7S3 plasmid. The primer sequences used to generate the mutant viruses are listed in appendix 3. All viruses were cultured to passage three on either IBRS-2, BTY, BHK or ZZR-127 goat tongue cells. A large number of cell lines were used to ensure that any residue changes that alter virus receptor usage had the best chance of being rescued. Following the rescue of each virus, the presence of the correct mutation was confirmed using Sanger sequencing as per section 2.4. Once each mutation was confirmed, the viruses were stored at -80°C until used.

6.2.2 Serological analysis

All mutant viruses were tested five times in both the VNT and the LPBE, as described in sections 2.2.4 and 2.2.6 respectively. The serum used in the analysis was the homologous O KIC and the heterologous O BFS sera (for more details on the serum used see section 2.2.2). Due to difficulties with culturing some viruses to acceptable titres on IBRS-2 cells, ZZR-127 (foetal goat tongue) cells were used to perform the VNT assay in this chapter (Brehm *et al.*, 2009).

6.2.3 Plaque assay

A plaque assay was performed as outlined in section 2.2.7 using ZZR-127 cells.

6.2.4 Statistical analysis

Statistical analysis was performed as outlined in section 2.7 with the virus as a fixed effect and the date of each test as a random effect.

6.3 Results

6.3.1 Serological comparison of the O KIC and O KWT viruses.

The O KWT was tested twice in the VNT and LPBE against the O KIC sera in the work conducted in Chapter three, with surprising results (Tab 6.1 and 6.2). The results revealed that antigen derived from the O KIC virus would not be a suitable vaccine for use in a situation where an outbreak was caused by the O KWT virus. This was unexpected as both these viruses were presumed to be very closely related. In the VNT, the difference in serum titre between the O KIC and O KWT viruses was 0.64 $\log_{10}$ and the r_1 value was 0.22 (Table 6.1). The difference in serum titres in the LPBE was even greater, at 0.78 $\log_{10,}$ the r_1 value was 0.17 (Table 6.2).

Virus strain	VNT titre 1	VNT titre 2	Mean VNT titre	r_1 value VNT
O KIC	2.06	2.06	2.06	1.00
O KWT	1.46	1.34	1.40	0.22

Table 6.1: Table showing the initial VNT results obtained with an O KIC antiserum.

Virus strain	LPBE titre 1	LPBE titre 2	Mean LPBE titre	r_1 value LPBE
O KIC	2.55	2.55	2.55	1.00
O KWT	1.77	1.77	1.77	0.17

Table 6.2: Table showing the initial LPBE results obtained with an O KIC antiserum.

6.3.2 Sequence comparison of the O KIC and O KWT viruses.

The full capsid sequences for both the O KIC and O KWT viruses were generated and aligned. Upon comparison, there were eight amino acid differences between the two viruses (Table 6.3 and Figure 6.1). Six differences were located on VP3, with only a single substitution occurring on each of VP1 and VP2. None of the changes were located at known antigenic sites (Figure 6.1), although on the 3D structure VP3 56, 60 and 85 are in close proximity to antigenic site four (Kitson *et al.*, 1990) and, along with VP3 173, are also in close proximity to residue VP1 198 of an adjacent protomer, previously defined as part of an epitope (Aktas & Samuel, 2000).

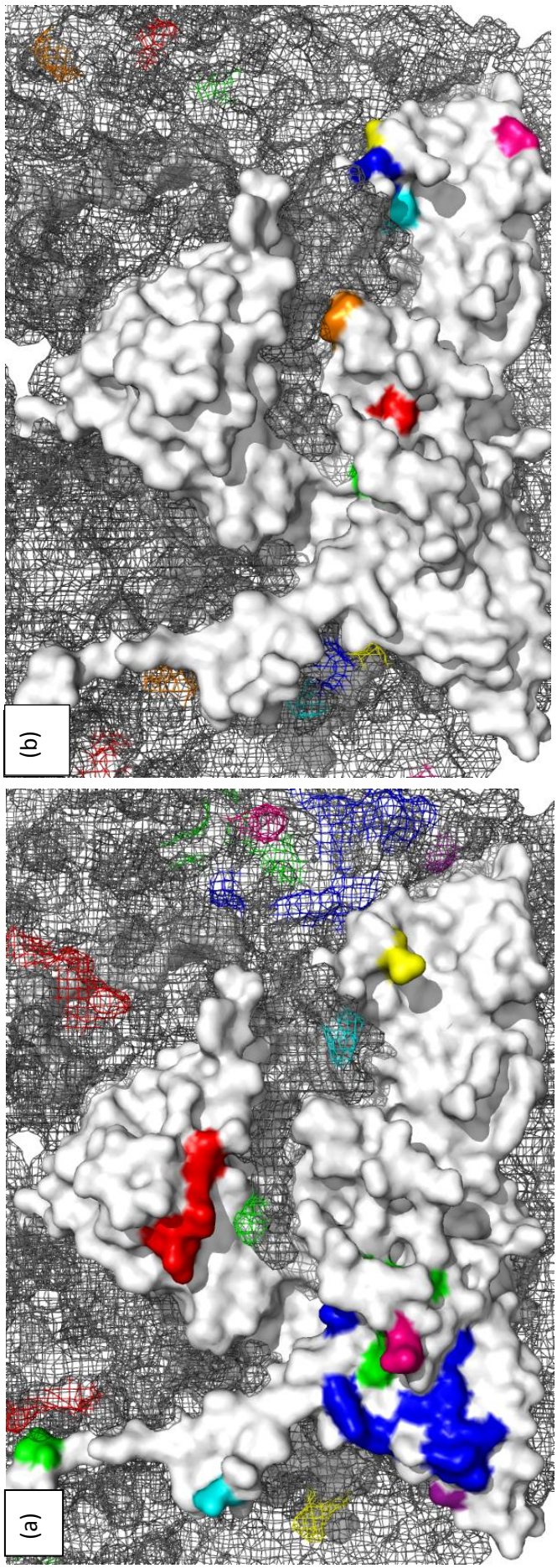

Figure 6.1: A side by side comparison of O BFS (PDB ID 1FOD) showing the molecular surface of the capsid, with a central protomer (in a reduced conformation with the GH loop present) represented in solid white and surrounding protomers as a grey mesh, highlighting the known antigenic sites (a) and the location of the amino acid differences between the O KIC and O KWT wild type viruses (b). On (a) Antigenic site one is coloured green, site two is blue, site three is red, site four is yellow and site five is pink. The additional residues identified from other studies are coloured purple (VP2 188) and teal (VP1 198). The substitutions between the O KIC and O KWT (b) are coloured as shown in table 6.3. Figure made using Pymol (Schrödinger LLC)

Position	O KIC	O KWT	Colour on Figure 6.1
VP1-133	Glutamic acid (E)	Lysine (K)	
VP2-130	Tyrosine(Y)	Cysteine (C)	
VP3- 56	Arginine (R)	Histidine (H)	
VP3- 60	Glycine (G)	Aspartic acid (D)	
VP3- 68	Threonine (T)	Methionine (M)	
VP3- 85	Glutamine (Q)	Histidine (H)	
VP3- 123	Valine (V)	Isoleucine (I)	Buried along the 2 fold axis
VP3- 173	Glycine (G)	Aspartic acid (D)	

Table 6.3: List of amino acid differences between the O KIC and O KWT viruses. The single letter amino acid code is listed in brackets.

VP3 56 and 60 are also involved in the binding of heparan sulphate (HS) *in vitro* (section 1.2.4) and also comes into very close proximity on the 3D structure with antigenic site two of the adjacent protomer. The substitution at VP3 56 has been identified previously as the key residue change for the interaction with HS (Fry *et al.*, 1999; Jackson *et al.*, 1996), so it seems likely that this change therefore occurred during the adaptation of the O KIC virus to tissue culture. When the location of these substitutions are observed on the virus structure it can been seen that VP3 56, 60 and 85 are all located in or around the HS receptor binding site (figure 6.2- coloured as table 6.3).

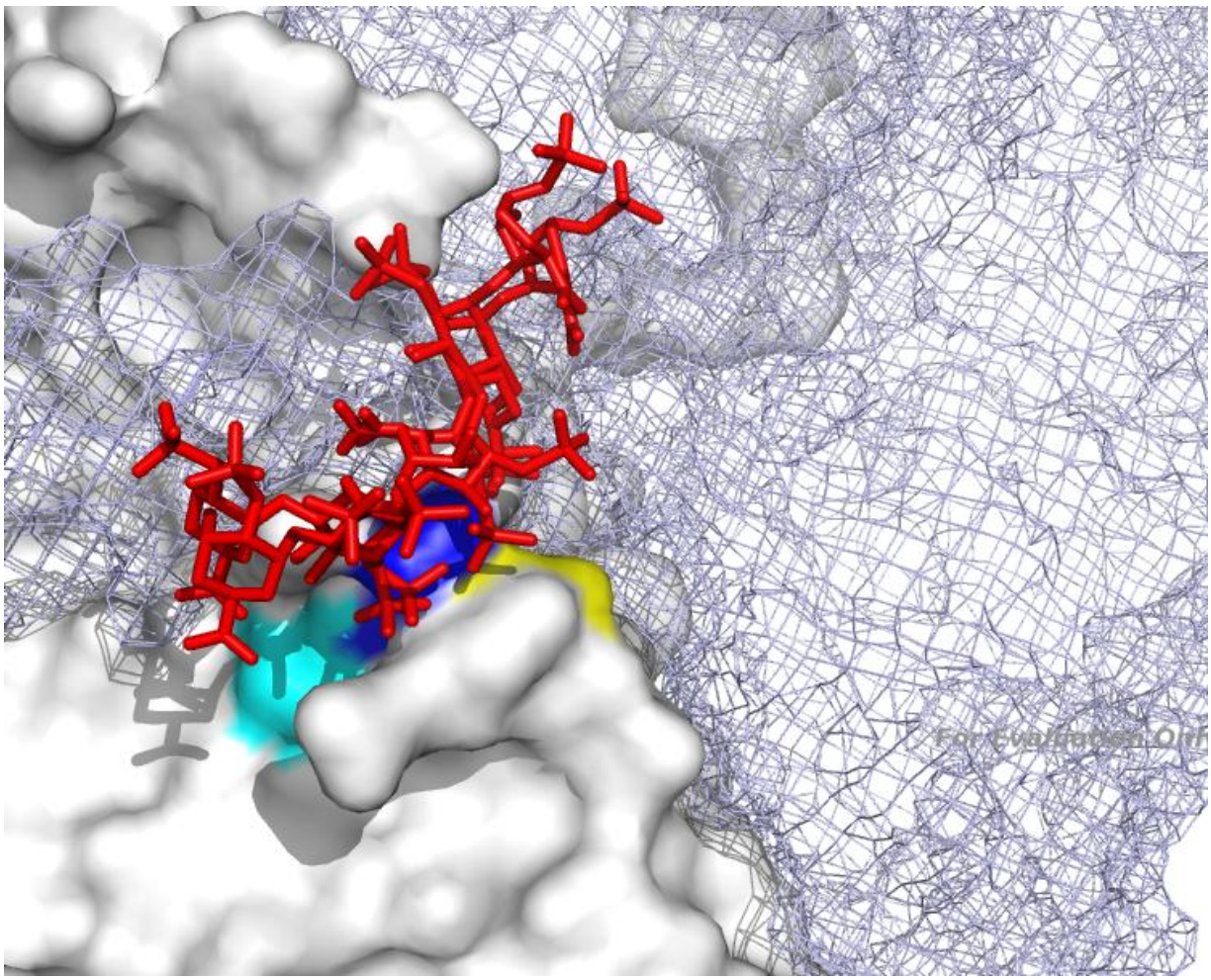

Figure 6.2: A zoomed in area of a serotype O capsid, in the same orientation and configuration as figure 6.1, showing the proximity of several of the residues substituted between the O KIC and O KWT virus to the binding site of Heparan sulphate. The heparin molecule is shown as a stick and coloured red with the residues coloured as outlined in table 6.3. Picture made use PDB 1QQP in pymol Pymol (Schrödinger LLC).

The only substitution on VP2 is located at a site normally occupied by a cysteine residue that forms a disulphide bridge with residue 134 of VP1 (Acharya *et al.*, 1989), which has previously been suggested to play a role in the antigenicity of serotype O FMDV by holding the highly mobile and antigenic GH loop in an "up" conformation ((Parry *et al.*, 1990)-see section 1.3 for more details on this disulphide bridge). In addition, VP2 130 lies next in sequence to VP2 131, which forms part of antigenic site 2. The initial hypothesis was that, for the reasons listed above, it was the change at this residue that was causative of the unusual sero-reactivity of the O KIC serum (the hypothesised impact of each residue on serum titre and receptor affinity is listed in table 6.4). The single difference

between the two sequences in VP1, VP1 133 is located next to the residue that forms the disulphide bridge with VP2 130 (VP1 134).

Substitution from the O KIC to O KWT	Hypothesised effect on titre
VP1 133 E-K	Change in charge-may affect titre
VP2 130 Y-C	Residue change will affect the conformation of the GH loop, suspected to be the change that is driving the unique antigenicity
VP2 130 Y-A*	Residue change will affect the conformation of the GH loop, to an even greater extent than a change from Y-C
VP3 56 R-H	Previous reports have suggested a role in antigenicity, should effect the ability of the virus to bind heparan sulphate.
VP3 56 R-A*	Should abrogate heparan sulphate binding
VP3 60 G-D	Change in charge- may affect serum titre
VP3 68 T-M	Neutral substitution- not expected to affect titre
VP3 85 Q-H	Neutral substitution- not expected to affect titre
VP3 123 V-I	Internal- not expected to affect titre
VP3 173 G-D	Change in charge- may affect serum titre

Table 6.4: Summary of the hypothesised role of each substitution found between the O KIC and O KWT virus.

Several substitutions have resulted in changes to the charge at a particular residue, this is particularly true of the change at VP1 133, from a glutamic acid to a lysine, and both VP3 173 and VP3 60 where substitutions resulted in a gain of a negative charge (table 6.4). These changes, along with the change at VP2 130 may be important in driving antigenic changes because both charged and aromatic residues are reported to be the main drivers of interactions between epitopes and paratopes, as discussed in section 1.7. Several substitutions that result in a change in the size and polarity of a residue, for example changes from cysteine to tyrosine of from aspartic acid to glycine, are also observed. These could result in subtle changes in the structure of an epitope and therefore diminish binding of antibodies.

When these eight substitutions are examined on the other 105 virus capsid sequences generated in this study (chapter 3) it becomes apparent that three of the changes are unique to the O KIC virus. These include the substitutions at residues VP2 130 and VP3 85, which are cysteine and histidine, respectively, on all other viruses. The glycine substitution at VP3 173 is also unique to the O KIC; however the 3 viruses belonging to the East Africa-2 topotype have a serine at this position, rather than the aspartic acid found on all other viruses.

Residues VP3 56 and 60, involved in heparan sulphate binding, vary on many viruses in the same way as the O KIC and O KWT, with either an arginine or a histidine at VP3 56 and either glycine or aspartic acid at VP3 60. However, this is not associated with viruses of a particular topotype (apart from the change to tyrosine at VP3 56 on the Cathay topotype viruses) and is therefore likely to be associated with the level of adaptation of each virus to tissue culture rather than reflecting naturally occurring substitutions in the field. As discussed previously, the O KIC virus is highly adapted to tissue culture, having been passaged over 64 times.

The O KIC virus shares the same amino acid (glutamic acid) at VP1 133 as 12 of the viruses from the European-South American topotype. The valine at VP3 123 on O KIC is shared with 13 viruses from the Cathay topotype. The final residue, VP3 68, is the only residue where the O KIC virus shares the same amino acid with the majority of viruses analysed; only four viruses clustered together in the Middle East-South East Asia topotype and the O KWT and Lausanne viruses, both closely related phylogenetically to the O KIC virus, have a different amino acid at this residue.

6.3.3 Site directed mutagenesis on the O KIC PT7S3 plasmid.

To identify the cause of the low cross-reactivity of O KIC BVS to the O KWT virus twenty full length genome constructs harbouring either single, double or multiple mutations, based on the observed changes between the O KIC and O KWT viruses (Table 6.5) were generated using standard site-directed mutagenesis, and were assigned a mutant (M) number. This included several of the substitutions in combination with a substitution at VP2 130 from a tyrosine to a cysteine, as this was hypothesised to be an important change (discussed above). Other combinations involving residues VP3 56, 60, 85 and 173 were also selected based on the proximity of these substitutions to each other, which could therefore be having a combined effect on cross reactivity. An additional two mutant viruses with a single substitution to an alanine were also constructed at residues of particular interest, VP2 130 (discussed above) and VP3 56, which is the key residue involved in HS binding *in vitro.* Alanine was selected as this has been suggested previously as the best residue to use for epitope scanning, as its small and neutral nature means that the residue does not impact on the overall backbone structure of a protein, enabling the specific effect of an individual residue to be examined (Cunningham & Wells, 1993).

