

Mapping of antigenic determinants on a SAT2 foot-and-mouth disease virus using chicken single-chain antibody fragments

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Running Title: Antigenic sites of a SAT2 virus

Abstract

Recombinant single-chain variable fragments (scFvs) of antibodies make it possible to localize antigenic and immunogenic determinants, identify protective epitopes and can be exploited for the design of improved diagnostic tests and vaccines. A neutralizing epitope, as well as other potential antigenic sites of a SAT2 foot-and-mouth disease virus (FMDV) were identified using phage-displayed scFvs. Three unique ZIM/7/83-specific scFvs, designated scFv1, scFv2 and scFv3, were isolated. Further characterization of these scFvs revealed that

only scFv2 was capable of neutralizing the ZIM/7/83 virus and was used to generate neutralization-resistant virus variants. Sequence analysis of the P1 region of virus escaping neutralization revealed a residue change from His to Arg at position 159 of the VP1 protein. Residue 159 is not only surface exposed but is also located at the C-terminal base of the G-H loop, a known immunogenic region of FMDV. A synthetic peptide, of which the sequence corresponded to the predicted antigenic site of the VP1 G-H loop of ZIM/7/83, inhibited binding of scFv2 to ZIM/7/83 in a concentration-dependent manner. This region can therefore be considered in the design of SAT2 vaccine seed viruses for the regional control of FMD in Africa.

Keywords: foot-and-mouth disease; SAT2; phage display; single-chain variable fragment; epitope; antigenic site.

Abbreviations: foot-and-mouth disease (FMD); FMD virus (FMDV); single-chain variable fragment (scFv); monoclonal antibody (MAb)

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of even-toed ungulates and ranks as one of the most important infectious animal diseases due to its economic impact. The causative agent, FMD virus (FMDV), is a single-stranded, positive-sense RNA virus and the prototype member of the *Aphthovirus* genus in the family *Picornaviridae* (King *et al.*, 2000). The South African Territories (SAT) types 1, 2 and 3 are confined to Africa where six of the seven FMDV serotypes occur (Brooksby, 1972; Ferris and Donaldson, 1992; Vosloo *et al.*, 2002). The SAT types are maintained within African buffalo (*Syncerus caffer*)

populations, which provide a potential source of infection for domestic livestock and other susceptible wildlife (Bastos *et al.*, 2000; Dawe *et al.*, 1994; Vosloo and Thomson, 2004), as well as the opportunity for antigenic and molecular evolution of the virus to occur (Bastos *et al.*, 2001, 2003a, 2003b; Vosloo *et al.*, 1996).

The FMDV capsid is composed of 60 copies of each of four structural proteins namely, VP1, VP2 and VP3, which are surface exposed, and the internally located VP4 protein (Acharya *et al.*, 1989; Morrell *et al.*, 1987). The capsid shares many structural features with that of other picornaviruses, including the similar orientation of the β -barrel structures of the three outer capsid proteins. It has been shown that the majority of FMDV-neutralizing antibodies are directed against epitopes located in the three surface-exposed capsid proteins of the virus, of which the flexible G-H loop in VP1 is antigenically important (Acharya *et al.*, 1989; Kitson *et al.*, 1990; Thomas *et al.*, 1988; Xie *et al.*, 1987). Antigenic variation results from changes to the viral capsid as a consequence of the high mutation rate of the virus (Domingo and Holland, 1997; Holland *et al.*, 1982), thus contributing to the generation of a spectrum of antigenic subtypes within each serotype. Furthermore, each FMDV isolate is antigenically unique in its fine epitopic composition such that the location of these antigenic sites and their antigenic features vary between the different serotypes. This results in decreased vaccine efficacy and effectiveness of vaccination programs in the field (Feigelstock *et al.*, 1996). Several studies have been carried out to delineate the neutralizing antigenic sites of representative viruses from serotypes A (Baxt *et al.*, 1989; Bolwell *et al.*, 1989; Thomas *et al.*, 1988), O (Crowther *et al.*, 1993a; Kitson *et al.*, 1990), C (Mateu *et al.*, 1990) and Asia-1 (Sanyal *et al.*, 1997). In these studies, monoclonal antibodies (MAbs) were pivotal in identifying critical amino acid (aa) residues of the different neutralizing antigenic sites.

Despite the fact that the SAT2 serotype has been responsible for most of the FMD outbreaks in domestic livestock in southern Africa (Thomson and Bastos, 2004), limited studies aimed at the production of MAbs specific for this serotype have been performed. These MAbs nevertheless allowed the identification of at least two neutralizing epitopes, one within the G-H loop of VP1 (Crowther *et al.*, 1993b; Davidson *et al.*, 1995; Grazioli *et al.*, 2006) and one at the C-terminus of VP1 (Grazioli *et al.*, 2006). Evidence from studies using FMDV serotypes C (Feigelstock *et al.*, 1992; Mateu *et al.*, 1995), A (Thomas *et al.*, 1988) and SAT1 (Grazioli *et al.*, 2006) indicated that antigenic sites on VP2 and VP3 are also important in the generation of an immune response following infection or vaccination. Therefore, all such antigenic determinants have to be considered in the design of vaccines for regional control of FMD.

Direct or structural approaches to identify antigenic sites on large protein structures like viral capsids include the use of X-ray crystallography, nuclear magnetic resonance (NMR) and electron microscopy (Guthridge *et al.*, 2001; Kleymann *et al.*, 1995). However, functional methods like competition assays, virus neutralization escape mutants, protein fragmentation methods and the use of synthetic peptides or peptide libraries are also essential in the mapping of neutralizing epitopes or antigenic sites on viral capsids (Bich *et al.*, 2008; Crowther *et al.*, 1993b; Irving *et al.*, 2001; Joseph *et al.*, 2002). Many recombinant antibodies have been produced and selected through the construction of phage display libraries (Carmen and Jermutus, 2002). Antibody libraries can be constructed from either immunized (immune library) or non-immunized (naïve library) sources. Although immune libraries are more specific (reviewed in Winter *et al.*, 1994) their construction is time-consuming (Brichta *et al.*, 2005), whereas naïve libraries are not confined to any specific target and therefore contain a broad range of specificities (Brichta *et al.*, 2005; Gram *et al.*, 1992; Van Wyngaardt *et al.*,

2004). Furthermore, access to naïve libraries may produce rapid results and could circumvent many of the complexities normally related to the conventional production of antibodies (Clackson *et al.*, 1991; Smith, 1985). Of particular interest for this study is the large semi-synthetic Nkuku[®] library. This single-chain variable fragment (scFv) phage display library based on the chicken immunoglobulin genes has yielded a panel of antibodies against a variety of antigens, including viruses of veterinary importance such as bluetongue virus (BTV) and African horse sickness virus (AHSV) (Fehrsen *et al.*, 2005; Rakabe, 2008; Van Wyngaardt *et al.*, 2004).