Live infectious virus was recovered from all the constructs, with the exception of the virus having only a single substitution from a glutamic acid to a lysine at VP1 133. All the viruses were grown on tissue culture at least three times and the identity of the recovered virus was confirmed by sequencing the capsid coding genes. For an abridged amino acid capsid sequence alignment showing the presence of the correct mutations see appendix 5. The residue (VP1 133) that was not successfully rescued is located adjacent to the cysteine (figure 6.3) that normally (in the case of serotype O) forms the disulphide bridge with a cysteine at VP2 130. However, the presence of a tyrosine at residue VP2 130 of VP2 on O KIC leads to a strong selective pressure for this residue to revert back to glutamic acid, as seen in the sequencing results from passage three on all cell lines evaluated (figure 6.4). When this mutation was made in combination with the substitution of the

tyrosine at VP2 130 to cysteine this selective pressure was not observed (data not shown). This suggests that there is some interaction between the tyrosine and glutamic acid.

Mutant name	Substitution(s) made to O KIC capsid	Rescued?
Single Mutants		
M6	VP1 133 E-K	-
M1[#]	VP2 130 Y-C	+
M1a *	VP2 130 Y-A	+
M7	VP3 56 R-H	+
M7a *	VP3 56 R-A	+
M8	VP3 60 G-D	+
M3	VP3 68 T-M	+
M4	VP3 85 Q-H	+
M9	VP3 123 V-I	+
M5	VP3 173 G-D	+
Double Mutants		
M1+M6	VP2 130 Y-C & VP1 133 E-K	+
M1+M7	VP2 130 Y-C & VP3 56 R-H	+
M1+M8	VP2 130 Y-C & VP3 60 G-D	+
M1+M3	VP2 130 Y-C & VP3 68 T-M	+
M1+M4	VP2 130 Y-C & VP3 85 Q-H	+
M1+M5	VP2 130 Y-C & VP3 173 G-D	+
M2	VP3 56 R-H & 60 G-D	+
M4+M5	VP3 85 Q-H & 173 G-D	+
Triple Mutants		
M2+M4	VP3 56 R-H, 60 D-G & 85 Q-H	+
M2+M5	VP3 56 H-R, 60 D-G & 173 D-G	+

Table 6.5: List of the single, double and triple mutants generated for serological testing. * Denotes an alanine mutant virus and not an amino acid substitution that occurred between the O KIC and O KWT viruses. [#]Mutant M1 rescued by Amin Asfor.

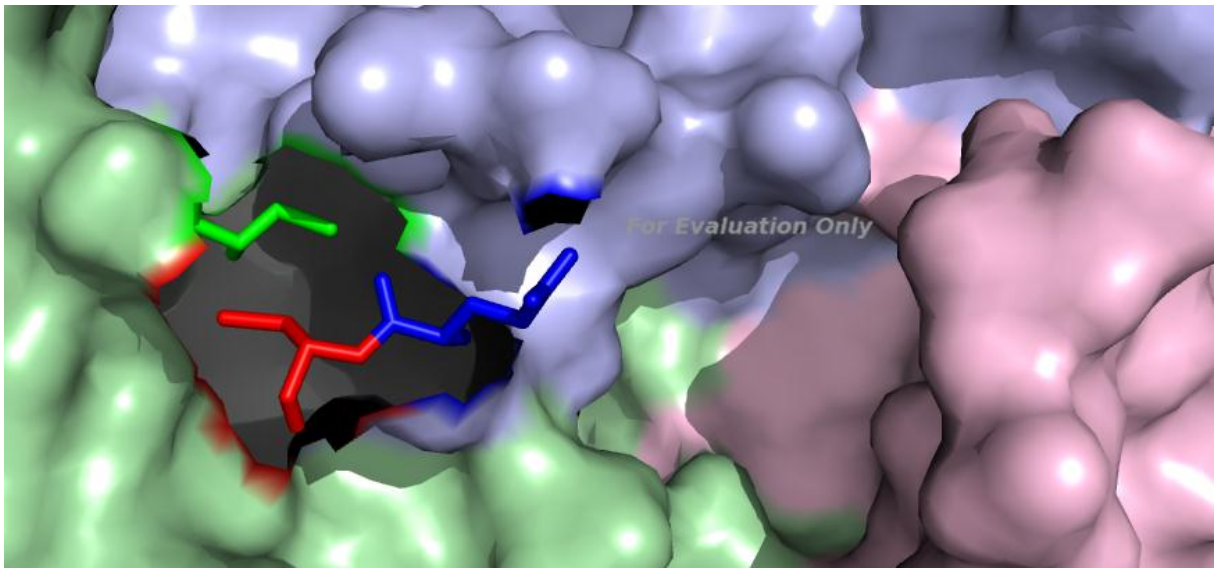

Figure 6.3: Close up of the molecular surface of an O BFS protomer (1BBT) with the GH loop absent. The location of the Disulphide bridge between VP2 130 (green) and VP1 134 (red) with the location of VP1 133 in blue. VP1 is coloured light blue, VP2 light green and VP3 mauve.

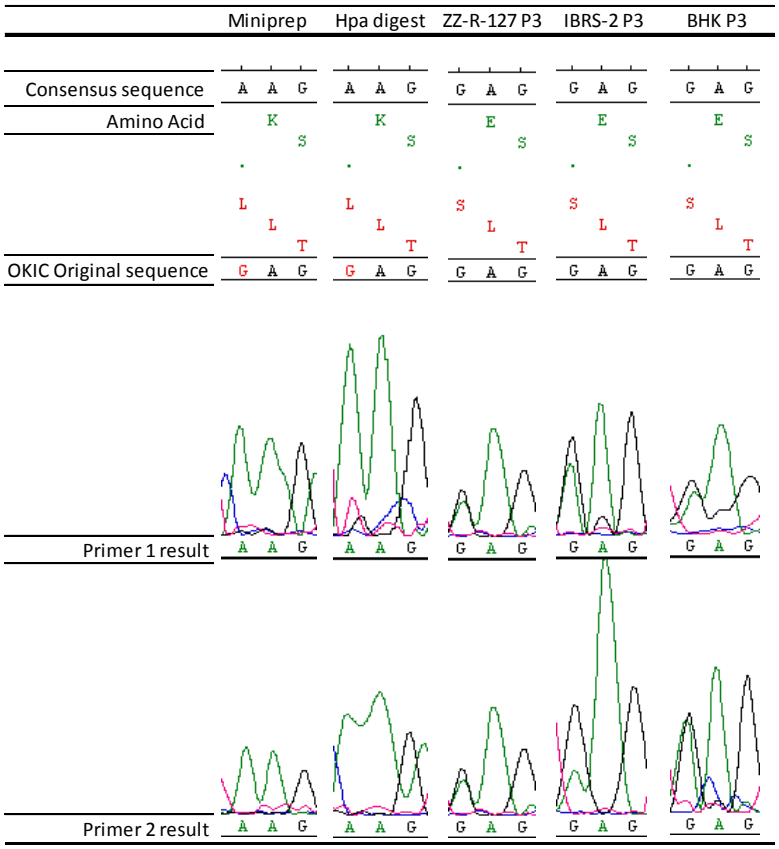

Figure 6.4: Mutant M6 sequence alignments showing the codon for VP1 133 at the consensus level, the original O KIC sequence and primer traces obtained from Sanger sequencing for the mutant virus M6 from miniprep, Hpa digested DNA prior to electroporation and RNA extracted from 3 different cell lines after three passages. Within the nucleotide traces a green line represents an adenine, a blue line represents cytosine, a pink line represents thymine and a black line guanine.

6.3.4 Plaque morphology

The plaque morphology for the two control viruses was as expected, with the O KIC virus, which contains the mutation at VP3 56 from histidine to arginine (considered the key mutation involved with HS binding *in vitro*) showing smaller plaques associated with HS binding (figure 6.5), when compared to the O KWT virus lacking this mutation, consistent with previous observations (Borca *et al.*, 2012; Jensen & Moore, 1993). Those viruses that possess a substitution at this residue from arginine to histidine (any virus containing M7 or M2 in the names given in Figure 6.5) also have a larger plaque phenotype more closely resembling the wild type virus. The mutation at VP3 60 from glycine to aspartic acid (M8) also appears to have the effect of abrogating binding to HS. Although this residue has been implicated as a ligand involved in the interaction with HS, a change at this residue has not previously been described as critical for this binding. Further to this, mutations at VP3 85 Q-H and 173 G-D (M4+M5 in figure 6.4) in combination also appear to have the same effect. However, the single substitution of VP3 85 Q-H (M4) appears to have an attenuating effect on virus growth. A summary of the main effects observed are displayed in table 6.6.

When observing the effects of other mutations (figure 6.5) it would appear that several have a mixed population of plaque morphologies. In some cases it appears that the viruses may be utilising the two different receptor types (integrin and HS), for example M1 and M1a (changes at VP2 130). Other mutant viruses have a large amount of heterogeneity in plaque size. This observation is consistent with previous reports for FMDV, where heterogeneity in plaque sizes in plaque purified viruses have been observed after only a small number of tissue culture passages (Gomez et al 1984). Further work could be done to establish the cause of this by picking and sequencing individual plaques.

Substitution between the O KIC to O KWT	Effect on plaque size
VP3 56 R-H (M7)	Abrogates heparan suplhate binding
VP3 56 R-A* (M7a)	Abrogates heparan suplhate binding
VP3 60 G-D (M8)	Abrogates heparan suplhate binding
VP3 85 Q-H (M4)	Has an attenuating effect on virus growth
VP3 173 G-D (M5)	Abrogates heparan suplhate binding when combined with VP3 85 Q-H

Table 6.6: The main effects reported for individual substitutions through plaque size observations

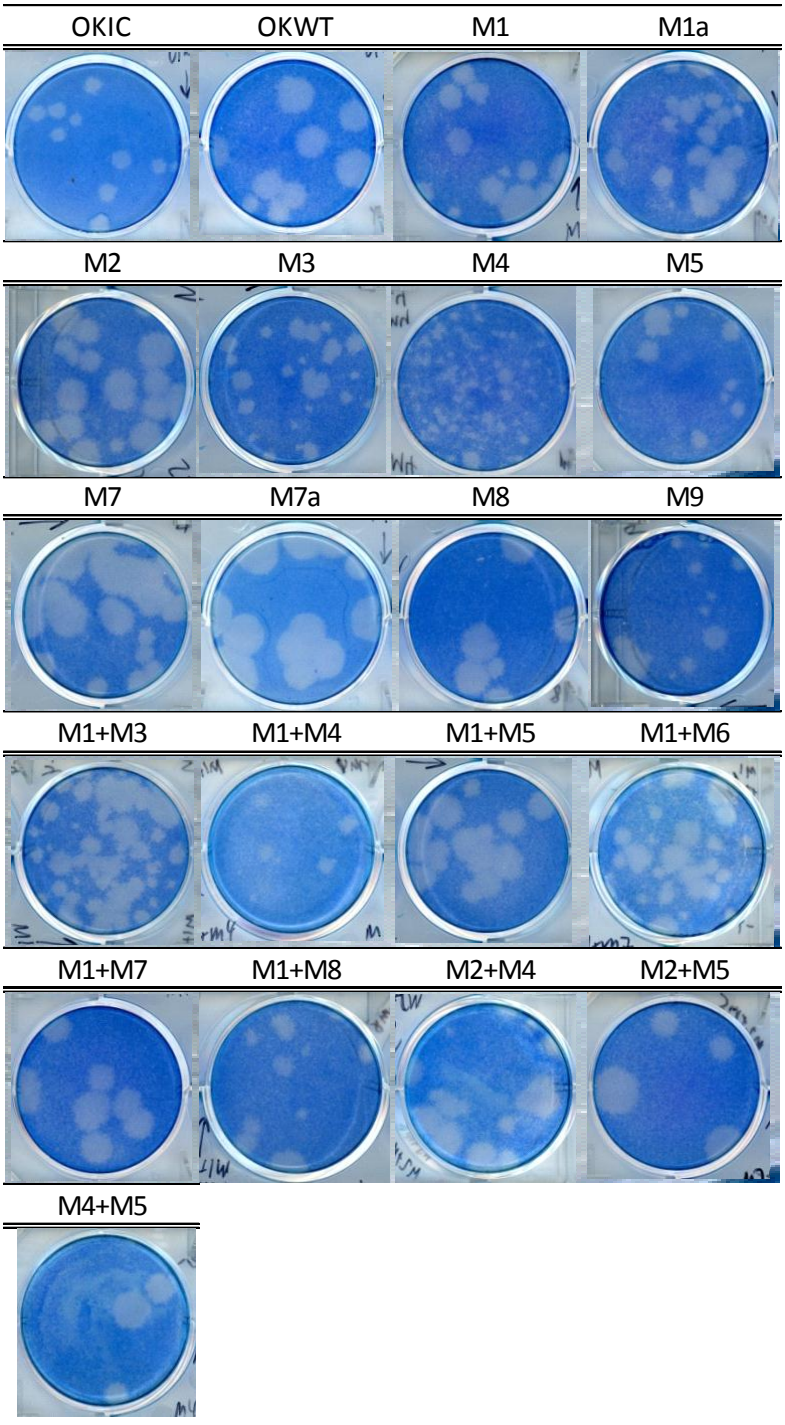

Figure 6.5: Plaque morphology of O KIC, O KWT and mutant viruses at 48 hours post infection.

6.3.5 Serological analysis

6.3.5.1 O KIC serum VNT and LPBE results

The statistical results demonstrated, for both the VNT and LPBE, that the general linear model selected for the analysis had a good fit for the data, with R^2 values (the percentage of variation accounted for by the model selected) reported of 86.73% and 70.23% respectively. Additionally, for both tests, the virus used and the date the test was conducted both had significant effects ($P<0.05$) on the results.

For both the VNT and the LPBE, the results obtained for the control viruses were similar to those from chapter three, with serum titres of 2.2 and 2.4 $\log_{10}$ for the O KIC and 1.5 and 1.9 $\log_{10}$ for the O KWT for the VNT and LPBE respectively (Table 6.8). For the VNT results, the cells used here were the ZZR-127 goat tongue cells and not IBRS-2 cells as in chapter three. These results suggest that changing the cell line did not have an impact on the results obtained.

After five repeats of the VNT, nine out of 19 rescued viruses were neutralised significantly less effectively than the parental O KIC virus with the O KIC serum ($P<0.05$), while for the LPBE ten of the 19 rescued viruses resulted in significant drops in serum titre compared to the parental O KIC virus with the O KIC serum ($P<0.05$). A summary of the results for each significant virus is listed in table 6.7- for the complete results see figure 6.6 and table 6.8 at the end of this section. Six of the mutant viruses caused significant drops in serum titre in both the LPBE and VNT assay. Four of which contained a substitution at VP3 173 G-D (M5, M1+M5, M4+M5 and M2+M5), meaning that any virus containing this mutation (either individually or in combination) caused a significant decrease in serum titre in both the VNT and LPBE assay. The remaining two viruses that caused significant decrease in serum titre in both the VNT and LPBE were the virus with a single substitution at VP2 130 from Y-A (M1a) and a double mutant virus with substitutions at VP1 130 from Y-C and VP3 85 from Q-H (M1+M4). The latter two of these substitutions (VP1 130 Y-C and VP3 85 Q-H) were also significant in combination with VP3 173 G-D, in both assays.

Virus	Mutations from O KIC	Drop in VNT serum titre (log_{10})	Drop in LPBE serum titre (log_{10})
M1+M3	VP2 130 Y-C & VP3 68 T-M		-0.6
M1+M4	VP2 130 Y-C & VP3 85 Q-H	-0.4	-0.5
M1+M5	VP2 130 Y-C & VP3 173 G-D	-0.5	-0.3
M1+M8	VP2 130 Y-C & VP3 60 G-D	-0.2	
M1a	VP2 130 Y-A	-0.4	-0.5
M2+M4	VP3 56 R-H, 60 D-G & 85 Q-H	-0.7	*
M2+M5	VP3 56 R-H, 60 D-G & 173 D-G	-0.7	-0.5
M3	VP3 68 T-M		-0.4
M4	VP3 85 Q-H		-0.7
M4+M5	VP3 85 Q-H & 173 G-D	-0.6	-0.4
M5	VP3 173 G-D	-0.2	-0.4
M7	VP3 56 R-H	-0.3	
M9	VP3 123 V-I		-0.6
O KWT	N/A	-0.7	-0.5

Table 6.7: Summary of the O KIC serology results, showing the size of the drop in serum titre (log_{10}) reported compared to the homologous O KIC virus, for each virus having a significant effect on serum titres ($P< 0.05$) and the O KWT virus, in both the VNT and LPBE. *Indicates that the virus did not trap in the LPBE.

In addition to those mutants affecting both assays three mutant viruses containing just a single substitution caused significant decreases in serum titre only in the LPBE (table 6.7); VP3 123 V-I (M9) is an anomalous results as this residue is buried on the intact capsid. VP3 68 T-M (M3) is significant both individually and as a double mutant with VP2 130 Y-C (M1+M3). The mutant virus with a single substitution at VP3 85 Q-H caused the most significant drop in LPBE titre, in addition to the significance of this substitution in both assays in varying combinations, as discussed above.

A further three of the mutant viruses caused significant decreases in serum titres when compared to the homologous O KIC titre only in the VNT (table 6.7). These were one mutant virus with only a single substitution, at VP3 56 R-H (M7), one mutant virus with a double substitution, at VP2 130 Y-C and VP3 60 (M1+M8), and a mutant virus with three substitutions, at VP3 56 R-H, 60 D-G & 85 Q-H (M2+M4). This final virus again contains the substitution at VP3 85 Q-H (M4), which resulted in significant drops in serum titre on may other occasions, mainly in the LPBE assay. However unfortunately this triple mutant virus did not trap in the LPBE assay, so it could not be determined if this virus also resulted in significant drops in LPBE titre.

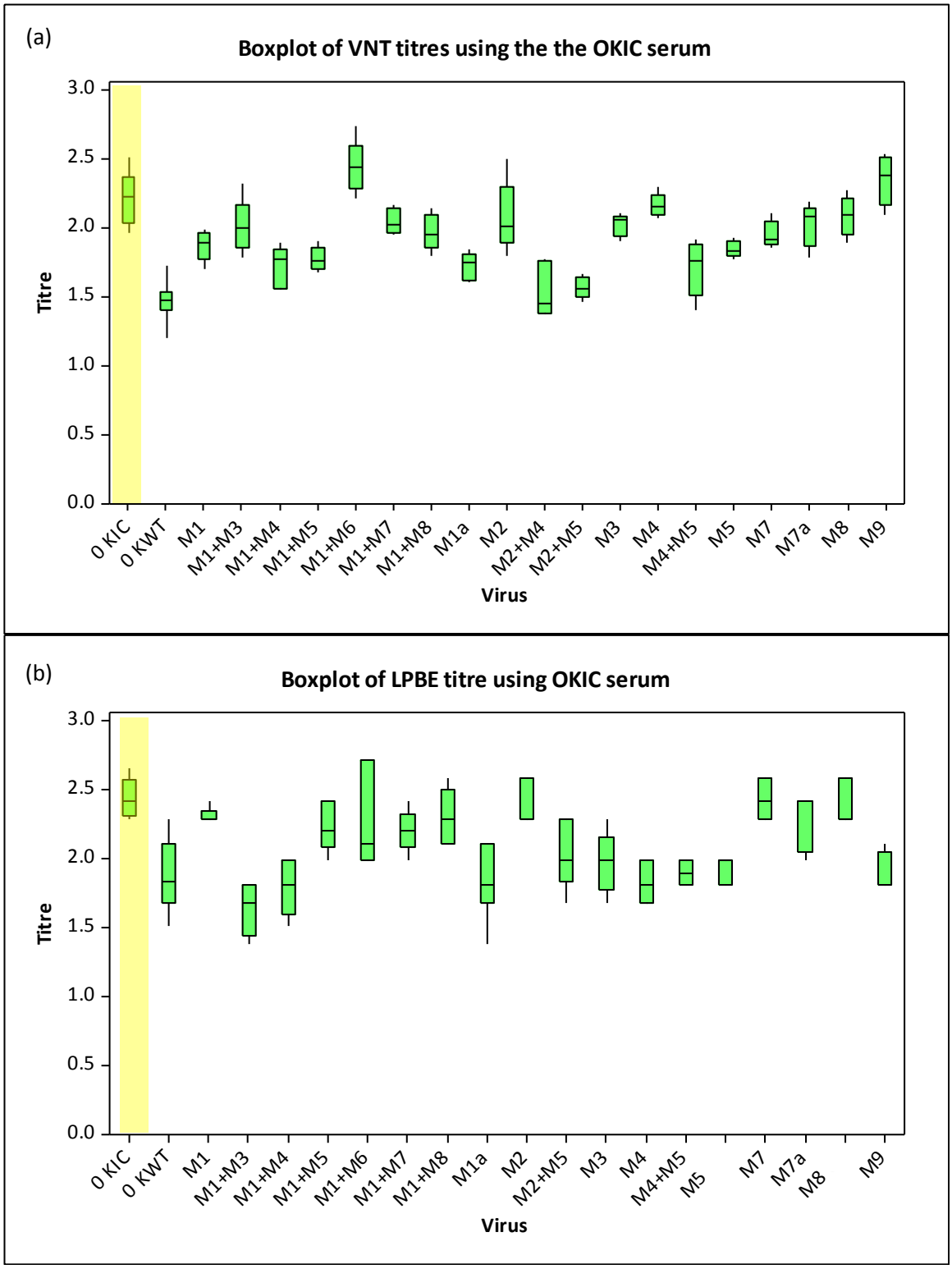

Figure 6.6: $\log_{10}$ serum neutralisation titres generated for each mutant virus against the O KIC serum in the LPBE (a) and the VNT (b). The interquartile ranges for each virus are represented as green boxes, with the horizontal black line within showing the mean value. The 95% confidence intervals are displayed as vertical black lines. The parental O KIC virus result is highlighted in yellow on both plots.

Virus	Mutations from O KIC	VNT Mean	VNT P value	LPBE Mean	LPBE P value
O KIC	N/A	2.2	N/A	2.4	N/A
M1	VP2 130 Y-C	2	0.0578	2.3	1
M1+M3	VP2 130 Y-C & VP3 68 T-M	2.1	0.8189	1.8	0.0001
M1+M4	VP2 130 Y-C & VP3 85 Q-H	1.8	0.0001	1.9	0.0001
M1+M5	VP2 130 Y-C & VP3 173 G-D	1.7	0.0001	2.1	0.0354
M1+M6	VP2 130 Y-C & VP1 133 E-K	2.4	0.3531	2.1	0.1387
M1+M7	VP2 130 Y-C & VP3 56 R-H	2	0.2287	2.3	0.9719
M1+M8	VP2 130 Y-C & VP3 60 G-D	2	0.0145	2.4	1
M1a	VP2 130 Y-A	1.8	0.0001	1.9	0.0001
M2	VP3 56 R-H & 60 G-D	2.1	0.6158	2.4	1
M2+M4	VP3 56 R-H, 60 D-G & 85 Q-H	1.5	0.0001		*
M2+M5	VP3 56 R-H, 60 D-G & 173 D-G	1.5	0.0001	1.9	0.0002
M3	VP3 68 T-M	2.1	0.9335	2	0.0014
M4	VP3 85 Q-H	2.2	0.9999	1.7	0.0001
M4+M5	VP3 85 Q-H & 173 G-D	1.6	0.0001	2	0.0026
M5	VP3 173 G-D	2	0.0162	2	0.0005
M7	VP3 56 R-H	1.9	0.0060	2.5	1
M7a	VP3 56 R-A	2.1	0.9779	2.3	0.9982
M8	VP3 60 G-D	2.1	0.7474	2.5	0.9996
M9	VP3 123 V-I	2.3	0.9774	1.8	0.0001
O KWT	N/A	1.5	0.0001	1.9	0.0001

Table 6.8: The VNT and LPBE mean $\log_{10}$ serum titres and Statistical significance compared to the O KIC virus using the O KIC serum. Those virus with serum titres that are significantly ($P<0.05$) different from the titre of the parental O KIC virus are coloured in Yellow. For each virus the given name from table 6.5 and the substitutions made compared to the O KIC sequence are shown.

6.3.5.2 O BFS serum VNT and LPBE results

The statistical results demonstrated, for both the VNT and LPBE, that the general linear model selected for the evaluation had a good fit for the data, with R^2 values (the percentage of variation accounted for by the model selected) reported of 72.98% and 62.11% respectively, although the LPBE model does appear to have a large amount of variation that is not accounted for by our model. As with the results for the O KIC serum, for both tests, the virus used and the date the test was conducted both had significant effects ($P<0.05$) on the results.

The serological results obtained for both the VNT and LPBE using the O BFS, O KIC and O KWT viruses against the O BFS serum were similar to those reported in chapter three (data not shown) with titres of 1.9, 2.2 and 1.7 $\log_{10}$ in the VNT and 2.6, 2.6 and 2.4 $\log_{10}$ for the LPBE respectively. Each of the viruses listed in table 6.5 were tested five times in both the VNT and LPBE and the serum titre compared to the parental O KIC virus to determine if any changes were responsible for any significant changes in titre towards a heterologous serotype O serum.

After five repeats of the VNT using the heterologous O BFS serum, only four out of 19 rescued viruses neutralised significantly less effectively than the parental O KIC virus ($P<0.05$), while for the LPBE five of the 19 rescued viruses resulted in significant decreases in LPBE titre when compared to the parental O KIC virus ($P<0.05$). A summary of the results for each significant virus is listed in table 6.9- for a complete set of results for each virus see figure 6.7 and table 6.10 at the end of this section. Unlike the results obtained using the O KIC serum none of the mutant viruses caused significant drops in serum titre both in the VNT and the LPBE assay. In addition the serum titre of the O KWT virus was not significantly lower than the serum titre of O KIC virus in the LPBE assay, using the O BFS serum.

Virus	Mutations from O KIC	Drop in VNT titre (log_{10})	Drop in LPBE titre (log_{10})
M1+M4	VP2 130 Y-C & VP3 85 Q-H		-0.4
M1+M7	VP2 130 Y-C & VP3 56 R-H		-0.6
M1a	VP2 130 Y-A	-0.5	
M2+M4	VP3 56 R-H, 60 D-G & 85 Q-H	-0.4	*
M2+M5	VP3 56 R-H, 60 D-G & 173 D-G	-0.5	
M3	VP3 68 T-M		-0.4
M4	VP3 85 Q-H		-0.6
M4+M5	VP3 85 Q-H & 173 G-D	-0.3	
M5	VP3 173 G-D		-0.6
O KWT	N/A	-0.5	

Table 6.9: Summary of the O BFS serology results, showing the size of the drop in serum titre (log_{10}) reported compared to the O KIC virus, for each virus having a significant effect on serum titres ($P< 0.05$) and the O KWT virus, in both the VNT and LPBE. *Indicates that the virus did not trap in the LPBE.

As with the results reported using the O KIC serum, the substitution at VP3 173 G-D (M5) again seems to be the most associated with significant decreases in serum titre (table 6.9). Two of the four viruses with reported drops in serum titre in the VNT had a substitution at this residue, although individually it was not significant in the VNT. However the virus possessing a single mutation at this residue did result in a significant drop in serum titre in the LPBE.

The remaining two viruses that caused significant decreases in VNT titre, when compared to the O KIC virus, was the virus with a single substitution at VP2 130 Y-A (M1a) and the triple mutant virus VP3 56 R-H, 60 D-G and 85 Q-H (M2+M4). The remaining viruses that caused significant decreases in the titre in the LPBE were the mutant viruses containing single substitutions, VP3 68 T-M (M3) and VP3 85 Q-H (M4) and two double mutant viruses, of which one virus again has a substitution at VP3 85 Q-H. Both of these double mutants also contained a substitution at VP2 130 Y-C.

Two of the substitutions that individually caused significant drops in LPBE titre (VP3 173 G-D and VP3 68 T-M) did not cause significant decreases in LPBE titre when in combination with an additional substitution at VP1 130 Y-C (table 6.10). This suggests that this substitution may be having some kind of compensatory effect, reducing the size of the decrease in serum titre

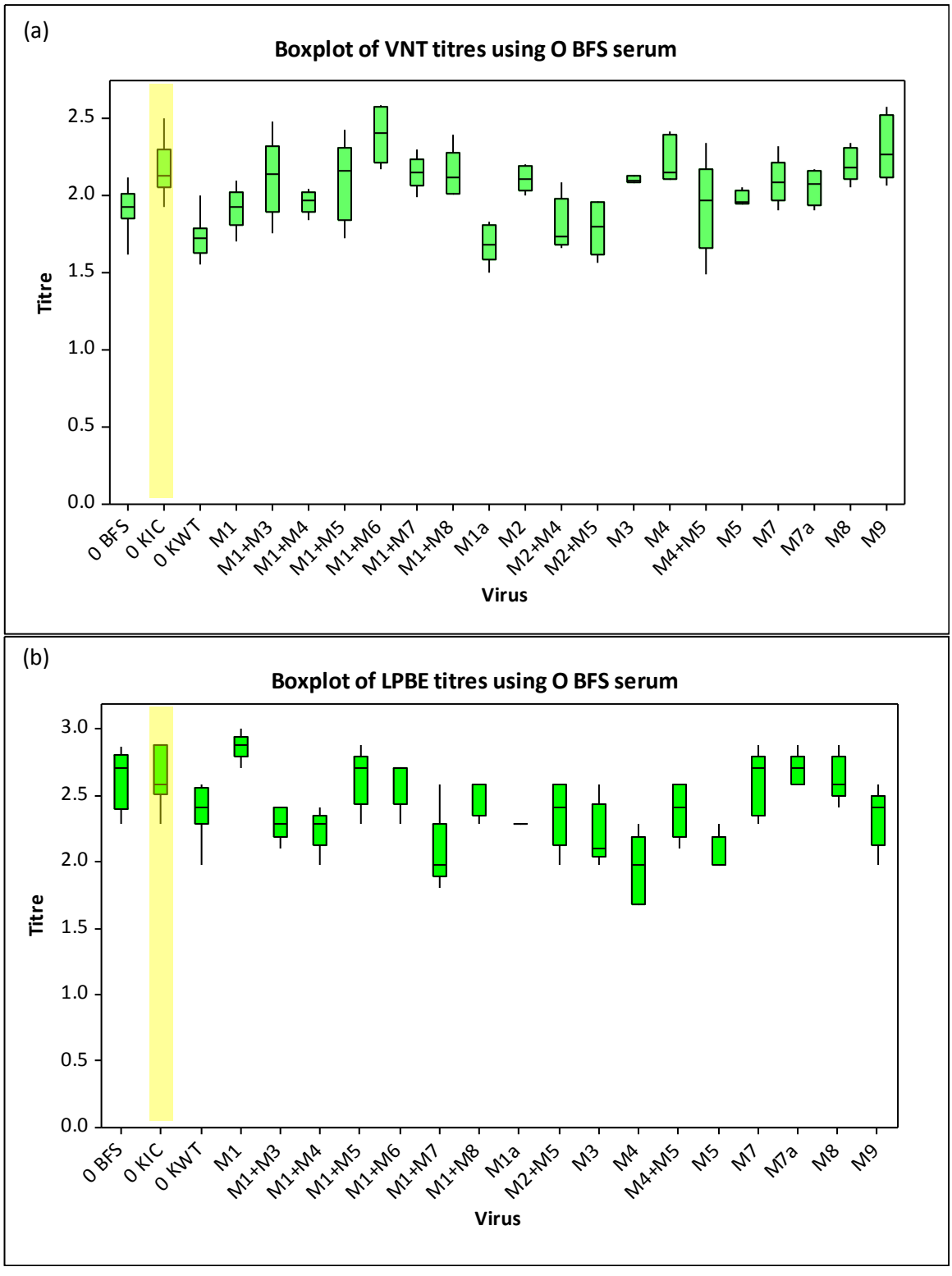

Figure 6.7: $\log_{10}$ serum binding titres generated in the VNT (a) and LPBE (b)for each mutant virus against the O BFS serum. The interquartile ranges for each virus are represented as green boxes, with the horizontal black line within showing the mean value. The 95% confidence intervals are displayed as vertical black lines. The O KIC virus is highlighted in yellow.