In this paper we aimed to identify antigenic sites of a SAT2 virus that could be used in the rational design of more effective vaccines for southern Africa. Of the three recombinant scFvs panned from the Nkuku[®] library, one was used to generate virus neutralization escape mutants that facilitated the identification of a neutralizing epitope of a SAT2 virus, while the two non-neutralizing scFvs were further analyzed to determine their putative binding sites to the virus. The binding profiles and specificity of these scFvs for representative SAT2 field isolates were also determined for application in the control of new and re-emerging FMDV outbreaks.

2. Materials and Methods

2.1 Cell cultures, virus propagation and purification

Baby hamster kidney (BHK) strain 21 clone 13 cells (ATCC CCL-10), used for virus propagation, were maintained as described by Van Rensburg *et al.* (2004). Instituto Biologico Renal Suino-2 (IB-RS-2) cells, used during virus neutralization tests (VNT), were maintained

in RPMI medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS; Invitrogen) and 1 × antibiotics (Invitrogen). The Mycl-9E10 hybridoma (ECACC 85102202) was cultured in protein-free hybridoma medium (Invitrogen).

The SAT2 FMDV vaccine strain ZIM/7/83 (passage history: B1BHK5B1; B=bovine) is a bovine virus, originating from an outbreak in western Zimbabwe during 1983 (Van Rensburg *et al.*, 2004). ZIM/7/83 146S virus particles were concentrated with 8% (w/v) polyethylene glycol (PEG)-8000 (Sigma-Aldrich) and purified on 10% to 50% (w/v) sucrose density gradients (SDG), prepared in TNE buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 mM EDTA), as described by Knipe *et al.* (1997). Peak fractions corresponding to the 146S virus particles were pooled.

The SAT2 viruses included in this study, *i.e.* ZIM/5/83, ZIM/7/89, ZIM/13/01, ZIM/8/94, ZIM/4/97, ZIM/44/97, ZIM/5/02 and ZIM/2/88, form part of the virus bank at the Transboundary Animal Diseases Programme, Onderstepoort, South Africa. The viruses were propagated in IB-RS-2 and BHK-21 cells prior to RT-PCR amplification or SDG purification of 146S particles.

2.2 Selection of scFvs against ZIM/7/83

Selection of ZIM/7/83-specific scFvs from the Nkuku[®] phage display library (provided by Dr D.H. du Plessis, Onderstepoort Veterinary Institute) was performed as described by Van Wyngaardt *et al.* (2004). Briefly, 2-ml immunotubes (Nunc Maxisorp), coated with 30 µg/ml of purified ZIM/7/83 and blocked with 1 × PBS containing 2% (w/v) casein, were incubated for 1.5 h with library phage particles (10^{12} - 10^{13} transducing particles)

diluted in 2% (w/v) casein and 0.1% (v/v) Tween-20. Phage-displayed scFvs that bound to ZIM/7/83 were eluted with 100 mM triethylamine (pH 12), neutralized with 1 M Tris (pH 7.4) and used to re-infect exponentially growing *Escherichia coli* TG1 cells (Stratagene, USA) before plating on TYE plates (15 g/l agar, 8 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) supplemented with 2% (w/v) glucose and 100 µg/ml ampicillin. Following overnight incubation at 30°C the bacteria were collected and the phagemids rescued by the addition of M13KO7 helper phage (helper phage: bacteria = 20:1). Infected bacterial cells were incubated overnight at 30°C in 2 × TY medium (16 g/l tryptone, 10 g/l yeast extract, 5 g NaCl/l) that contained 100 µg/ml ampicillin and 25 µg/ml kanamycin. Phage-displayed scFvs were precipitated from the cell-free culture supernatant with one-fifth of the original culture volume of 20% (w/v) PEG-8000 in 2.5 M NaCl and were then suspended in 1 × PBS for use in the next selection round. Five such selection rounds were performed.

Enrichment was monitored by titration of the input and output phages from each selection round, as well as a polyclonal ELISA of the outputs of the consecutive selection rounds. Monoclonal soluble scFvs from selection rounds four and five were tested for specific binding to ZIM/7/83.

2.3 Polyclonal phage ELISA

The polyclonal phage ELISA (Van Wyngaardt *et al.*, 2004) was performed by coating 96-well Maxisorp immunoplates (Nunc) overnight at 4°C with purified ZIM/7/83 (30 µg/ml), diluted in 1 × PBS. Casein (2% w/v) in 1 × PBS was used as blocking reagent and negative control to verify the ZIM/7/83 specificity of the phage-displayed scFvs. The PEG-precipitated phage-displayed scFvs, produced at each selection round, were detected with the

MAB B62-FE2 (100 ng/ml; Progen Biotechnik) and horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse IgG (PO260; Dako). The substrate/chromogen solution consisted of 4 mM 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich) in substrate buffer (0.1 M citric acid monohydrate, 0.1 M tri-potassium citrate, pH 4.5) and 0.015% H₂O₂. The colour reaction was stopped after 10 min with 1 M H₂SO₄ and the absorbance values were recorded at 450nm.

2.4 Monoclonal soluble scFv ELISA

Van Wyngaardt *et al.* (2004) described the monoclonal soluble scFv ELISA. In short, soluble scFvs were obtained by inoculating overnight cultures of infected bacterial cells into 2 × TY medium containing 100 µg/ml ampicillin and 0.1% (w/v) glucose. Following incubation for 3 h at 37°C, IPTG was added to a final concentration of 1 mM and incubation continued for a further 16 h at 30°C. Cells were removed by centrifugation and the supernatant tested as follows. Antigen coating of the ELISA plates was performed as described for the polyclonal phage ELISA (Section 2.3) and all other steps were performed as described by Van Wyngaardt *et al.* (2004). Secreted soluble scFvs were detected with the anti-c-Myc MAb 9E10, expressed from the murine hybridoma Myc1-9E10 (CAMR, UK), and polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (P0260; Dako).

2.5 Large scale purification of soluble scFvs

Large scale purification of soluble scFvs from 1-L cultures was performed by the Biotechnology Division of the National Bioproducts Unit in Kwa-Zulu Natal, South Africa,

on an affinity column that contained 75 ml Sepharose coupled to 100 mg of the Myc tag-specific MAb, 9E10.

2.6 DNA sequencing and sequence analysis

2.6.1 Phage-displayed scFvs

Phagemid DNA was isolated with a QIAprep[®] Spin Miniprep Kit (Qiagen) from positive clones picked from selection rounds four and five. These single clone inserts were sequenced with the OP52 forward (Van Wyngaardt *et al.*, 2004) and M13 reverse primers, and an ABI PRISM[™] Big Dye[™] Terminator Cycling Ready Reaction Kit v.3.0 (Applied Biosystems). The extension products were resolved on an ABI 3100 automated sequencer and the nucleotide sequences were assembled and translated using BioEdit v.7.0.9 (Hall, 1999).