Virus	Mutations from O KIC	VNT Mean	VNT P value	LPBE Mean	LPBE P value
O KIC	N/A	2.2	N/A	2.6	N/A
M1	VP2 130 Y-C	2	0.4740	2.9	0.5882
M1+M3	VP2 130 Y-C & VP3 68 T-M	2.2	1.0000	2.3	0.0765
M1+M4	VP2 130 Y-C & VP3 85 Q-H	2.1	0.9984	2.2	0.0181
M1+M5	VP2 130 Y-C & VP3 173 G-D	2	0.5967	2.5	1
M1+M6	VP2 130 Y-C & VP1 133 E-K	2.4	0.1180	2.5	1
M1+M7	VP2 130 Y-C & VP3 56 R-H	2.1	0.9987	2	0.0001
M1+M8	VP2 130 Y-C & VP3 60 G-D	2.2	1.0000	2.4	0.9519
M1a	VP2 130 Y-A	1.7	0.0001	2.3	0.193
M2	VP3 56 R-H & 60 G-D	2.1	1.0000		*
M2+M4	VP3 56 R-H, 60 D-G & 85 Q-H	1.8	0.0004		*
M2+M5	VP3 56 R-H, 60 D-G & 173 D-G	1.7	0.0001	2.3	0.2318
M3	VP3 68 T-M	2.2	1.0000	2.2	0.0108
M4	VP3 85 Q-H	2.2	1.0000	2	0.0001
M4+M5	VP3 85 Q-H & 173 G-D	1.9	0.0059	2.3	0.2705
M5	VP3 173 G-D	2.1	0.9878	2	0.0001
M7	VP3 56 R-H	2.1	1.0000	2.5	1
M7a	VP3 56 R-A	2.1	1.0000	2.7	1
M8	VP3 60 G-D	2.2	1.0000	2.6	1
M9	VP3 123 V-I	2.2	1.0000	2.2	0.0572
O BFS	N/A	1.9	0.0667	2.6	1
O KWT	N/A	1.7	0.0001	2.4	0.2105

Table 6.10: The VNT and LPBE mean $\log_{10}$ serum titres and Statistical significance compared to the O KIC virus using the O BFS serum. Those viruses with serum titres that are significantly ($P<0.05$) different are coloured in Yellow. For each virus the given name from table 6.5 and the substitutions made compared to the OKC sequence are shown. * Means virus not tested.

6.3.5.3 Comparison of the O KIC and O BFS serum results

Upon comparison of the results there appear to be several substitutions that are consistently significantly decreasing serological titres across both sera tested. The substitution that comes up most consistently is VP3 173 G-D, which is significantly affecting titres, both individually and in combination with other substitutions, in the VNT and LPBE against the O KIC and O BFS sera. The substitution at VP2 130 Y-A is also significant in the VNT against both sera. The VP3 85 Q-H substitution significantly reduces titres, when in combination with other substitutions, in the VNT and individually in the LPBE, against both sera. The substitution at VP3 68 T-M also significantly decreased titres against both serum, but only in the LPBE assay. Other substitutions are associated with significant drops in both LPBE and VNT titres across both sera tested. However these are generally significant in combination with the other substitutions listed above.

6.4 Discussion.

The initial discovery that a loss of cross reactivity (and hence predicted vaccine coverage) between the O KWT virus and serum raised towards the O KIC virus was based on amino acid substitutions at just 8 sites across the capsid was surprising. None of the amino acid sequence differences occurring on the capsid between the viruses were located at previously described antigenic sites of serotype O (see chapter one for complete list); however, some residues were in close proximity to these sites. As discussed previously, the substitutions at VP3 56 and 60 are also associated with HS binding *in vitro* (Fry *et al.*, 1999) and, along with the substitution at VP2 130 have been previously implicated as having a potential role in the antigenicity of serotype O FMDV (Jensen & Moore, 1993; Kitson *et al.*, 1990; Lea *et al.*, 1995; Parry *et al.*, 1990; Sa-Carvalho *et al.*, 1997)- see section 1.8 for additional information). A selection of 20 viruses with either individual or multiple substitutions based on the eight observed changes between O KIC and O KWT were isolated, characterised and tested by the classical “gold standard” serological approaches. For a summary of the effects of the eight mutations on serum titres and receptor affinity see table 6.11

Substitution from the O KIC to O KWT	Summary of main effects reported for each substitution
VP1 133 E-K	Only rescued in combination with a change at VP2 130 Y-C, Suggesting an interaction between the two residues.
VP2 130 Y-C	No effect reported on titre individually.
VP2 130 Y-A*	Significant reductions in titre reported both in the VNT and LPBE.
VP3 56 R-H	Abrogated heparan sulphate binding. Only significantly affected titre a single time individually in the VNT against the O KIC sera.
VP3 56 R-A*	Abrogated heparan sulphate binding. No effect on serum titres individually.
VP3 60 G-D	Abrogated heparan sulphate binding. No effect on serum titres individually.
VP3 68 T-M	Significant reductions in LPBE titre individually and in combination. No effect VNT titre individually.
VP3 85 Q-H	Significant reductions in LPBE titre individually and in combination. No effect VNT titre individually. Also appears to have an attenuating effect on virus growth.
VP3 123 V-I	Only significantly affected titre a single time individually in the LPBE against the O KIC sera.
VP3 173 G-D	Significant reductions in both the VNT and LPBE titre individually and in combination against both serum tested. Likely to be the main residue responsible for the unique sero-reactivity of the O KIC serum. Also, in combination with VP3 85, abrogates heparan sulphate binding.

Table 6.11: Summary of the main effects of the eight substitutions that had occurred between the O KIC and O KWT (and the two additional alanine mutants made, labelled with an *) on both serum titres and receptor affinity reported in the results.

Upon observation of the virus plaques it was clear that the O KIC and O KWT viruses had distinct morphologies. The O KIC was smaller in size akin to plaques observed for viruses binding to HS, which is also consistent with the sequencing results indicating the virus had undergone a key change at VP3 56 from histidine to arginine, previously described as the key residue for HS binding *in vitro*. Several of the other substitutions to O KIC virus also appeared to affect the ability of the virus to use HS. VP3 60 has been demonstrated as a ligand involved in this interaction but the results here indicate that a change at this region results in plaques more similar to those of the O KWT virus, as does a change at VP3 173 G-D (in combination with VP3 85 Q-H), suggesting that changes at these residues may abrogate HS binding. In addition, the single substitution at VP3 85 Q-H appeared to have an attenuating effect on the growth of the virus, with a smaller plaque morphology observed.

These results are interesting, but more work needs to be done to determine the true effects of these substitutions on receptor usage. This could be done by observing whether viruses carrying these mutations are still able to infect cells possessing heparan sulphate through immunofluorescence studies. Growth kinetics and crystallographic studies should also be performed to observe the exact effect of these substitutions on the growth rates of the viruses and to determine the subtle changes in structure that are causative of these changes. Finally more work should be done to determine the cause of the heterogeneity in plaque sizes observed though picking and sequencing isolated plaques of different sizes.

The initial hypothesis was that the substitution at residue 130 of VP2 from a tyrosine to a cysteine was the cause of the loss in cross-reactivity between the O KIC and O KWT, as in serotype O this residue forms a disulphide bridge with VP1 residue 134. As discussed previously, this disulphide bridge is believed to keep the antigenic and mobile GH loop on VP1 in an "up" conformation and It has been suggested that the alteration of the conformation of this loop, so that it lies across different parts of the capsid surface, is a novel mechanism for antigenic variation in serotype O FMDV (Parry *et al.*, 1990). The results of the serological assays did not demonstrate any antigenic variation caused by the individual substitution of the VP2 130 from tyrosine to cysteine, but did demonstrate large changes in antigenicity if the tyrosine was substituted with an alanine. This could be because a bulky amino acid such as tyrosine may keep the GH loop in the same "up" conformation as that achieved by the formation of the disulphide bridge that is usually present (see section 1.3), whereas the substitution to a small amino acid like alanine results in perturbation in the position of the GH loop, potentially altering GH loop interactions with other parts of the capsid. This change was also not serum specific (at least in the VNT) and therefore suggests that changes to this residue could result in changes to serotype O antigenicity. However, as no heterologous virus investigated in Chapter three has an alanine at this site, this change is clearly not driving any of the antigenic diversity observed in the O serotype across the heterologous viruses within that chapter. Additionally, there does also

appear to be some interaction of this residue with VP1 133 when VP2 130 is occupied by a tyrosine and it would be interesting to explore this interaction further by testing the stability of other substitutions at these sites and through the use of crystallographic studies**.**

For VP3 56 R-H and VP3 60 G-D individually, only the single substitution at VP3 56 R-H was significant, and then only on a single occasion when tested against the O KIC serum in the VNT. For the double mutant virus with just these two substitutions there was no significant effect reported against either serum in the LPBE or VNT. This indicates that these two changes, associated with adaptation to HS *in-vitro,* do not affect virus antigenicity, contrasting with some previous reports. However, when an additional substitution was added to this double mutant at either VP3 85 Q-H or VP3 173 G-D, making a triple mutant, a large effect was observed on the results obtained, with these combinations bringing about a significant decreases in the serum neutralising titre in the VNT against both sera tested. The combination of VP3 56 R-H, 60 G-D and 173 G-D was also significant in the LPBE against the O KIC serum but not the O BFS serum. Unfortunately, the combination of VP3 56 R-H, 60 G-D and 85 (Q-H) resulted in an antigen that did not trap in the LPBE, so could not be tested. Although this does not imply a big change in binding to the O BFS serum it does imply that there was a large decrease in binding of this virus to the O Philippines capture and trapping antibodies used in the LPBE.

The substitution at VP3 173 G-D significant individually in the VNT assay against the O KIC serum and against both sera in the LPBE. This substitution, in any other combination tested, was also significant in the LPBE and VNT against the O BFS and O KIC serum. The frequency of the significance of this substitution suggests that this residue may be playing a role in both the whole antibody and neutralising antibody response in Serotype O. This substitution lies in close proximity to the C terminus of VP1 from the adjacent protomer and the change is also to a charged residue, which is found to be most involved in epitope/paratope interactions (see section 1.7). It could also be that

this substitution perturbs the VP1 C terminus and therefore has an impact on antigenic site one of serotype O FMDV.

VP3 85 Q-H is causative of significant drops in VNT serum titre when in combination with other substitutions in the VNT, and both individually and in combination with VP2 130 Y-C, in the LPBE, with the mutant virus containing a single substitution at this site having the most significant effect against both sera in the LPBE assay. The substitution at VP3 68 T-M is only significant in the LPBE, but again against both sera. Both VP3 68 T-M and 85 Q-H have been suggested to play a role in mAb sensitivity (Kitson *et al.*, 1990). This substitution at VP3 68 has also been associated with small perturbations in the surface structure of serotype O FMDV (Lea *et al.*, 1995) and is adjacent to a previously described antigenic site on serotype A (VP3 69). In addition, VP3 85 is conformationally close to VP3 58 on the structure and therefore this residue may modulate antigenic site four. The substitution at VP3 85 was also located in one of the regions found to significantly correlate with the changes in serum titre in the first vaccine match model developed in chapter 4, however this would not have been identified as belonging to an epitope in this chapter as this change occurred only on a single branch of the phylogenetic tree. The results described here suggest that both of these substitutions may individually play a role in the whole antibody response to serotype O FMDV, with VP3 85 important in the neutralising response in combination with other residues.

There were also several anomalous results reported. The significance of the change at VP3 123 V-I in decreasing the LPBE titre against the O KIC serum was not expected as this residue is normally buried at the two fold axis of symmetry between pentamers. It may be that there are a number of antibodies directed towards this region that have been produced during the immune response to vaccination, if the antigen dissociates into pentamers *in vivo*, exposing this residue. Because of the nature of the LPBE test these antibodies may be detected. In addition five of the mutant viruses caused significant drops in LPBE titre against the O BFS sera but the O KWT and O KIC virus titres were not significantly different.

Some interesting observations were seen in the effects that different combinations of substitutions had on the serological results obtained. Several of the mutant viruses with multiple substitutions bring about a bigger drop in serum titres than those seen from the individual residues alone. The reason for this could be fairly straight forward, in that the residues may be interacting with others, either resulting in larger perturbations to the virus surface or resulting in greater changes in charge. However, some other combinations of residues appear to diminish the size of the effects seen when a substitution was present individually. This is particularly observed with the O BFS LPBE results, where two individually significant residues (VP3 68 T-M and 173 G-D) are no longer significant when in combination with the substitution at VP2 130 from tyrosine to cysteine. The reason for this effect is not understood, however, it could potentially be because of some compensatory effect this additional substitution is having. Indeed, the O BFS LPBE serum titre of the virus with a single mutation at VP2 130 from tyrosine to cysteine is 0.3 $\log_{10}$ higher than the parental O KIC virus. Although this is not individually significant after five repeats it could indicate that this residue is increasing the avidity of the virus for the serum, a concept that relates directly to the appearance of those high avidity viruses discussed in chapter three. It would be interesting to test this by performing more repeats of this assay to determine the validity of this hypothesis.

This chapter has demonstrated a role for several novel residues in contributing to the antigenicity of serotype O FMDV and identifies four new antigenic residues. The most interesting of which are the substitution at VP2 130 from a tyrosine to an alanine and the substitution at VP3 173 from a glycine to an aspartic acid, which are significant in several of the tests conducted against both sera. As discussed previously, VP2 130 Y-A is likely to be affecting the antigenicity of the virus by altering the GH loop conformation while VP3 173 G-D may modulate antigenic site one. The results for VP2 130 Y-A have demonstrated the importance of changing this residue to virus antigenicity, which can have a large effect by amplifying structural changes at other regions of the capsid, in a mechanism analogous to that previously reported (Parry *et al.*, 1990). The principle residue that

appears to have caused the unique sero-reactivity of the O KIC serum is residue 173, as the glycine at this residue is unique to the O KIC virus. This is compatible with the finding that all of the viruses tested in Chapter three were antigenically distant from the O KIC serum. The results here have demonstrated that residue this site is an important modulator of the antibody response more broadly across the O serotype and, although the O KIC virus is unique in having a glycine at this residue, the residue does vary in it composition between some of the other O viruses studied in Chapter three meaning it could be a modulator of antigenic diversity occurring in the field. Two other substitutions, VP3 68 and VP3 85, are also playing a role in the antigenicity in the whole antibody response as defined by the LPBE. Again none of the viruses tested in Chapter three shares the same residue as O KIC at VP3 85, meaning that this residue may also contribute the unique sero-reactivity of the O KIC serum observed, at least in the LPBE. However, as this residue does not vary across the other 104 viruses tested in chapter three this residue will not contribute more generally to the antigenic diversity in the O serotype seen in Chapter three. Finally the results shown here again highlight the differences between the results obtained in the LPBE and VNT and between different sera, as discussed in chapter three, and demonstrate that different parts of the capsid are involved in the whole antibody and neutralising antibody responses respectively (See section 1.10.2). These results also highlight the necessity of using a broad range of different sera to determine the reactivity of different epitopes within and between serotypes.

Chapter 7: Use of a reverse genetics system to evaluate some predictions made in chapters four and five.

Part of the work conducted within this chapter will be submitted for publication along with the results from chapter 4:

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7.1 Summary.

The analyses conducted in chapters four and five have predicted several residues as potentially novel antigenic residues for serotype O FMDV. The aim of this chapter is to determine through reverse genetics the validity of these approaches. A small subset of the predicted antigenic residues were selected and point mutations were inserted into the O UKG/O KIC chimeric full length infectious copy plasmid. Viable viruses were rescued and tested to determine whether each mutation had an effect on recognition and neutralisation by FMDV specific antisera compared to the parental virus.

All three mutant viruses selected for evaluation were successfully rescued and stable after three passages. These were subsequently tested in the VNT and LPBE assays. None of the mutations (VP2 191 T-N, VP2 219 T-A and VP3 69 D-A) had a significant effect on the titres obtained for the VNT. However, two of the mutations made, one at VP2 191 T-N and the other at VP3 219 T-A, caused significant ($p<0.01$) drops in whole antibody titre when measured in the LPBE against the homologous O UKG serum and the mutation at VP2 191 also had a significant effect on the titre obtained against one of the two heterologous sera tested. Both of these mutations were identified by the LME modelling method described in chapter four and VP2 191 was also predicted by the structure based method described in chapter five. However, residue VP3 69, also predicted by this model, did not cause any significant changes in cross-reactivity.

These results validate the use of the LME model for epitope prediction, suggesting that of the two methods this is the more reliable. However, the results also partially validated the use of the structure based prediction and the comparative ease of use of this method over the LME modelling suggests that this may still be an appropriate method for epitope prediction on multiple serotypes; at least in the absence of serological and sequence data.

7.2 Methods

7.2.1 Generation of mutant viruses

All mutant viruses were generated using standard site-directed mutagenesis, as described in section 2.5 from the infectious copy of the O UKG/OKIC chimeric virus. The primer sequences used to generate the mutant viruses are listed in appendix 4. All viruses were cultured to passage three on IBRS-2 cells. Following the rescue of each virus, the presence of the correct mutation was confirmed using Sanger sequencing as per section 2.4. Once each mutation was confirmed, the viruses were stored at -80°C until used.