2.6.2 FMDV outer capsid-coding region

The Leader-P1-2A coding regions of the SAT viruses were obtained via RT-PCR of viral genomic RNA, as described previously (Bastos, 1998; Van Rensburg *et al.*, 2002). Direct DNA sequencing of amplicons yielded a consensus sequence representing the most probable nucleotide for each position. Sequences of the *ca.* 2.2-kb P1-coding region were compiled and edited using BioEdit v.7.0.9 software, and the amino acid sequences were deduced and aligned. The GenBank accession numbers of the SAT2 viruses are summarized in Table 1. Phylogenetic trees were constructed in MEGA 4.0 (Tamura *et al.*, 2007) using neighbour-joining algorithms and node reliability is supported by 1000 bootstrap replications.

Table 1. FMDV isolates used in this study.

Serotype	Virus strain	Species of origin	Year of sampling	Passage history	GenBank accession nos.
SAT1	KNP/196/91	Buffalo	1991	PK1RS4B1BHK6	DQ009716
SAT2	ZIM/7/83	Bovine	1983	B1BHK5B1BHK4	DQ009726
	ZIM/5/83	Bovine	1983	BTY4RS2BHK5	JQ639289
	ZIM/7/89	Buffalo	1989	B1PK1RS2	JQ639296
	ZIM/13/01	Bovine	2001	RS2BHK5	JQ639292
	ZIM/8/94	Buffalo	1994	BTYRS4BHK5	JQ639290
	ZIM/4/97	Bovine	1997	B1PK1RS2BHK5	JQ639293
	ZIM/44/97	Buffalo	1997	BTY1RS4BHK5	JQ639291
	ZIM/5/02	Bovine	2002	PK1RS4BHK7	JQ639295
	ZIM/2/88	Buffalo	1988	CFK1RS1BHK8	JQ639294
SAT3	KNP/10/90	Buffalo	1990	PK1RS1BHK5	AF286347

2.7 Binding specificity of the secreted soluble scFvs

The specificity of the soluble scFv binders was tested on representative SAT1, SAT2 and SAT3 viruses (Table 1), propagated and purified as described in Section 2.1. ELISA plates were coated with 30 µg/ml of purified SAT2/ZIM/7/83, SAT1/KNP/196/91, SAT3/KNP/10/90, as well as with 30% (w/v) sucrose, 2% (w/v) casein and a BHK-21 cell extract as negative controls. ELISAs were performed as described for the monoclonal soluble scFv ELISA (Section 2.4). The binding specificity of the soluble scFvs to the SAT2 viruses, ZIM/5/83, ZIM/7/89, ZIM/13/01, ZIM/8/94, ZIM/4/97, ZIM/44/97, ZIM/5/02 and ZIM/2/88 (Table 1), was determined at various antigen concentrations. ELISA plates were coated with two-fold dilutions (from 30 to 0.94 µg/ml) of the antigens, and the results for the 7.5 µg/ml coating concentration were compared.

2.8 Neutralization assays and generation of virus escape mutants

The 50% tissue culture infective dose (TCID₅₀) of ZIM/7/83 was determined on IB-RS-2 cells (Esterhuysen *et al.*, 1988) and the resulting virus titre was used to calculate the dilutions subsequently used in the VNT. Triplicate repeats of the appropriate ZIM/7/83 dilutions in RPMI medium, containing *ca.* 500, 50 and 5 infectious particles, were applied across a microtitre plate and diluted two-fold down the plate. scFvs, *ca.* 0.167 mg/ml, were added to each well and a control plate without soluble scFvs was also included. Following incubation for 1 h at 37°C in an atmosphere of 5% CO₂, IB-RS-2 cells (0.3 × 10⁶ cells/ml) in RPMI medium supplemented with 1% (v/v) FCS and antibiotics (virus growth medium; VGM), was added. The microtitre plates were incubated for 72 h at 37°C, examined microscopically and the cytopathic effect (CPE) was scored as a measure of neutralization.

Virus neutralization escape mutants were generated as described by Crowther *et al.* (1993b). For this purpose, *ca.* 25 infectious virus particles of ZIM/7/83 were diluted two-fold in RPMI medium containing antibiotics before being mixed with an equal volume of scFv2 (17 µg). The suspension was incubated for 30 min at 37°C and applied to an IB-RS2 cell monolayer. Following incubation for 1 h at 37°C the virus-scFv complexes were removed. The monolayer was washed twice with RPMI medium and then VGM, containing a 1:50 dilution of scFv2 (3 µg/ml), was added. The ZIM/7/83 virus was passaged three consecutive times under scFv pressure.

2.9 Synthetic peptide blocking ELISA

Two different synthetic peptides, synthesized by GenScript Corp., were used in a blocking ELISA to confirm the presence of the predicted antigenic site on ZIM/7/83. The sequence of the 40-mer peptide (KYTQQSTAIRGDRAVLA AKYANT **KRK** LPSTFNFGYV TADK), designated EpiR, was derived from the VP1 G-H loop region of ZIM/7/83 that contains the predicted epitope (marked in bold and underlined). A 30-mer peptide, which had the sequence AAVESAAVESAAVESAAVESAAVESAAVES, was used as a non-competitive negative control in the assays. ELISA plates were coated overnight at 4°C with purified ZIM/7/83 (*ca.* 25 µg/ml) and blocked with 2% (w/v) casein. Equal volumes of scFv2 (*ca.* 5.3 µg/ml) in 4% (w/v) casein and peptides in 1 × PBS (400 µM to 50 µM) were mixed. As a non-inhibition control the peptides were replaced with 1 × PBS and mixed with the soluble scFvs. The soluble scFv and peptide solutions were incubated for 1 h at 30°C with shaking (100 rpm) before being added to the pre-blocked ELISA plates. ELISA plates were incubated for 1 h and binding of soluble scFv2 fragments to the immobilized ZIM/7/83 was detected as described in the monoclonal soluble scFv ELISA (Section 2.4).

2.10 Structural modeling

A homology model of the SAT2 capsid proteins was built using Modeller 9v3 (Sali and Blundell, 1993) with O₁BFS (Logan *et al.*, 1993) as template. The model was based on the optimal alignment of the SAT2 virus, ZIM/7/83, P1 sequence to the corresponding sequence of O₁BFS. The satisfaction of spatial restraints, as described by empirical databases, was used to calculate homology modeled structure. Structures were visualized and the surface-exposed residues identified with PyMol v1.1rc2pre (DeLano Scientific LLC).

3. Results

3.1 Selection and identification of phage-displayed scFvs against ZIM/7/83

The Nkuku[®] library, a large semi-synthetic phage display library based on chicken immunoglobulin genes, was panned by exposing the recombinant antibody repertoire to purified virions of the SAT2 virus ZIM/7/83. Phage-displayed scFvs that bound to ZIM/7/83 were eluted with triethylamine. Enrichment of phage-displayed scFvs specific for the purified ZIM/7/83 virus was monitored by a polyclonal phage ELISA. Polyclonal phage outputs from the five consecutive selection rounds were tested and an aliquot of the library prior to panning was included as a non-enriched control (Fig. 1). The non-enriched control produced an absorbance at 450nm of below 0.180, whereas the output from selection round five produced an absorbance of 2.14, thus indicating that phage pools were enriched with ZIM/7/83-specific scFvs during consecutive selection rounds.