7.2.2 Serological analysis

All mutant viruses were tested 12 times in both the VNT and the LPBE, as described in sections as 2.2.4 and 2.2.6 respectively. The sera used in the analyses had been raised against the O UKG, O BFS and O KIC viruses (for more details on the sera, see section2.2.2). The homologous virus used was the O UKG chimeric virus rescued with an additional substitution at VP3 56 from histidine to arginine, making the capsid sequence identical to that of the O UKG 34/01 virus used as the homologous one in chapter 3. This virus is subsequently referred to as O UKG 34/01 throughout the remainder of this chapter.

7.2.3 Plaque assay

A plaque assay was performed as outlined in section 2.2.7, using IBRS-2 cells.

7.2.4 Statistical analysis

Statistical analysis was performed as outlined in section 2.6, with each virus as a fixed variable and the date performed (or plate for the LPBE) as a random effect.

7.3 Results

7.3.1 Selection of residues for site directed mutagenesis.

Prior to the substitution of any residues of interest into the O UKG/OKIC chimeric virus, an initial substitution was made to change residue VP3 56 from histidine to arginine. This ensured that the backbone of the capsid from the chimeric virus was identical to the original O UKG 34/01 virus that was used in chapter three to generate the serological data.

A small number of residues were selected for preliminary evaluation. The first (VP3 219 T-A) was predicted by the LME method developed in chapter four alone. The second residue (VP2 191 T-N) was one that was predicted by both the LME model in chapter four and the structure based prediction from chapter five. Both of the residues selected from the results of chapter four were those residues about which the model was most confident. The final residue (VP3 69 D-A) was one predicted by the structural based prediction alone; this residue was selected because upon observation, this residue was completely conserved across all the serotype O viruses investigated in chapter three. For a list of the residues, see table 7.1 and for the location on the surface of the capsid, see figure 7.1.

For the two mutants selected by the LME model, the substitutions that were made were as suggested by the model. For the substitution selected by the structure prediction alone, where no other residue was suggested, an alanine mutation was made. Alanine was selected as it has the least impact on the overall structure of an epitope (Cunningham & Wells, 1993), allowing the assessment of the contribution of the individual residue of interest to be evaluated.

Mutant name	Residue	Amino acid on O UKG 34/01 plasmid	Subsituted to	Method predicted by	Colour on figure 7.1
UKG1*	VP3 56	Histidine	Arginine	None	
UKG2	VP2 191	Threonine	Asparagine	Both	
UKG3	VP3 69	Aspartic acid	Alanine	Structure prediction	
UKG4	VP3 219	Threonine	Alanine	LME prediction	

Table 7.1: The residues substituted on the original O UKG 34/2001 plasmid. The method that each was predicted by is also indicate,d along with the colour coding for each residue as used in figure 7.1. *Indicates that this mutation was made to the plasmid prior to inclusion of the other residues to ensure 100% homology with the original O UKG 34/01 virus used to generate the initial serology.

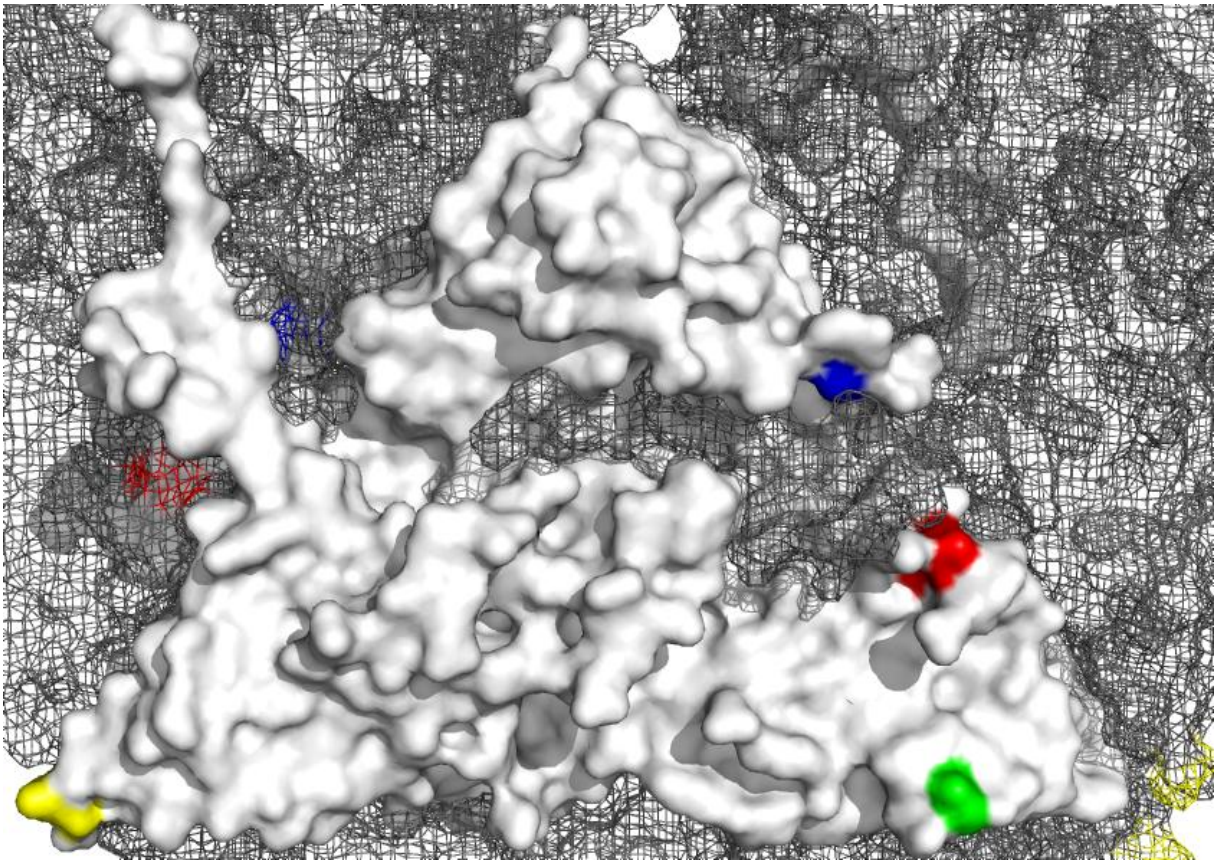

Figure 7.1: The molecular subunit of a reduced O BFS (PDB ID 1FOD) asymmetric protomer with surrounding protomers shown as a grey mesh. The locations of the substitutions made to the O UKG virus are shown. The substitutions are coloured as shown in table 7.1.

7.3.2 Confimation of the identity of the mutant viruses.

In order to ensure that the correct viruses were recovered, the whole capsid sequence for each new virus was determined, this confirmed that the right mutations were still present and stable after three passages in tissue culture (figure 7.2).

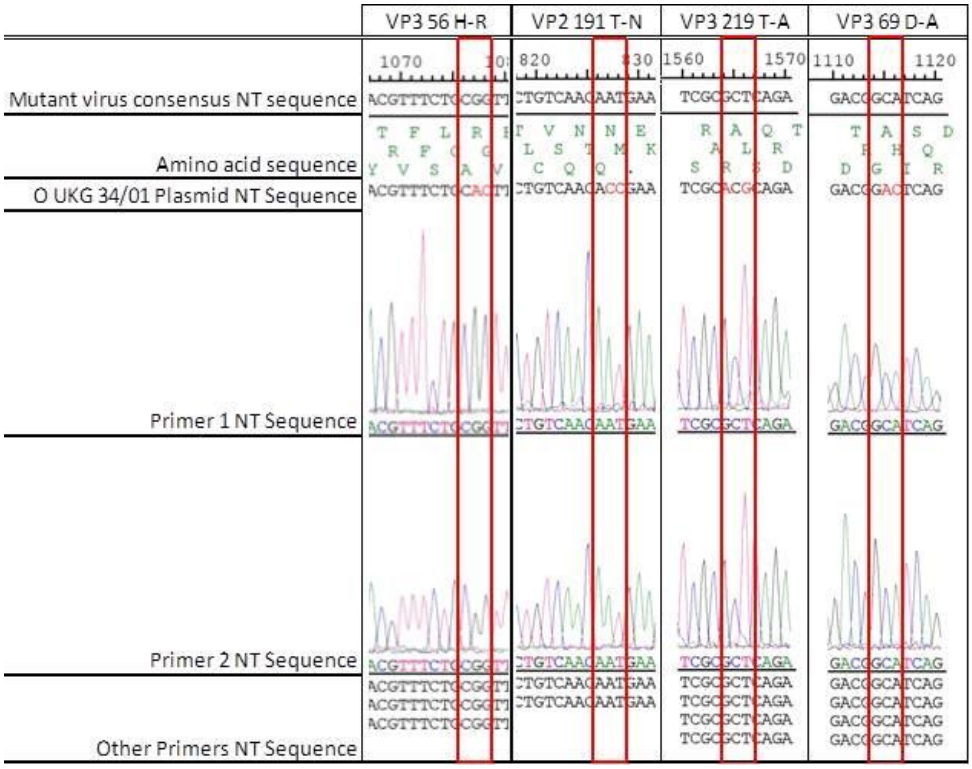

Figure 7.2: Sequence comparisons between the original O UKG 34 plasmid, the rescued mutant viruses and the mutation inducing primers. Each of the substituted codons are outlined in red.

7.3.3 Impact of mutations on plaque morphology.

All of the viruses generated for this chapter exhibited a similar plaque morphology at 48 hours (figure 7.3). The morphology seems typical of a virus using the Heparan sulphate receptor (see discussion of plaque sizes in chapter 6)

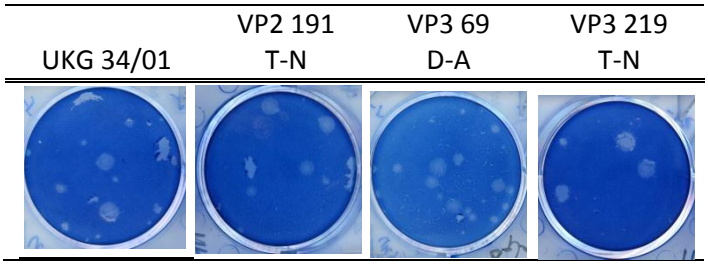

Figure 7.3: Plaque morphology at 48 hours post infection of viruses used in this chapter.

7.3.4 Impact of mutations on virus antigenicity.

7.3.4.1 Virus neutralisation test using O UKG serum.

The VNT results for all the viruses tested indicated that none of the mutations inserted had caused a significant (P=<0.01) drop in serum neutralising titre (figure 7.4), with the p values for each virus in relation to the parental O UKG 34/01 virus (the chimeric virus with a H-R substitution) ranging from 0.58 (UKG3- VP3 69 D-A) to 1 (UKG4- VP3 219 T-A). The statistical modelling (using an ANOVA model) showed that for this dataset, the overall effect of virus used on the titre was not significant (0.36). However, the date of the assay did have a significant effect on the titres obtained (P<0.05). The mean serum titres were 2.9 Log_{10} for the O UKG 34/01, VP2 191 T-N and VP3 219 T-A viruses and 2.8 $log_{10}TCID_{50}$/ml for VP3 69 D-A (figure 7.4). These titres all correspond well with the mean titre of the O UKG serum in the VNT ascertained in chapter 3 (2.75 log_{10} $TCID_{50}$/ml).

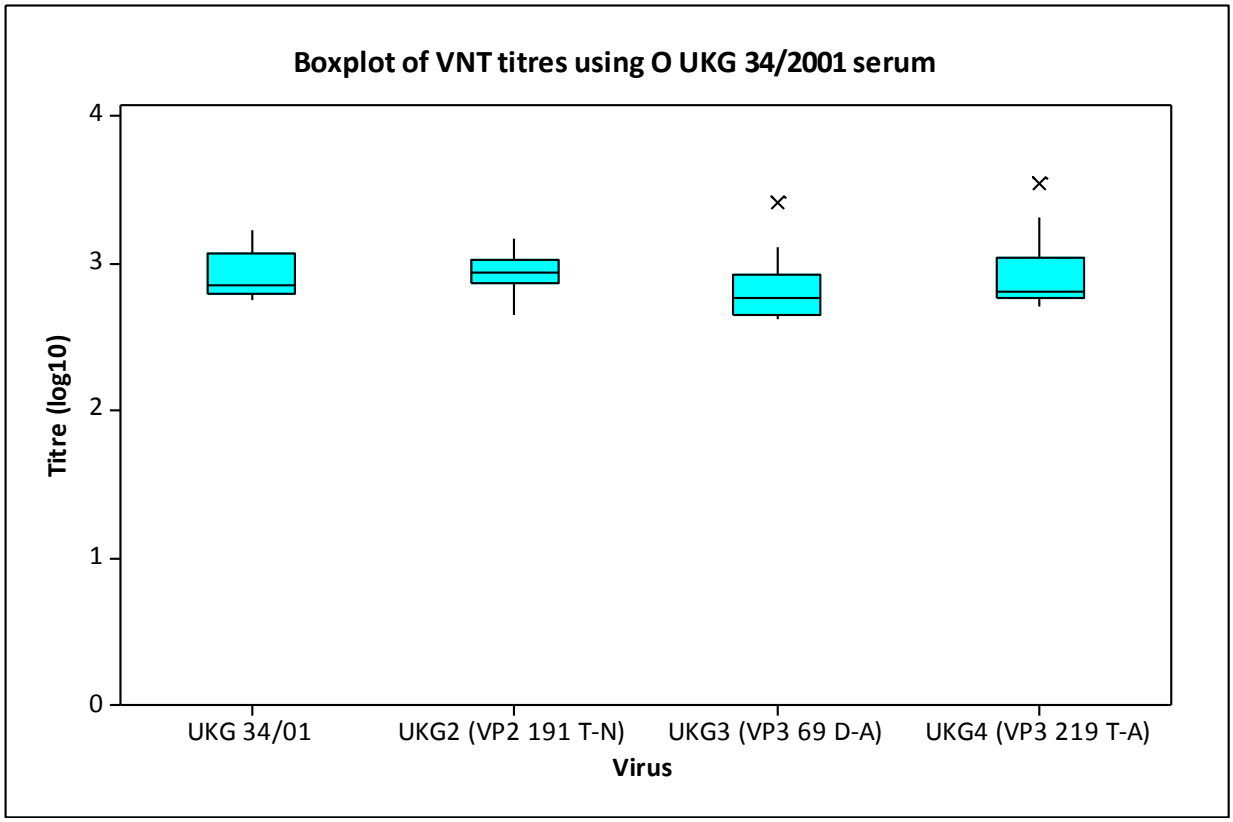

Figure 7.4: Boxplot of the log_{10} serum neutralisation titres of the three mutant viruses in the VNT at 100 $TCID_{50}$/ml of virus after twelve repeats. The coloured boxes represents the interquartile range spanning 50% of the data, the horizontal line through this box represents the median value, the vertical bars projecting from the top and bottom of the box denotes the limits of the data range included and x denotes an outlier.

7.3.4.1 Liquid phase blocking ELISA assay using O UKG serum.

The LPBE results showed that both the mutation at VP2 191 T-N and the mutation at VP3 219 T-A had caused a significant loss of cross reactivity, with a p value of <0.001 reported in both instances (the serological values are plotted in figure 7.5 and shown in table 7.3). This contrasts with the results for the mutation at VP3 69 D-A predicted from the structural modelling alone, which was not significant (p=0.63). The ANOVA demonstrated a good fit of the model for the data (R^2 = 71.9%) with an overall significant effect of the virus used (p=<0.01). In this case, the date the test was conducted had no significant impact on the titres obtained (p=0.325).

	UKG sera		OKIC sera		O BFS sera	
Virus	Mean	P-Value	Mean	P-Value	Mean	P-Value
UKG 34/01	2.9	N/A	1.7	N/A	2.0	N/A
UKG2 (VP2 191 T-N)	2.5	<0.01	1.6	0.1883	1.7	<0.01
UKG3 (VP3 69 T-A)	2.9	0.626	NT	NT	NT	NT
UKG4 (VP3 219 T-A)	2.7	<0.01	1.7	0.875	2.0	0.9979

Table 7.3: Summary of the LPBE results against each serum for the parental O UKG 34/01 serum. The the chimeric O UKG/ O KIC virus with a H-R substitution was used as parental O UKG 34/01 virus and this is the case wherever the O UKG parental virus is mentioned.