Single phage clones picked randomly from round four and five titre plates were subsequently tested in an ELISA to identify monoclonal ZIM/7/83-specific binders. The scFvs were expressed in phage-displayed and soluble formats. A total of 188 single clones were screened of which 90 clones expressed phage-displayed scFvs specific to ZIM/7/83 with ELISA signals two-fold greater than that of the negative casein controls. Of these, 73 clones secreted soluble scFvs that bound to ZIM/7/83. Sequencing of the 73 clones revealed the presence of three unique scFvs binders (Table 2), designated scFv1 (27 identical clones), scFv2 (43 identical clones) and scFv3 (3 identical clones) that produced ELISA signals of 0.239, 1.770 and 1.23, respectively, when measured at 450nm. These three representative soluble scFvs were further characterized.

Table 2. Amino acid sequence alignment of the complementary determining regions (CDR) of the heavy and light chains of the three ZIM/7/83-specific soluble scFvs panned from the Nkuku[®] library.

	Heavy chain			Light chain		
	Complementary determining region ^a			Complementary determining region ^a		
	CDR 1	CDR2	CDR3	CDR1	CDR2	CDR3
scFv 1	SYEM Q	AGIIDDGSSTYYAPAV KG	SGYDSSYYYS- TAADIIDA	SGGGRYTY G	YNDKRP S	GSYGSS- TGI
scFv 2	SYDM Q	AGIDDGGSFTGYGAAV KG	TTGSNMGYS--- AHLIDA	SGSS- GSYG	YNDKRP S	GGYDSSTYA I
scFv 3	SYGM G	AAIQNDGSSTYYGAAV KG	DVYDCSSSCGIYGHRI DA	SGGS- SSYG	YNDKRP S	GSRDSSN- PI

a

The CDR regions were annotated according to Andris-Widhopf et al. (2000) and sequence gaps were introduced to facilitate optimal alignment.

3.2 Characterization of ZIM/7/83-specific scFvs

The ZIM/7/83-specific scFvs were characterized on the basis of their ability to bind to complete 146S virions of representative viruses from the SAT 1, 2 and 3 serotypes. The binding of the various scFvs was determined by an indirect ELISA. The soluble scFvs bound to ZIM/7/83; however, none of the scFvs bound to the SAT1 virus KNP/196/91 or the SAT3 virus KNP/10/90 (Fig. 2A-C), suggesting that the identified scFvs may therefore be specific to SAT2 viruses. This specificity to the SAT2 FMDV serotype was further investigated by making use of a panel of SAT2 viruses. The results, also indicated in Fig. 2 (A-C), showed that the three soluble scFvs bound to the eight SAT2 viruses, albeit with apparent different binding profiles. Soluble scFv2 bound to all the SAT2 viruses with similar ELISA absorbance signals, whereas the ELISA signal for soluble scFv1 and 3 was generally reduced

with a few exceptions, *i.e.* ZIM/13/01 (Fig. 2A, indicated by number 3), ZIM/8/94 (Fig. 2A, number 4), ZIM/7/89 (Fig. 2A, number 5) for scFv1 and ZIM/7/89 (Fig. 2C, number 5) for scFv3.

3.3 Neutralization studies and the generation of virus escape mutants

Towards mapping of the binding sites of the three soluble scFv binders to the SAT2 virion, the respective soluble scFvs were first evaluated for their ability to neutralize ZIM/7/83 *in vitro*. In contrast to soluble scFv1 and 3, soluble scFv2 showed neutralizing activity of ZIM/7/83. Considerably less CPE was observed for the soluble scFv assay compared to the control assay that lacked soluble scFvs. Subsequently, virus neutralization escape mutants were generated by serial passage of the ZIM/7/83 virus in the presence of soluble scFv2, and the P1 nucleotide sequence of two viruses that escaped neutralization was determined.

Comparative analysis of the deduced amino acid sequences indicated that both virus neutralization escape mutants harboured two amino acid changes located in the VP1 protein. The first escape mutant (EscM1) contained a threonine (T) to serine (S) change at position 4 (T4→S) and an arginine (R) to histidine (H) change at position 159 (R159→H) of VP1. The second escape mutant (EscM2) also contained the T4→S change, but at position 159 one of two amino acids were present, either an R, as seen in the parental virus, or a H, as seen in EscM1 (Fig. 3A). The two amino acid residues observed at this position can be accounted for by the genetic heterogeneity due to the quasispecies nature of the FMDV genome. Each FMD viral population consists of a “mixture” of genomes (Domingo and Holland, 1997), allowing for the selection of variants upon environmental changes, *e.g.* immune pressure.

Mapping of the location of the observed variant amino acids on a model of the ZIM/7/83 protomer indicated that the T4→S change was hidden from the virion surface, and distantly located from any of the sequences known to be involved in antigenicity of the virus. Notably, the R159→H change was located on the surface of the pentamer, at the C-terminal base of the G-H loop (Fig. 3B).

3.4 Binding of soluble scFv2 to ZIM/7/83 is reduced by a synthetic peptide

To confirm the role of residue 159 of VP1 as part of the binding site of soluble scFv2, a synthetic peptide (EpiR), of which the sequence was derived from the predicted epitope site on ZIM/7/83, was used in a blocking ELISA together with soluble scFv2. Binding of the soluble scFv2 to ZIM/7/83 was not noticeably inhibited at concentrations of 50-200 μ M of the EpiR peptide, as compared to binding of the soluble scFv2 to ZIM/7/83 in the absence of EpiR. However, the ELISA signal was reduced by 24% and 45% (Fig. 4) in the presence of higher concentrations of EpiR (300 μ M and 400 μ M, respectively). A control peptide, which sequence displayed no homology to the putative binding site, did not inhibit binding of soluble scFv2 to ZIM/7/83. Taken together, the results indicate that the binding of soluble scFv2 to ZIM/7/83 was reduced in a concentration-dependent manner by pre-incubation with EpiR.

3.5 Identification of putative antigenic sites

The different binding profiles observed for scFv1 and 3 to the panel of SAT2 viruses (Fig. 2) were used to identify their potential binding footprints on the SAT2 capsid. The data

presented in Fig. 2 relied on there being a saturating concentration of virus antigen (30 µg/ml) available for the soluble scFvs to bind to. Therefore, the binding profiles of scFv1 and 3 were further examined using an indirect ELISA and nine SAT2 viruses with decreasing virus antigen concentrations. The differences in binding observed for a particular soluble scFv to the panel of SAT2 viruses were then compared to differences observed in the deduced amino acid sequence of the P1 region of the SAT2 viruses in order to predict the potential binding footprint of each soluble scFv. It has previously been shown that surface-exposed amino acid differences can be used to predict a change in antigenicity and may provide information regarding antibody footprints on the virus capsid (Maree *et al.*, 2011; Reeve *et al.*, 2010).