The change in serum titre was largest with the VP2 191 T-N mutation, with an observed drop in titre of 0.4 $\log_{10}$/ml when compared to the parental O UKG 34/01 virus. Results shown in table table 7.3. This compares to a drop of 0.2 $\log_{10}$/ml for the VP3 219 T-A mutation. The titre obtained with the VP3 69 mutant was identical to that of the parent virus, at 2.9 $\log_{10}$/ml. This was slightly lower than the mean titre of the O UKG LPBE results from chapter three, which was 3.19. The drops in cross reactivity observed from this analysis were much larger than those that were predicted from the analysis performed in chapter four of -0.081 $\log_{10}$/ml for both VP2 191 T-N and VP3 219 T-A.

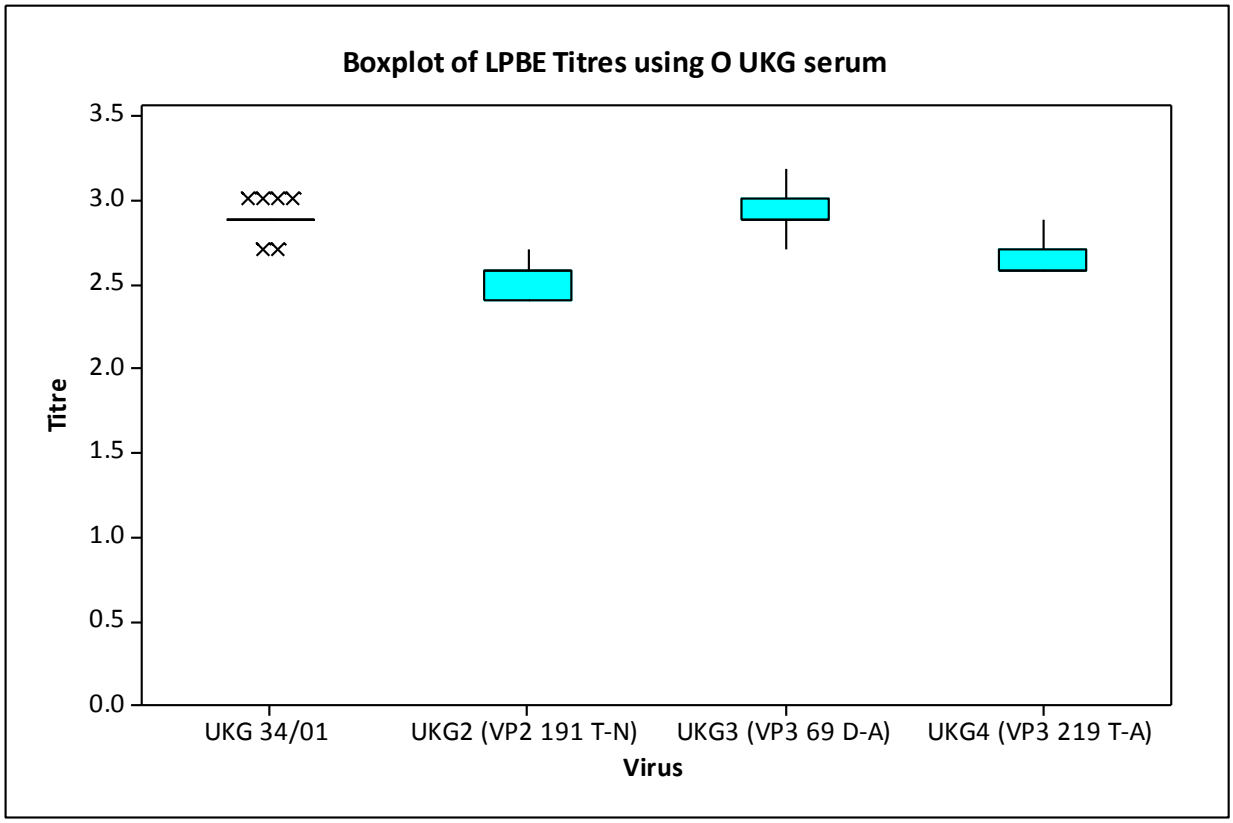

Figure 7.5 Boxplot of the $\log_{10}$ serum titres of the three mutant viruses in the LPBE using the Homologous O UKG serum after twelve repeats. Boxplot representation as described in figure 7.3.

7.3.4.1 Liquid phase blocking ELISA assay using heterologous O BFS and OKIC serum

In light of the LPBE results for the mutant viruses against the O UKG serum, where larger than expected drops in cross reactivity were reported, the viruses with mutations at VP2 191 T-N and VP3 219 T-A were taken forward for further analysis to determine the effect on cross reactivity of each mutation on heterologous serum titres. For this analysis, both the O BFS and OKIC sera were used, as from the initial work conducted in chapter three, these two sera had been tested against a larger number of heterologous viruses (89 for the O BFS and 104 for the OKIC) than either the O Uga 2002 or the O Ken 1978 sera (48 viruses tested against both serum). Hence, the O BFS and OKIC results may have had a greater effect on the predicted changes in cross reactivity. The LPBE results are summarised in table 7.3. Neither mutation resulted in significant changes in cross reactivity when compared to that of the parental O UKG 34/01 virus against the OKIC serum (figure 7.6), with P values of 0.18 and 0.88 for VP2 191 T-N and VP3 219 T-A respectively. Again, the ANOVA

demonstrated a good fit of the model for the data (R^2 = 88.27%) and there was an overall significant effect observed for the virus used in this model, presumably due to the inclusion of the OKIC virus as a control. The date the test was conducted had no significant impact. The mean serum titre for the OKIC control virus was 2.3 $\log_{10}$/ml, similar to that observed in chapter three. Additionally, the serum titres for the O UKG viruses were 1.7 $\log_{10}$/ml for the UKG 34/01 parent virus and VP3 219 T-A and 1.6 $\log_{10}$/ml for VP2 191 T-N, (figure 7.6) which are also similar to the previous titres reported for the O UKG virus (data not shown).

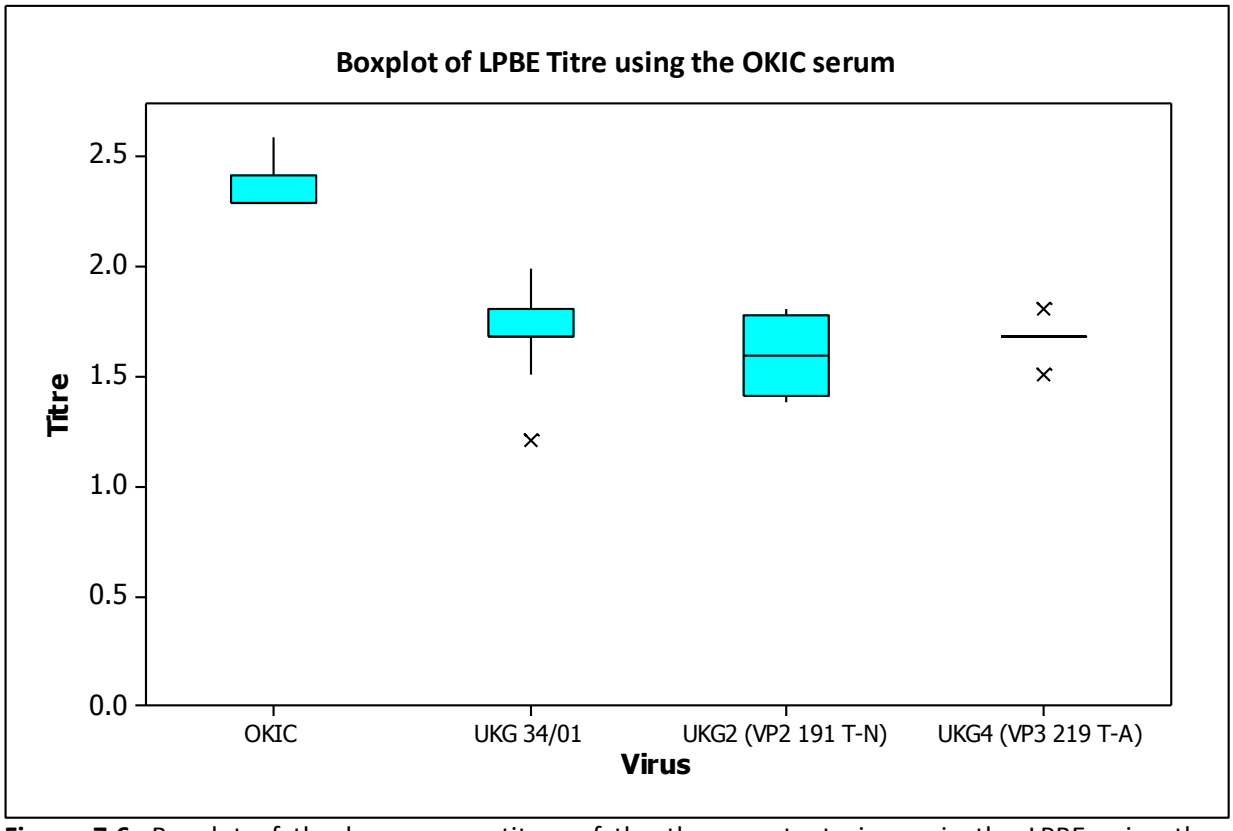

Figure 7.6: Boxplot of the $\log_{10}$ serum titres of the three mutant viruses in the LPBE using the Heterologous O KIC serum after twelve repeats. Boxplot layout as described in figure 7.3.

The mutation at VP2 191 did result in a significant loss in cross reactivity (p=< 0.01) compared to the parental virus against the O BFS serum, however, the drop in titre of 0.3 $\log_{10}$ was not as large as that seen against the homologous O UKG serum (figure 7.7). The substitution at residue VP3 219 T-A did not cause any changes in the cross reactivity of the O UKG virus to the O BFS serum with the serum titre of the UKG 34/01 virus and the mutant virus being 2 $\log_{10}$/ml. Finally, the ANOVA

demonstrated a good fit of the model for the data (R^2 = 85.83%) with a significant effect observed for the virus used but not for the date the test was conducted. Again, the serology results for the O UKG parent virus and the O BFS virus were all comparable to those previously reported.

The results reported here that show smaller changes in the cross reactivity of each mutant when compared to the parental O UKG 34/01 virus against both the O KIC and O BFS sera and may explain (at least for VP3 219 T-A) the larger than expected loss in cross reactivity observed in the homologous O UKG serum compared to the expected loss in cross reactivity reported in chapter four, as the predicted changes in cross reactivity in this chapter were averaged out from the serological results for all sera tested in chapter three.

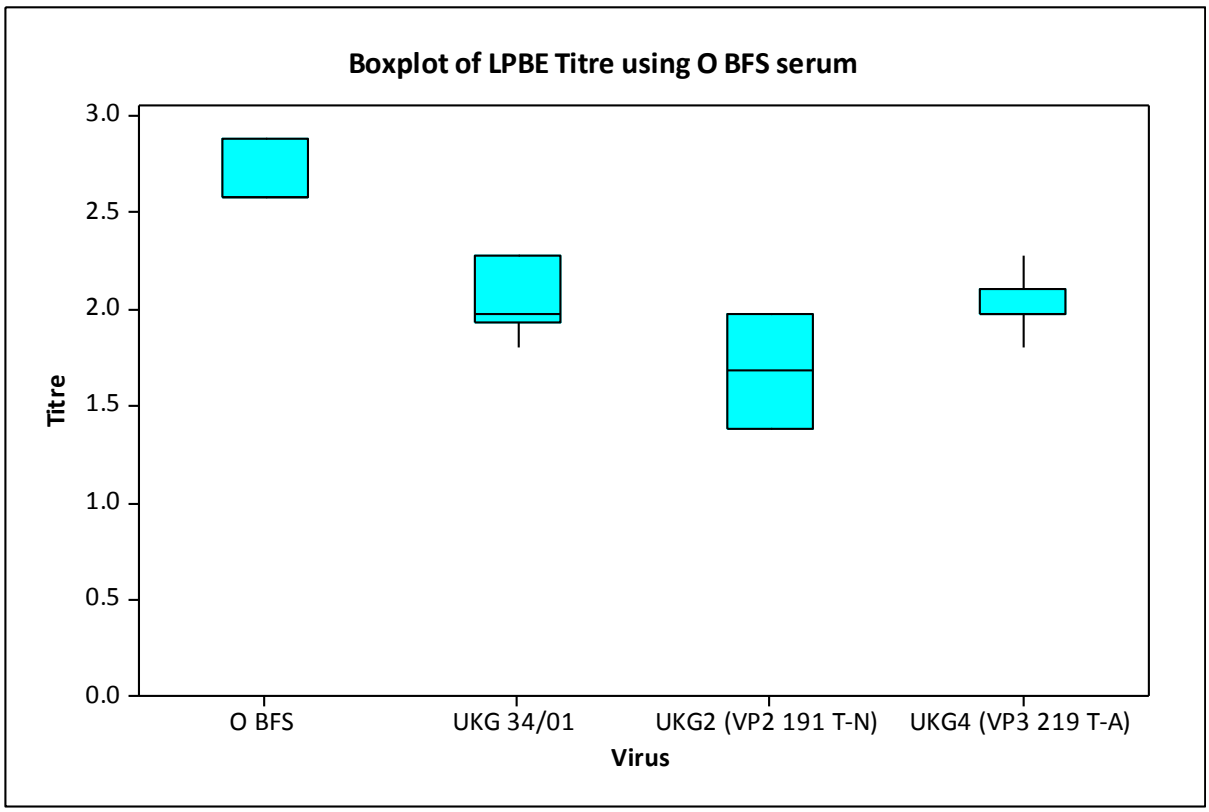

Figure 7.7: Boxplot of the $\log_{10}$ serum titres of the three mutant viruses in the LPBE using the Heterologous O BFS serum after twelve repeats. Boxplot layout as described in figure 7.3.

7.4 Discussion

The use of the modelling approaches described in chapters four and five predicted several novel amino acid residues as residues that could play a role in the antigenicity of serotype O FMDV. The validity of these predictions have now been tested with a small subset of residues, using reverse genetics techniques and serology assays. Residue VP2 191 has not previously been described as part of an epitope for any serotype of FMDV in the published literature, while residue VP3 219 is located next to a residue (218) that has previously been defined as part of an epitope of serotype Asia-1 (Grazioli *et al.*, 2004), and VP3 69 is part of an epitope on serotype A (Thomas *et al.*, 1988). The results demonstrate that each mutation was successfully rescued and was stable after three passages. Each virus also had a similar plaque morphology, indicating that none of the mutations made had an obvious effect on the receptor usage of the virus.

Of those selected, both VP2 191 T-N and VP3 219 T-A had significant effects on the serum titre and are therefore identified as residues modulating serotype O antigenicity. The effects observed were larger than predicted in chapter four against the O UKG serum; however a smaller effect on cross reactivity was observed when tested against two heterologous sera. This may explain the greater than expected changes in cross reactivity observed, as the original predictions had been made using the results for all the sera and not just the homologous serum. It would be interesting to test these mutant viruses against the remaining heterologous sera to explore this relationship further.

Of the two mutations, the one that had the largest effect on the serum titre was VP2 191 T-N, with a drop in titre of 0.4 $\log_{10}$/ml against the homologous serum and 0.2 $\log_{10}$/ml against the heterologous O BFS serum. This compares to the mutation at VP3 219 T-A where an observed drop in titre of 0.3 $\log_{10}$/ml was reported against the homologous serum and no changes in cross reactivity were observed against any of the other sera tested. VP3 219 is slightly obscured on the surface and it seems likely that this residue is therefore acting indirectly, as the alanine is smaller than the

threonine it may be modulating the C terminus of VP3 that protrudes next to the C terminus of VP1, which has previously been defined as antigenic. It is interesting that both of these residues involved a change from a threonine residue. Threonine is a residue that has been described to be overrepresented on the surface of Picornaviruses (Lea & Stuart, 1995). Threonine substitutions have been reported in mAb escape studies against several serotypes of FMDV (Grazioli *et al.*, 2004; Grazioli, 2006; Lea *et al.*, 1994; Thomas *et al.*, 1988), including serotype O, where a change at VP2 71 from T-N, the same as the substitution reported here for VP2 191, was observed (Kitson *et al.*, 1990). VP2 71 makes up part of antigenic site two, which as discussed in section 1.8, appears to be the immunodominant antigenic site.