At a concentration of 7.5 µg/ml, soluble scFv1 reacted similarly to ZIM/7/83, ZIM/44/97, ZIM/8/94, ZIM/2/88, ZIM/13/01 and ZIM/5/02. The binding profile of soluble scFv1 to ZIM/4/97 and ZIM/5/83 was reduced by *ca.* 80% and increased by *ca.* 37% to ZIM/7/89 when compared to ZIM/7/83 (Fig. 5; supplementary data A). In contrast, soluble scFv3 reacted similarly to ZIM/7/83, ZIM/7/89 and ZIM/2/88. The binding of scFv3 was reduced by *ca.* 55% to ZIM/5/02, *ca.* 75% to ZIM/4/97, ZIM/44/97, ZIM/5/83 and ZIM/13/01, and *ca.* 80% to ZIM/8/94 (Fig. 5; supplementary data A).

At least 10% (75 of 740) of the amino acid residues were variable when comparing the P1 regions of the nine SAT2 viruses. A systematic analysis of the capsid proteins revealed the variation was mostly focused in local regions of hypervariability. The hypervariable regions were mapped to a modelled structure of a SAT2 capsid, using O₁BFS (Acharya *et al.*, 1989) as a template. The variability was found to be concentrated in β-β loops surrounding the 5-fold and 3-fold axis of the virion (supplementary data C). With the exception of the

immunodominant VP1 β G- β H loop (VP1 aa residues 158-160), an additional four surface-exposed hypervariable sites were located within VP1, *i.e.* β B- β C loop (aa residues 43-48), β E- β F loop (aa residues 85/98-99), β F- β G loop (aa residues 108-111) and the C-terminus (aa residues 198/200-201). At least two other variable loops were identified, one located in VP2 [β E- β F loop (aa residues 133-134)] and one in VP3 [β B- β C loop (aa residues 56; 58)] (Table 3).

Since soluble scFvs 1 and 3 bind to complete 146S virions of SAT2 viruses but do not cause neutralization of the ZIM/7/83 virus *in vitro*, they may recognize the surface-exposed variable β - β structure of VP1, VP2 or VP3 without interfering with the RGD-integrin interaction. In this regard, ZIM/5/02, ZIM/4/97, ZIM/44/97, ZIM/13/01 and ZIM/8/94 differ from ZIM/7/83, ZIM/7/89 and ZIM/2/88 at the β B- β C, β E- β F and β F- β G loops surrounding the 5-fold axis of VP1. Amino acid differences here may contribute to the reduced binding of soluble scFv3 to these viruses. Similarly, amino acid differences in the G-H loop and the C-terminus of VP1 may explain the reduced binding of soluble scFv1 to ZIM/4/97 (supplementary data B).

4. Discussion

Here we describe the selection of three unique soluble SAT2-specific scFv binders from the Nkuku[®] chicken scFv phage-displayed library. Of the three binders identified, only scFv2 was capable of neutralizing the ZIM/7/83 virus and its binding position was successfully mapped to involve residue position 159 of the VP1 protein. Previously, unique scFv binders have been obtained following panning of the Nkuku[®] library with other pathogens like BTV and the 16-kDa antigen of *Mycobacterium tuberculosis* (Fehrsen *et al.*,

2005; Rakabe, 2008; Sixholo, 2008; Wemmer *et al.*, 2010). This is the first time the selection of SAT2-specific scFvs from this highly diverse and naïve, semi-synthetic IgY library is being described.

Two point mutations occurred in the VP1 protein of the ZIM/7/83 virus that escaped neutralization by scFv2. The T4→S change at the N-terminal of VP1 is 4 aa downstream of the VP3/VP1 cleavage site. These residues at the N-terminal of VP1, which have similar polar, hydroxyl-group containing side chains, are located in the internal surface of the capsid in close proximity to the N-terminal of VP2 and VP4. Neutralizing antibodies to the internal epitopes of VP1 and VP4 have been reported in poliovirus and rhinovirus (Katpally *et al.*, 2009; Li *et al.*, 1994; Roivainen *et al.*, 1993). However, neutralizing antibodies to internally located epitopes of FMDV have not been reported. Panning and analysis of binding specificity were performed using intact 146S particles. Therefore, it is unlikely that soluble scFv2 is directed to internally located residues and T4→S was discarded as a possible antigenic site. The T4→S residue change could be accounted for either as a result of a compensatory mutational event due to possible structural rearrangements following a distant effect (Grazioli *et al.*, 2006; Mateo and Mateu, 2007) or due to the quasispecies nature of FMDV. Since the majority of virus escape mutants involve the interaction of a surface-orientated side-chain with a MAb, the surface exposed residue change R159→H in VP1, located at the C-terminal base of the G-H loop, was thought to be involved in binding of the soluble scFv2. Additionally, a synthetic peptide with its sequence derived from the VP1 G-H loop of ZIM/7/83, corresponding to the region of the predicted epitope, reduced binding of soluble scFv2 to ZIM/7/83. This led us to believe that the epitope to which scFv2 binds to has been successfully mapped and that residue 159 of VP1 forms part of it.

The likelihood that the residue change at position 159 of VP1 forms part of an antigenic site on the SAT2 capsid is supported by the fact that residue 159 of VP1 forms part of the base of the G-H loop and the G-H loop residues 140-160 have been shown to play an important role in antigenicity in most FMDV serotypes (Barnett *et al.*, 1989; Bolwell *et al.*, 1989; Crowther *et al.*, 1993a; Parry *et al.*, 1989; Pfaff *et al.*, 1988; Thomas *et al.*, 1988). The antigenic sites Ia and Ib of SAT1 viruses, for example, involve residues on both sides of the RGD motif in the G-H loop (Grazioli *et al.*, 2006). In addition, plotting the observed variant amino acid residue on a model of the capsid pentamer of ZIM/7/83 indicated that the proposed epitope would be surface exposed and this position coincides with an antigenic region predicted by Reeve *et al.* (2010) and Maree *et al.* (2011) on ZIM/7/83. Added to this, the region has previously been identified as an antigenic area in the SAT2 viruses RHO/1/48 (Crowther *et al.*, 1993b), as well as in ZIM/5/81 (Grazioli *et al.*, 2006). Crowther and co-workers (1993b) identified antigenic sites, the most important of which is a conformation-dependent epitope involving amino acids at position 156 or 158 of VP1. Mice MAbs used to identify this site bound to RHO/1/48 only and not to any of the 73 SAT2 field isolates tested, including ZIM/7/83, indicating that this site is unique to RHO/1/48 (Crowther *et al.*, 1993b). Two neutralizing antigenic sites, both of which are linear, have been identified for SAT2/ZIM/5/81 and one mapped in the C-terminus of VP1 (position 210) and the other within the G-H loop region (position 154) (Grazioli *et al.*, 2006). Our results indicated that randomly-linked variable heavy and light chains of IgY amplified from naïve chickens bound to a region on the SAT2 virion that corresponds to that of MAbs generated from vaccinated mice, and it has the additional advantage that it bound to a panel of SAT2 viruses with a similar profile.