The change at VP3 69 D-A did not result in any change in the cross reactivity when tested against the homologous O UKG serum, which is disappointing as this residue is completely conserved within serotype O. It is also surprising because this residue was previously identified as part of an epitope for serotype A (Thomas *et al.*, 1988). VP3 70 was also identified as part of an epitope in this paper, with the change being identical to the one made here for VP3 69. There could be several reasons for this discrepancy. Firstly, although for serotype O a correlation between antigenic sites identified by bovine and murine mAbs has been reported (Barnett *et al.*, 1998) there has been no such correlation reported for serotype A, this could mean that this site identified using murine mAbs is not important in cattle. There has also been no work quantifying the proportion of the polyclonal bovine sera response to each serotype A antigenic site. It could therefore be the case that although this residue can elicit monoclonal antibodies the size of its contribution is not large enough to be detected using current serological methods with polyclonal serum.

None of these residues had an effect on the neutralising titres obtained. This indicates that the two residues identified are essentially non-neutralising sites towards which a large component of the antibody response is generated. The importance of these residues in the *in vivo* response could be sizable because, as discussed in section 1.5.2.1, direct neutralisation may not be as important in the

antibody response to FMDV as other effector mechanisms, such as opsonisation enhanced phagocytosis.

The LME model was the most accurate of the methods with both the predictions tested here correct. The structural approach, although not as successful as the LME approach, did still correctly identify as an epitope one of the two residues tested here. This model also suggested that this residue may be an epitope on several different serotypes of FMDV, which will be interesting to test. Although the results here demonstrated an incorrect prediction, it is interesting that an adjacent residue, confirmed as an antigenic residue by reverse genetics (VP3 68- data not shown) was predicted as an epitope for both serotype A and SAT-1 by this method (see chapter five). The C terminus of VP3 was also predicted as an epitope on serotypes C and SAT-1 but unfortunately not for serotype O. In conclusion, the best method for prediction based on the results presented here is the LME model with a 100% success rate, although this conclusion is based on the very small subset of predictions tested. However, the comparative ease of using the more rapid and cheaper method of prediction using structural data alone, especially in terms of looking at multiple serotypes, seems to also be a valid approach to predict epitopes, at least for initial evaluation.

Chapter 8: General Discussion

8.1 Thesis overview

The overall goal of this thesis was to increase our understanding of the antigenicity of serotype O FMDV, by identifying novel epitopes and evaluating the dominance of these new epitopes, with the hope of potentially identifying some novel, more broadly cross reactive epitopes for use as targets in the development of more cross reactive FMDV vaccines A further goal was to try and develop a vaccine matching model based on sequence data, with the goal of providing a cheaper and more rapid method for identifying the correct vaccine for use in an outbreak situation.

Examination of FMDV O serotype viruses conducted in chapter three yielded valuable insights into the interactions of several polyclonal sera from vaccinated cattle with a broad range of viruses, representative of all of the currently circulating topotypes. This work highlighted the complex nature of cross reactivity that can occur within the O serotype, with several viruses that appear distantly related through phylogenetic analysis being serologically similar to the vaccine strains, and other phylogenetically closely related viruses having starkly different antigenic profiles. Additionally r_1 values were observed that indicated several heterologous viruses were more reactive towards some sera than the actual homologous virus used to generate the sera, consistent with previous reports (Barnett *et al.*, 2001).

Although none of these 'high avidity' r_1 values reported in chapter three are likely to be of statistical significance, due to the limited number of repeats, the titre reported in chapter six of the O KIC was 0.5 $\log_{10}$ higher than the O BFS virus using the O BFS derived serum in the VNT, which was which, although not significant after five repeats ($p = 0.067$) may become significant after more repeats. Therefore, it would be interesting to test these two viruses further. The results in chapter three again highlight the generally good cross reactivity associated with vaccines from the O serotype (Doel, 2003), with the majority of viruses matching the vaccine strains evaluated; however, there were large differences in r_1 value agreement between the two serological tests reported. The O KIC

vaccine strain was poorly cross reactive; not even phylogenetically closely related viruses were of a suitable cross reactivity to be a vaccine match.

Collaboration with the Institute of Biodiversity, Animal Health and Comparative Medicine at the University of Glasgow provided the appropriate analytical tools to examine the potential of using the LPBE dataset from chapter three to develop models for both vaccine match and epitope prediction, similar to those developed previously for the SAT serotypes (Reeve *et al.*, 2010). These models predicted four residues as epitopes, one of which had been previously described in the literature. The importance of two of the predicted epitopes was investigated by reverse genetics in chapter seven, validating the approach used for epitope prediction. The work to develop a sequence-based model for vaccine matching was less successful; the best performing model developed here was not significantly better at predicting vaccine match than the individual titres from the day a test was conducted. Indeed the model using linear stretches of sequence performed significantly worse than serology. However, adaptation of the model to use just changes occurring in four residues predicted as epitopes did result in predictions that were comparable to those of serology, eliminating the need for serology.

Chapter five provided an alternative approach to epitope prediction, which was used to complement and extend upon the results obtained from the work in chapter four. Using virus structural data enabled the identification of completely conserved epitopes and also the examination of several serotypes at the same time. This work, the first to use these readily available immunoinformatics approaches to try and identify viral epitopes, demonstrated the generally poor reliability of current B cell epitope prediction tools when used individually with virus structures. However, the method developed for predicting novel epitopes using a consensus of the three best performing programs appears, at least notionally, to have overcome some of these limitations and could be a useful tool for rapid and cheap epitope prediction. This

method predicted two of the same residues as parts of epitopes that were identified by the modelling in chapter four, one of which was the previously described epitope and the other was subsequently found to be part of an epitope (this epitope is also predicted on all other serotypes evaluated). An additional predicted epitope that was completely conserved was found not to be having an effect on serological titres, thereby resulting in an incomplete validation of this approach (although many other predicted regions have also previously been defined as epitopes). However, this method could certainly be used to guide the search for epitopes using other techniques.

The work in chapter three showed there was little or no correlation between amino acid changes at the level of the whole capsid, the individual capsid proteins, the antigenic sites or the most diverse residues. However the work on vaccine matching from chapter four did find three regions of the capsid that were correlated with the antigenic drift. Although one of these regions, with a single amino acid substitution at VP3 85 (Q-H), is not likely to be a real effect as this change does not occur on any heterologous viruses examined. However this change occurred on the homologous O KIC virus, which was antigenically distinct from all other viruses, which would therefore provide a strong signal. Both of the remaining two regions contain known epitopes and are on some of the most exposed parts of the capsid. That the G-H loop of VP1 was the most significant of the regions is interesting. As discussed, this area is freer from the structural constraints of the other parts of the capsid and is known to contain a hyper-variable region. This region was also the main correlate with changing antigenicity observed in the SAT-1 vaccine match model, lending support to the idea that this region could be a molecular clock, the accumulation of substitutions within which occurs concurrently to, but is not responsible for, the antigenic drift of FMDV. Although whether knowledge of this molecular clock can be of a practical technical use seems doubtful as changes in this region alone are not sufficient to enable the prediction of vaccine match.

8.2 The identification of novel epitopes

The work conducted in chapters six and seven, based on the results from chapters three, four and five identified several new epitopes playing a role in the antigenicity of serotype O FMDV, the majority of which were targeted by the whole antibody response, measured in the LPBE.

The substitution playing the largest role in the unique sero-reactivity of the O KIC sera, both in the VNT and LPBE was VP3 173 (G-D), with two other substitutions on VP3 (85 (Q-H) and 68 (T-M)) more important in the whole antibody response (chapter six). As discussed previously, while both VP3 173 (G-D) and 68 (T-M) vary in amino acid composition and therefore may be residues driving the antigenic diversity across the O serotype, VP3 85 does not. However one important point to note is that some of the actual residues identified previously as antigenic sites show very limited variability across the serotype O viruses tested here and also do not necessarily ever contain the particular substitution identified. For example, the particular substitution on the mAb escape mutant that identified site five does not occur on any O serotype virus investigated and there is only a single virus out of the 105 examined here that has any substitution at this site (data not shown) this calls into question the relevance of the substitutions identified to serological variation observed in the field.

The role of the disulphide bridge between the G-H loop of VP1 and the G-H loop of VP2 in serotype O antigenicity was also investigated in chapter six. It appears that the effect this residue has on the antigenicity is directly linked to the size of the amino acid that replaces the cysteine. The change from a tyrosine to a cysteine did not, in most instances lead to a significant effect on serum titre, however the change to an alanine did. The significance of this modulation on the diversity seen within the serotype is debatable as although all of the serotype O viruses tested here (with the exception of O KIC) have a cysteine at residue VP2 130, the cysteine at VP1 134, with which residue VP2 130 interacts is not highly conserved; all of the viruses of the Cathay topotype have a substitution to a serine, which is similar in size (although polar) to an alanine and so may perturb the

G-H loop in an analogous fashion. It would be interesting to look at this further to see if this affects the antigenicity of the Cathay topotype viruses.

Two other antigenic residues were also identified in chapter seven, again directed toward the whole antibody response, these also validated (partially in the case of the structural modelling for chapter 5) the use of the predictive approaches used for epitope identification. Both of these sites were novel to the O serotype. These epitopes are likely to be important as they were identified by looking broadly across the O serotype. The work in chapter five also predicts that one of these regions is likely to be an epitope on other FMDV serotypes.

A further predicted epitope, VP3 69 (D-A), identified using the structural method in chapter five and tested in chapter seven was either not an epitope or the effect of the substitution was too small to be observed. This is surprising as this residue is a known epitope on serotype A (Thomas *et al.*, 1988). It is also interesting to note that VP3 68 does appear to have a significant effect on the titres obtained in the LPBE for serotype O FMDV (chapter six). This may be because a change from an aspartic acid to an alanine would not necessarily result in structural changes to the capsid (Cunningham & Wells, 1989) although the change in the charge would perhaps have been expected to have an effect. However, a change from a small polar threonine residue to a larger, non-polar methionine that was observed at VP3 68 in chapter 6 may result in alterations in the capsid structure.

All of the epitopes identified resulted in large decreases in serological titres. This is consistent with previous studies that have demonstrated large drops in cross reactivity through a small number of substitutions (see sections 1.6.2 and 1.8) and suggests that these residues make up part of the small number of dominant epitopes that drive the antigenic diversity of the O serotype. However, it is likely that the type of amino acid involved in the substitution is important in determining the size of the effect as a simple count of the substitutions at the known antigenic sites does not correlate with changes observed in serology.

The above findings and the identification of 'high avidity' r_1 values does lead to a conundrum; how can some viruses which are genetically distant be so closely related antigenically? It could be assumed based on the data above that a virus carrying a substitution at any of these novel antigenic sites would be antigenically distant to other viruses but this is not always the case. One possible explanation regarding the diminished effect of these single substitutions involves the introduction of compensatory substitutions, either by moderating the level of structural alteration brought about or by compensating for any changes in the overall charge or hydrophobicity brought about by a substitution. The high avidity r_1 values reported could be brought about by substitutions that enhance immunoglobulin avidity in a heteroclitic manner or by removing antigenic competition between different antigenic sites, resulting in the uninhibited binding of antibodies at sites to which immunoglobulins with a higher affinity have been generated.

8.3 Conclusions

The work conducted in this thesis, along with previously published data, demonstrates the role of a small number of dominant substitutions in mediating the antigenic diversity of serotype O foot-and-mouth disease virus. Two alternative but complementary methods for identifying epitopes were validated by the use of reverse genetics, which enabled the identification of two novel serotype O epitopes, with a further three novel epitopes identified by looking in depth at the interactions between two viruses. This increased understanding of the antigenic composition of viruses is of immense importance to increasing our understanding of the nature of vaccine efficacy and breadth of protection, as this could lead to the development of more broadly reactive vaccines

The mathematical tool developed in chapter four is of particular importance because it enables the examination of interactions between viruses and the polyclonal serum response generated within a natural host species. Looking at the polyclonal response in this fashion provides clear advantages over the traditional methods for epitope mapping using monoclonal antibodies, which have been used previously to identify FMDV epitopes, as the residues identified are naturally

occurring changes within a host species that are directly relevant to the evolution of FMDV in the field. This technique also quantifies their relative importance. Although the structural method is less precise than the serological method and does not look directly at actual serological relationships it forms the basis of a quick and simple method for making predictions across multiple serotypes (and also identifying conserved epitopes) which can be used to inform and extend the serological analysis. A combination of the models could form the basis of a technique that can greatly enhance our understanding of the humoral aspect of host-pathogen interactions that maybe a valuable tool aiding in the selection and rational design of new, more broadly reactive vaccines, with the additional benefit that these techniques could be applied to many other clinically important viruses.

Although it is clear that the nature of the antigenic relationships of FMDVs to one another is incredibly complex and further work is needed to fully understand the interactions involved (especially in identifying any factors that may lead to high avidity r_1 values or compensatory interactions between substitutions) the work in this thesis provides a big step forward in our understanding of serotype O Foot-and-Mouth disease antigenicity.

8.4 Future work

The biggest aspect of any future work on this topic would be to refine the modelling used to identify epitopes in chapter four. Currently the candidate regions used for both vaccine matching and epitope prediction are linear loop elements of the genome. It would be more appropriate to use, as candidate regions, an area centred around each surface exposed residue including those residues that come in close enough proximity to this residue to form an antigenic site, based on the size of an immunoglobulin 'footprint'. An algorithm similar to those used in many of the structure prediction programs would be suitable in defining these areas, and this would also allow for the interactions of residues that may be forming conformational epitopes (which make up >90% of all epitopes) to be taken into account, which at present cannot be done. These models should be taken forward and applied to the other serotypes of FMDV, both in the LPBE and VNT (which should also be done for

the O serotype). It would be interesting to observe the levels of similarity across the serotypes in terms of the antigenicity within a natural host. More data should also be generated for the LPBE to improve the vaccine matching model, which at present is not good enough to replace the current serological techniques performed.

It is difficult to draw too many conclusions on the relative performance of each of the epitope prediction models at present, as it is based on a very small number of tested predictions. It would be nice to test many more of the predicted epitopes through reverse genetics. The structural prediction method did identify several more epitopes which can be tested to more accurately gain a better understanding of how effective this method is. It would also be nice to test the same epitope residues identified here on other FMDV serotypes to determine if they are antigenic across serotypes and to look at some of the more conserved antigenic residues to determine if they can be of any use in developing more broadly cross reactive vaccines. In addition some crystallographic analysis should be performed on those viruses found to be affecting both the serum titres and receptor affinity to determine what kind of changes are occurring across the virus structure that may be driving these changes.

The final area that needs to be investigated further is the 'high avidity' r_1 values, and the role of virus avidity in general. More work is needed to determine both the significance and the causes of these. As discussed, the O KIC virus was consistently (but after five repeats not significantly) of a higher titre in the VNT in chapter 6 than the O BFS virus. If after more repeats this proved to be significant, then the interactions between these two viruses would provide an ideal starting point for investigations, as there is only a small number of changes between the two viruses this could be used as a model system to determine which residues are responsible for increasing serological titres.