It is proposed that the “mechanism” of virus neutralization by soluble scFv2 is due to steric hindrance caused by soluble scFv2 binding to the C-terminal region of the G-H loop. As a result of the binding the presentation of the flexible external loop could be altered in such a way that the highly conserved three-amino-acid-sequence Arg-Gly-Asp (RGD) at its apex, which is involved in binding of FMDV to host cells (Curry *et al.*, 1996; Fox *et al.*, 1989; Lea *et al.*, 1994; Logan *et al.*, 1993; Mason *et al.*, 1994), is blocked from cell surface receptors. This mechanism of neutralization has also been proposed for the site A antigenic site, positioned C-terminal to the G-H loop RGD motif for serotype C (Domingo *et al.*, 1999; Verdaguer *et al.*, 1997). The G-H loop of VP1 has for many years been regarded as an immunodominant antigenic site of FMDV. However, ongoing research has indicated that other antigenic sites are also important in providing protection against FMDV and eliciting good antibody responses (Fowler *et al.*, 2010; and references therein). Nevertheless, this study has shown that the scFv2 intratypic conserved “footprint”, which is located in the C-terminal region of the VP1 G-H loop where high variation is found in field isolates, may be important as an antigenic site for SAT 2 viruses.

Although we did not provide direct proof of the exact binding sites of soluble scFv1 and 3 to the virion, we used the observed variation in the binding profiles of soluble scFv1 and 3 to the panel of SAT2 viruses, the P1 sequences of the SAT2 viruses, as well as structural data as an indirect way to predict potential footprints of soluble scFv1 and scFv3 on the SAT2 capsid. Comparison of the P1 amino acid sequences of the SAT2 viruses indicated that the variation observed was confined to hypervariable loops of which five were identified in VP1 and one each in VP3 and VP2. The structural orientation of these variable loops on the capsid correlates strongly with previously identified neutralizing epitopes of type A (Baxt *et al.*, 1989; Thomas *et al.*, 1988) and O (Barnett *et al.*, 1989; Crowther *et al.*, 1993a; Kitson

et al., 1990; Parry *et al.*, 1989) viruses. The hypervariable regions may act as topographical regions for binding of soluble scFv1 or scFv3 on the virus capsid and the variation observed in reactivity of these soluble scFvs to the panel of SAT2 virus might be a reflection of the amino acid variation in these regions. For example, soluble scFv1 and scFv3 consistently bind to ZIM/7/83 with a high binding profile but to ZIM/5/83 with a low binding profile. The P1 regions of these two ancestral SAT2 viruses vary with less than 1% at an amino acid level. The exact positions of the seven varying residues have been mapped to a modelled structure of the ZIM/7/83 capsid. Three of these varying residues are substituted by amino acids similar in character and four of the changes are surface exposed. Thus, it can be suggested that scFv1 or 3 could be binding to a region on the capsid that overlaps with one of the four surface exposed variable residues of ZIM/7/83 and ZIM/5/83. By including binding profiles and P1 sequences of more viruses the topographical regions for binding of soluble scFv1 or scFv3 on the virus capsid will become more apparent. Direct proof of the role of the above-mentioned regions in binding scFv1 and scFv3 will come from competition assays using appropriately designed overlapping synthetic peptides.

The sensible design of vaccines, in which potent and broadly neutralizing antibodies are elicited, is fast becoming a reliable way to address the vast genetic and antigenic diversity seen in certain pathogens, *e.g.* HIV (Burton *et al.*, 2005; Walker and Burton 2008). Towards this end, epitope-based vaccine technology has become very appealing in recent years (Purcell *et al.*, 2007; Sette and Fike, 2003; Shao *et al.*, 2011). Infectious cDNA technology (Blignaut *et al.*, 2011; Fowler *et al.*, 2008; Rieder *et al.*, 1993), used in the development of chimeric FMDV vaccines, may also be used to display identified epitopes for use in epitope-based vaccines. Use of the currently identified epitope, and possibly more epitopes identified

in the future, as a first step towards a SAT2-specific epitope-based vaccine is therefore appealing.

In conclusion, our study revealed that the antigenic site identified at the C-terminal end of the G-H loop of the VP1 protein is not only neutralizing but is specific to the SAT2 FMDV serotype. This conclusion is supported by the finding that soluble scFv2 binds to SAT2 serotype viruses but not to representative SAT1 and SAT3 viruses. Furthermore, the finding that the three SAT2-specific FMDV soluble scFvs bind to a panel of SAT2 viruses with different binding profiles indicates that these scFvs have the potential to be used in a scFv-based ELISA for rapid matching of vaccines with outbreak isolates. The availability of such a diagnostic test will greatly aid in vaccine matching procedures. However, more than the three SAT2-specific scFvs described here will be needed for an effective assay.

Acknowledgements

The authors would like to thank Mrs J Frischmuth (Biotechnology Division of the National Bioproducts Unit in Kwa-Zulu Natal, South Africa) for assistance with the purification of the soluble scFvs and the following people from the Onderstepoort Veterinary Institute; Dr D.H. du Plessis for kindly providing the Nkuku[®] phage display library, Ms C. Mashau and Dr J. Fershen, from the New Generation Vaccines Programme and staff of the Transboundary Animal Disease Programme. Drs Mans and Koekemoer from the OVI for critical reading of the manuscript. Funding was provided by the South African Department of Science and Technology (DST) and SA-UK collaborative initiative.

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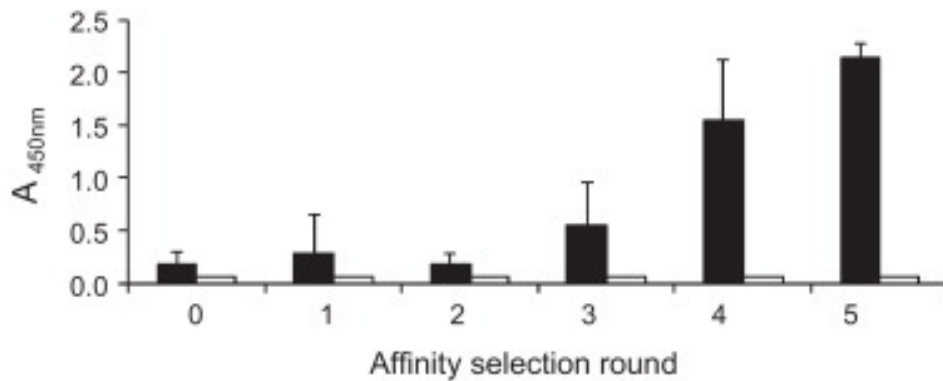


Fig. 1. Enrichment of phage-displayed scFvs specific for ZIM/7/83. Phage-displayed scFvs were selected by panning on immobilized ZIM/7/83 and enrichment of virus-specific phage-displayed scFvs (black bars) was monitored by a polyclonal phage ELISA of the outputs of five consecutive selection rounds (indicated as 1-5). An aliquot of the Nkuku[®] phage display library prior to panning was included as a non-enriched control (indicated as 0) and 2% (w/v) casein was included as a negative control (white bars). The data are means \pm SD of three experiments.

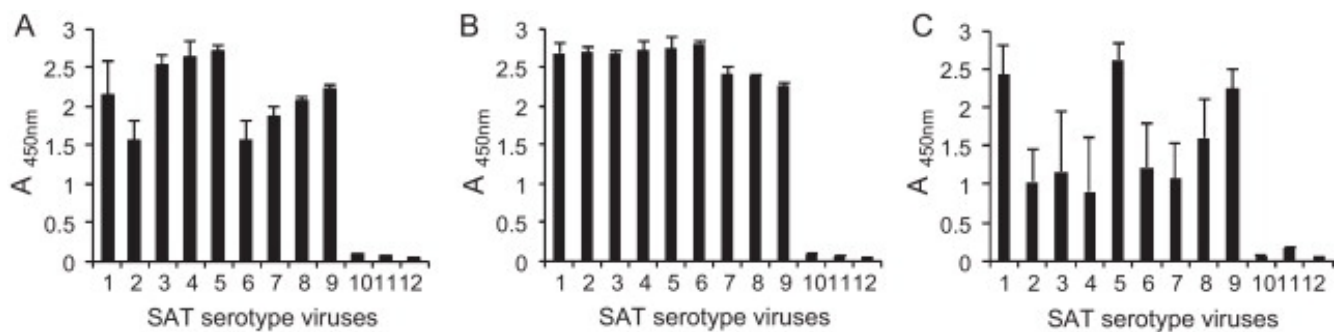


Fig. 2. Binding of soluble scFvs to representative SAT serotype viruses. An indirect ELISA was performed to determine binding of soluble scFv1 (A), scFv2 (B) and scFv3 (C) to purified 146S virions of the SAT2 serotype viruses ZIM/7/83 (1), ZIM/5/83 (2), ZIM/13/01 (3), ZIM/8/94 (4), ZIM/7/89 (5), ZIM/4/97 (6), ZIM/44/97 (7), ZIM/5/02 (8), ZIM/2/88 (9), as well as the SAT1 virus KNP/196/91 (10) and the SAT3 virus KNP/10/90 (11). As a negative control, 2% (w/v) casein (12) was included in the assays. The data are means \pm SD of two independent experiments.

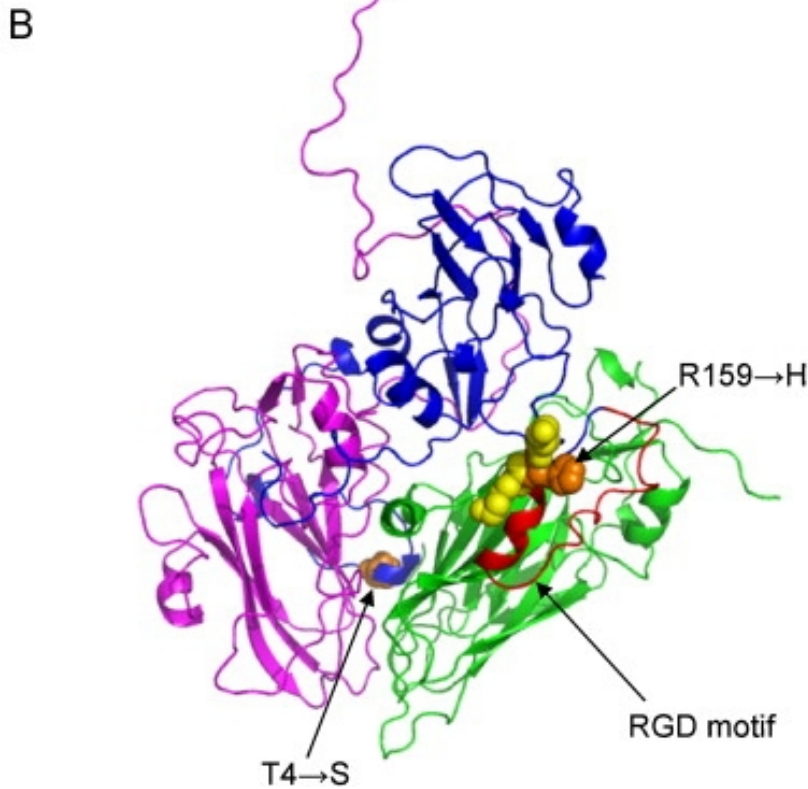
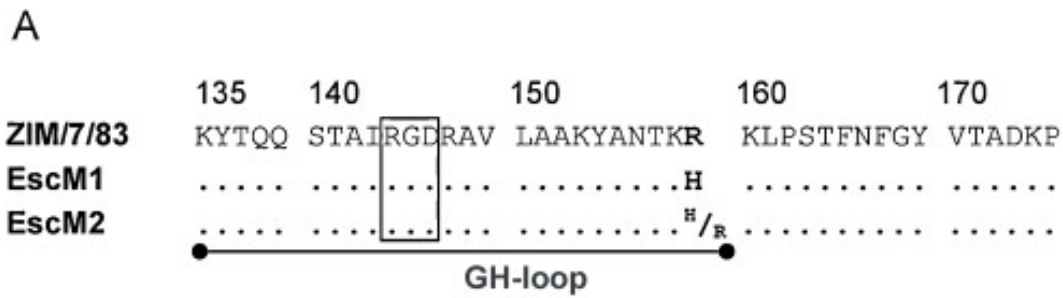


Fig. 3. Amino acid alignment and structural depiction of the amino acid substitutions observed for the ZIM/7/83 virus neutralization escape mutants. (A) The alignment of the VP1 G-H loop of the parental ZIM/7/83 virus and the two virus neutralization escape mutants, EscM1 and EscM2, indicating the Arg (R) to His (H) change at position 159 in bold. The RGD motif is blocked. (B) A 3D structure of the ZIM/7/83 biological protomer showing the position of the T4→S and R159→H changes in VP1 (orange spheres). Positively charged amino acids, situated either side of the R159→H change, are indicated as yellow spheres and the G-H loop, with the RGD motif indicated, is shown in red. The VP1 protein is indicated in blue, VP2 in green and VP3 in magenta.