Appendix 1: Details of the serotype O viruses used in this study

Virus strain (chapter 3)	WRLFMD_REF	COUNTRY	YEAR	SPECIES
O KIC (Kau IC/1966)	Vaccine strain	Germany	1967	Cattle
Alg 02/1999	ALG/02/1999	Algeria	1999	Cattle
Arg 02/2000	ARG/02/2000	Argentina	2000	Cattle
Arg 03/2000	ARG/03/2000	Argentina	2000	Cattle
BFS 18/1960	BFS 1860/ 1967	United Kingdom	1967	Cattle
Bhu 02/2008	BHU/2/2008	Bhutan	2008	Cattle
Bol 17/1990	BOL 17/1990	Bolivia	1990	Cattle
Bra 01/1992	BRA/01/1992	Brazil	1992	Cattle
Bra 01/1994	BRA/01/1994	Brazil	1994	Cattle
Bra 01/1995	BRA/01/1995	Brazil	1995	Cattle
Bra 02/1994	BRA/02/1994	Brazil	1994	Cattle
Bra 03/1994	BRA/03/1994	Brazil	1994	Cattle
Bra 04/1994	BRA/04/1994	Brazil	1994	Cattle
Bra 05/1994	BRA/05/1994	Brazil	1994	Cattle
Bra 06/1994	BRA/06/1994	Brazil	1994	Cattle
Bra 08/1994	BRA/08/1994	Brazil	1994	Cattle
Bul 01/1993	BUL/01/1993	Bulgaria	1993	Cattle
Bun 03/2003	BUN/03/2003	Burundi	1993	Cattle
Cam 03/1992	CAM/03/1992	Cambodia	1992	Cattle
Cam 06/1998	CAM/06/1998	Cambodia	1998	Pig
Cas 00/1993	Vaccine strain	Argentina	1993	Not known
Equ 01/2010	ECU/01/2010	Ecuador	2010	Cattle
Equ 09/2010	ECU/09/2010	Ecuador	2010	Cattle
Eth 01/1995	ETH/01/1995	Ethiopia	1995	Cattle
Eth 02/1993	ETH/02/1993	Ethiopia	1993	Cattle
Eth 03/2004	ETH/3/2004	Ethiopia	2004	Cattle
Eth 30/1994	ETH/30/1994	Ethiopia	1994	Cattle
Eth 58/2005	ETH/58/2005	Ethiopia	2005	Cattle
Gre 21/1994	GRE/21/1994	Greece	1994	Cattle
Gre 04/1994	GRE/04/1994	Greece	1994	Cattle
Hkn 03/2003	HKN/03/2003	Hong Kong	2003	Pig
Hkn 03/2004	HKN/03/2004	Hong Kong	2004	Pig
Hkn VS/1983	HKN/06/1983	Hong Kong	1983	Pig
Hkn WT/1983	HKN/06/1984	Hong Kong	1983	Pig
Hkn 01/2010	HKN/1/2010	Hong Kong	2010	Pig
Irn 15/1997	IRN/15/1997	Iran	1997	Cattle
Irn 05/2010	IRN/5/2010	Iran	2010	Cattle
Irn 08/2010	IRN/8/2010	Iran	2010	Cattle
Irn 30/2010	IRN/30/2010	Iran	2010	Cattle
Irn 33/2010	IRN/33/2010	Iran	2010	Cattle

Irn 89/2010	IRN/89/2010	Iran	2010	Sheep
Irn 174/2010	IRN/174/2010	Iran	2010	Sheep
Irn 187/2010	IRN/187/2010	Iran	2010	Cattle
ITA 01/1993	ITA/1/1993	Italy	1993	Cattle
OK WT (KauWT/1966)	N/A	Germany	1966	Cattle
O Ken 77/1978	KEN/77/1978	Kenya	1978	Cattle
KUW 02/2006	KUW/2/2006	Kuwait	2006	Cattle
Kuw 03/1988	KUW/3/1988	Kuwait	1988	Cattle
Kuw 04/2008	KUW/4/2008	Kuwait	2008	Cattle
Lau 00/1966	Vaccine strain	Switzerland	1965	Cattle
Man 00/1969	Vaccine strain	Turkey	1969	Cattle
Mog 01/2010	MOG/1/2010	Mongolia	2010	Cattle
Mog 09/2010	MOG/9/2010	Mongolia	2010	Mongolian gazelle
Mya 03/2010	MYA/3/2010	Myanmar	2010	Cattle
Mya 13/2010	MYA/13/201	Myanmar	1020	Cattle
Pak 07/2003	PAK/7/2003	Pakistan	2003	Cattle
Pak 16/2005	PAK/16/2005	Pakistan	2005	Cattle
Pak 17/2003	PAK/17/2003	Pakistan	2003	Cattle
Pak 20/2007	PAK/20/2007	Pakistan	2007	Cattle
Pak 50/2007	PAK/50/2007	Pakistan	2007	Cattle
Pak 67/2003	PAK/67/2003	Pakistan	2003	Cattle
Pak 68/2003	PAK/68/2003	Pakistan	2003	Cattle
Pak 71/2007	PAK/71/2007	Pakistan	2007	Cattle
Pak 01/2010	PAK/1/2010	Pakistan	2010	Cattle
Pak 20/2010	PAK/20/2010	Pakistan	2010	Cattle
Pak 25/2010	PAK/25/2010	Pakistan	2010	Cattle
Pak 42/2010	PAK/42/2010	Pakistan	2010	Cattle
Par 01/2002	PAR/1/2002	Paraguay	2002	Cattle
Par 02/2002	PAR/2/2002	Paraguay	2002	Cattle
Per 10/1993	PER/10/1993	Peru	1993	Cattle
Per 14/1993	PER/14/1993	Peru	1993	Cattle
Per 05/1994	PER/5/1994	Peru	1994	Cattle
Per 06/1994	PER/6/1994	Peru	1994	Cattle
Per 07/1994	PER/7/1994	Peru	1994	Cattle
Phi 09/1995	PHI/9/1995	Phillipines	1995	Pig
PHI 02/1995	PHI/2/1995	Phillipines	1995	Pig
Phi 03/1995	PHI/3/1995	Phillipines	1995	Pig
Sau 72/1994	SAU/72/1994	Saudi Arabia	1994	Cattle
Sau 01/2008	SAU/1/2008	Saudi Arabia	2008	Cattle
Sau 01/2009	SAU/1/2009	Saudi Arabia	2009	Cattle
Skr 04/2010	SKR/4/2010	South Korea	2010	Cattle
Srl 01/2009	SRL/1/2009	Sri Lanka	2009	Buffalo
Sud 04/2008	SUD/4/2008	Sudan	2008	Cattle
Sud 08/2008	SUD/8/2008	Sudan	2008	Cattle
TAI 12/1997	TAW/112/1997	Taiwan	1997	Pig
Tan 02/2004	TAN/2/2004	Tanzania	2004	Cattle

TAI 14/1997	TAW/114/1997	Taiwan	1997	Pig
Taw 01/2009	TAW/1/2009	Taiwan	2009	Pig
TUR 03/1987	TUR/3/1987	Turkey	1987	Not known
TUR 03/1994	TUR/3/1994	Turkey	1994	Cattle
TUR 07/2000	TUR/7/2000	Turkey	2000	Cattle
Tur 35/2008	TUR/35/2008	Turkey	2008	Cattle
Tur 05/2009	TUR/5/2009	Turkey	2009	Cattle
Tur 18/2010	TUR/18/2010	Turkey	2010	Cattle
Tur 39/2010	TUR/39/2010	Turkey	2010	Cattle
UAE 03/2008	UAE/3/2008	United Arab Emirates	2008	Sand Gazelle
UAE 04/2009	UAE/4/2009	United Arab Emirates	2009	Blackbuck
Uga 03/2002	UGA/3/2002	Uganda	2002	Not known
UKG 10/2001	UKG/10/2001	United Kingdom	2001	Pig
UKG 33/2001	UKG/33/2001	United Kingdom	2001	Pig
UKG 34/2001	UKG/34/2001	United Kingdom	2001	Pig
VIT 12/2005	VIT/12/2005	Vietnam	2005	Pig
VIT 03/2004	VIT/3/2004	Vietnam	2004	Pig
VIT 03/1997	VIT/3/1997	Vietnam	1997	Pig
VIT 04/1997	VIT/3/1997	Vietnam	1997	Cattle
Yem 29/2006	YEM/29/2006	Yemen	2006	Cattle
Yem 04/2006	YEM/4/2006	Yemen	2006	Cattle

Appendix 2: Universal primers used to sequence viruses

Primer Name	Sequence
EUR2B52R	GAC ATG TCC TCC TGC ATC TGG TTG AT
L463F	ACC TCC RAC GGG TGG TAC GC
L490F	GAYGAGGAITTYTACCCITGGAC
NK61	GACATGTCCTCCTGCATCTG
NK72	GAAGGGCCCAGGGTTGGACTC
01A67F	AACAAYTACTACATGCARCA
O1B190R	GTGACCCARTCRAAIARRTGGGT
01B253R	GCCGTAGACRCCYTTCTGGTCAGT
01B454F	ACGAACATGACRGCICACATC
01B439R	GTCATGTTCGTICKIGGGTTRATGAA
O1B535F	CTIGTGGTYATGGTYGTRGC
O1C244F	GCAGCAAAACACARGRCAAACACCTT
01C272F	TBG CRG GNC TYG CCC AGT ACT AC
01C283F	GCCCAGTACTACACACAGTACAG
01C286R	GTGCCRCTGTACTGKGTGTAGTACTG
01C361R	GGI GGG GCR TAI GCA AYC ATG TA
01C583F	GACGGYGAYGCICTGGTCGT
01D296F	ACAACACCACCAACCCAAC
01D628R	GTTGGGTTGGTGGTGTTGT
MH2	CACACAACCAACAACACCCAGAACAAT
ROD2	AACTGAGTAAGCACCCTGTCCC
O1C564F	AATTACACATGGCAAGGCCGACACGG

Appendix 3: O KIC Mutagenic primers used in Chapter 6

Primer name	Primer sequence	NT substituted*	Subsitution	AA substituted*	Substitution	Residue ID
O KIC M1F	GTA CCA GAG CTT TGT TCT ATC CAA AAG AGG G	644	A-G	215	Y-C	VP2-130
O KIC M1R	CCC TCT TTT GGA TAG AAC AAA GCT CTG GTA C					
O KIC M1aF	GTA CCA GAG CTT GCT TCT ATC CAA AAG AGG	644	TA-GC	215	Y-A	VP2-130
O KIC M1aR	CCT CTT TTG GAT AGA AGC AAG CTC TGG TAC					
O KIC M2F	CGT TTC TGC ACT TCG AGG GTG ACG TAC CGT ACG	1076	G-A	359	R-H	VP3-56
O KIC M2R	CGT ACG GTA CGT CAC CCT CGA AGT GCA GAA ACG	1088	G-A	363	G-D	VP3-60
O KIC M3F	GTG ACC ACG AAA ATG GAC TCG GAC AGG	1112	C-T	371	T-M	VP3-68
O KIC M3R	CCT GTC CGA GTC CAT TTT CGT GGT CAC					
O KIC M4F	GGC AGC AAA ACA CAT GTC AAA CAC C	1164	A-C	388	Q-H	VP3-85
O KIC M4R	GGT GTT TGA CAT GTG TTT TGC TGC C					
O KIC M5F	GTA CAC CGC GTC TGA CGT GGC CGA GAC	1427	G-A	476	G-D	VP3-173
O KIC M5R	GTC TCG GCC ACG TCA GAC GCG GTG TAC					
O KIC M6F	CGT GTA CAA CGG TAA GTG CAG GTA	1966	G-A	656	E-K	VP1-133
O KIC M6R	TAC CTG CAC TTA CCG TTG TAC ACG					
O KIC M7F	CGA CGT TTC TGC ACT TCG AGG GTG G	1076	G-A	359	R-H	VP3-56
O KIC M7R	CCA CCC TCG AAG TGC AGA AAC GTC G					
O KIC M7aF	ACG TTT CTG GCC TTC GAG GGT G	1075-1076	CG-GC	359	R-A	VP3-56
O KIC M7aR	CAC CCT CGA AGG CCA GAA ACG T					
O KIC M8F	GCT TCG AGG GTG ACG TAC CGT ACG	1088	G-A	363	d-g	VP3-60
O KIC M8R	CGT ACG GTA CGT CAC CCT CGA AGC					
O KIC M9F	GGCGCGTTACATGATTGCCTAC	1276	G-A	426	V-I	VP3-123
O KIC M9R	GTAGGCAATCATGTAACGCGCC					

*both nucleotide (NT) and amino acid (AA) numbering started from the First residue of VP4

Appendix 4: O KIC Mutagenic primers used in Chapter 7

Primer name	Primer sequence	NT substituted*	Subsitution	AA substituted*	Substitution	Residue ID
O UKG 1F	CCT ACG TTT CTG CGG TTT GAG G	1076-1077	AC-GG	359	H-R	VP3 56#
O UKG 1R	CCT CAA ACC GCA GAA ACG TAG G					
O UKG 2F	GAC TGT CAA CAA TGA AGG TGC	828-829	CC-AT	276	T-N	VP2 191
O UKG 2R	GCA CCT TCA TTG TTG ACA GTC					
O UKG 3F	CAA AGA CGG CAT CAG ACA GG GTG	1115-1116	AC-CA	372	D-A	VP3 69
O UKG 3R	CAC CCT GTC TGA TGC CGT CTT TG					
O UKG 4F	GTT GAC GCT CGC GCT CAG ACC AC	1564&1566	A_G-G_T	522	T-A	VP3 219
O UKG 4R	GTG GTC TGA GCG CGA GCG TCA AC					

*both nucleotide (NT) and amino acid (AA) numbering started from the First residue of VP4. # indicates that this primer was used to first to ensure that the sequence of the plasmid was as the O UKG 34/2001 virus used as a homologous in chapter 3. All other mutations then made to this plasmid

Appendix 5: Partial amino acid alignment of O KIC O KWT and Mutants constructed in Chapter 6

```
                    210       220       230       240       250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O KIC               FNGGCLLVAMVPELYSIQKRELYQLTLFPHQFINPRTNMTAHITVPFVGVNRYDQYKVHKPWTLVVMVVAPLTVNTEGAPQIKVYANIAPTNVHVAGEFP
O KWT               ............C.....................................................................................
M1                  ............C.....................................................................................
M1a                 ............A.....................................................................................
M1a Regrown         ............A.....................................................................................
M2                  ..............................................................................................
M3                  ..............................................................................................
M4                  ..............................................................................................
M5                  ..............................................................................................
M7                  ..............................................................................................
M7a                 ..............................................................................................
M8                  ..............................................................................................
M9                  ..............................................................................................
M1+M3              ............C.....................................................................................
M1+M4              ............C.....................................................................................
M1+M5              ............C.....................................................................................
M1+M6              ............C.....................................................................................
M1+M7              ............C.....................................................................................
M2+M4              ..............................................................................................
M2+M5              ..............................................................................................
M4+M5              ..............................................................................................
M1+M8              ............C.....................................................................................
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                    310       320       330       340       350       360       370       380       390       400
                    ....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
O KIC               SKEGIFPVACSDGYGGLVTTDPKTADPVYGKVFNPPRNQLPGRFTNLLDVAEACPTFLRFEGGVPYVTTKTDSDRVLAQFDMSLAAKQMSNTFLAGLAQY
O KWT               ..........................................H...D.....M.....................H.....................
M1                  ....................................................D.....M.....................H.....................
M1a                 ....................................................D.....M.....................H.....................
M1a Regrown         ....................................................D.....M.....................H.....................
M2                  ....................................................H...D.....................H.....................
M3                  ....................................................D.....M.....................H.....................
M4                  ....................................................D.....M.....................H.....................
M5                  ....................................................D.....M.....................H.....................
M7                  ....................................................H.....M.....................H.....................
M7a                 ....................................................A.....M.....................H.....................
M8                  ....................................................D.....M.....................H.....................
M9                  ....................................................D.....M.....................H.....................
M1+M3               ....................................................D.....M.....................H.....................
M1+M4               ....................................................D.....M.....................H.....................
M1+M5               ....................................................D.....M.....................H.....................
M1+M6               ....................................................D.....M.....................H.....................
M1+M7               ....................................................H.....M.....................H.....................
M2+M4               ....................................................H...D.....................H.....................
M2+M5               ....................................................H...D.....................H.....................
M4+M5               ....................................................D.....M.....................H.....................
M1+M8               ....................................................D.....M.....................H.....................
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                     410       420       430       440       450       460       470       480       490       500
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O KIC         YTQYSGTINLHFMFTGPTDAKARYMVAYAPPGMEPPKTPEAAAHCIHAEWDTGLNSKFTFSIPYLSAADYAYTASGVAETTNVQGWVCLFQITHGKADGD
O KWT         .........................I.................................................D........................
M1            ....................................................................................................
M1a           ....................................................................................................
M1a Regrown   ....................................................................................................
M2            ....................................................................................................
M3            ....................................................................................................
M4            ....................................................................................................
M5            ...........................................................................D........................
M7            ....................................................................................................
M7a           ....................................................................................................
M8            ....................................................................................................
M9            .........................I..........................................................................
M1+M3         ....................................................................................................
M1+M4         ....................................................................................................
M1+M5         ...........................................................................D........................
M1+M6         ....................................................................................................
M1+M7         ....................................................................................................
M2+M4         ....................................................................................................
M2+M5         ...........................................................................D........................
M4+M5         ...........................................................................D........................
M1+M8         ....................................................................................................
```

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                                  610       620       630       640       650       660       670       680       690       700
                         ....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
O KIC                    IAVKHEGDLTWVPNGAPEKALDNTTNPTAYHKAPLTRLALPYTAPHRVLATVYNGECRYNRNAVPNLRGDLQVLAQKVARTLPTSFNYGAIKATRVTELL
O KWT                    ....................................K.....................................................
M1                       ....................................................................................
M1a                      ....................................................................................
M1a Regrown              ....................................................................................
M2                       ....................................................................................
M3                       ....................................................................................
M4                       ....................................................................................
M5                       ....................................................................................
M7                       ....................................................................................
M7a                      ....................................................................................
M8                       ....................................................................................
M9                       ....................................................................................
M1+M3                    ....................................................................................
M1+M4                    ....................................................................................
M1+M5                    ....................................................................................
M1+M6                    ....................................K.....................................................
M1+M7                    ....................................................................................
M2+M4                    ....................................................................................
M2+M5                    ....................................................................................
M4+M5                    ....................................................................................
M1+M8                    ....................................................................................
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