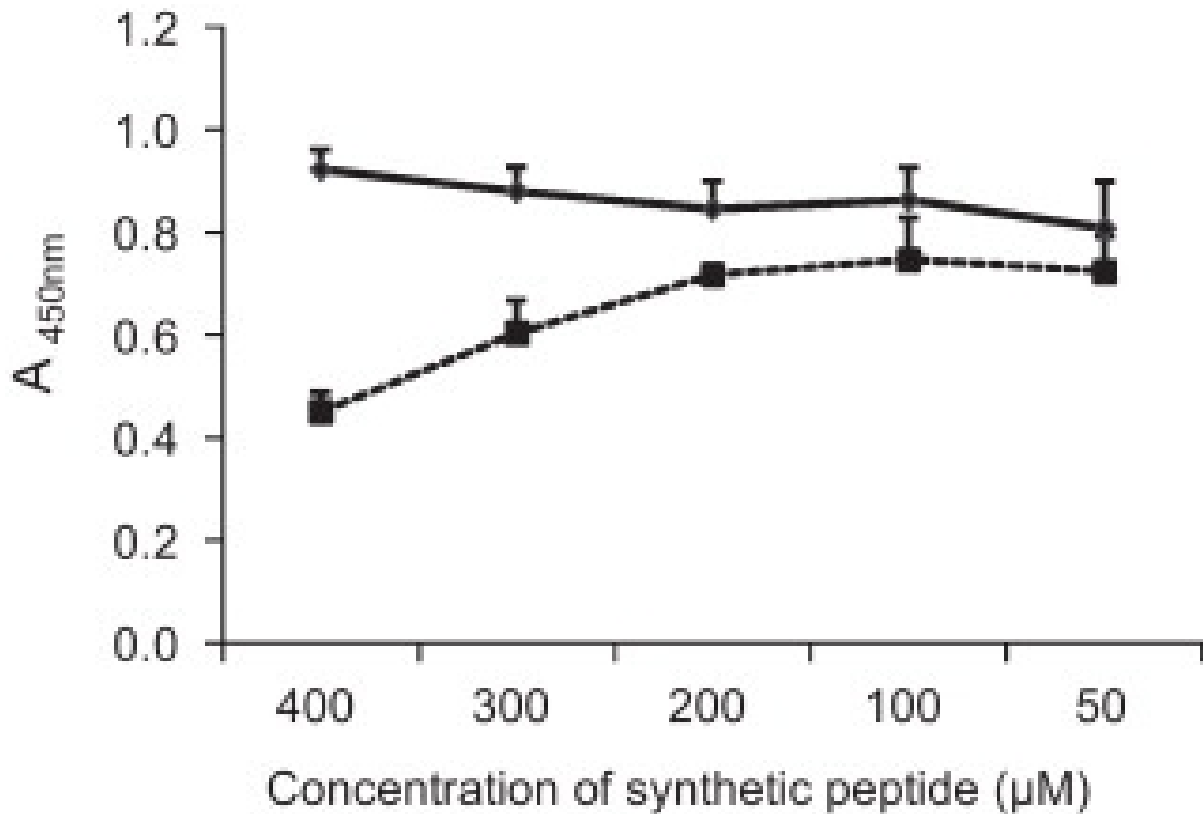


Fig. 4. Binding of soluble scFv2 to immobilized ZIM/7/83 in a competitive blocking ELISA in the presence of the EpiR synthetic peptide (■). A competitive blocking ELISA was performed to determine whether the synthetic peptide, EpiR, inhibits binding of soluble scFv2 to immobilized ZIM/7/83. Various concentrations (400-50 µM) of the synthetic peptide EpiR (■) were tested. A non-inhibition control (♦), in which the peptide was replaced with 1 × PBS and mixed with the soluble scFv2, was included. The data are means ± SD of three independent experiments.

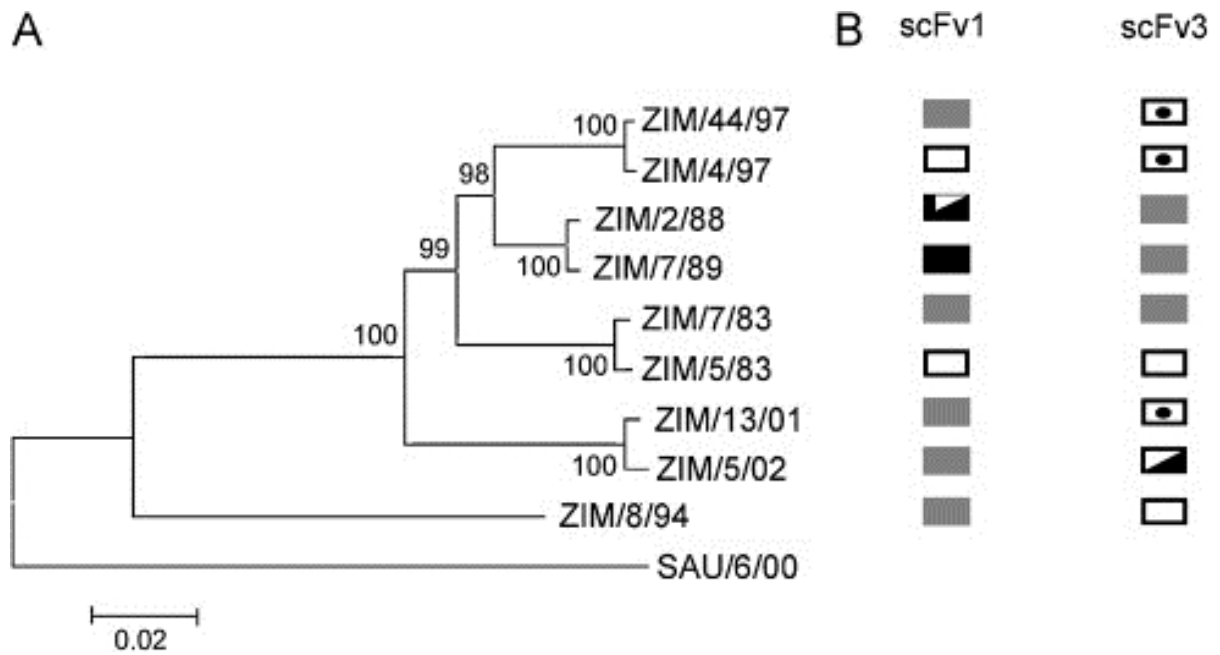


Fig. 5. Binding of soluble scFv1 and scFv3 to SAT2 FMD viruses. (A) Phylogenetic tree depicting the gene relationship of the P1 region of the SAT2 viruses included in this study. The tree was constructed using a neighbor-joining algorithm and node reliability is supported by 1000 bootstrap replications. The phylogenetic tree was rooted using the SAT2 isolate SAU/6/00 as an outgroup for the P1 phylogenies. (B) Binding of soluble scFv1 and scFv3 to a panel of SAT2 viruses as compared to ZIM/7/83. □ <25%, ◻ 25-45%, ▤ 45-65%, ▥ 65-85%, ▧ 85-100%, ▨ >100% binding.