Arch Virol (2006) 151: 37–54
DOI 10.1007/s00705-005-0623-9

Archives of Virology
Printed in Austria

Characterization of epitopes for neutralizing monoclonal antibodies to classical swine fever virus E2 and E^{rns} using phage-displayed random peptide library

F. Zhang[1,2], **M. Yu**[1], **E. Weiland**[3], **C. Morrissy**[1], **N. Zhang**[2], **H. Westbury**[1], and **L.-F. Wang**[1]

[1] CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Australia
[2] Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory, Kunming, P.R. China
[3] Friedrich-Loeffler-Institut, Institute for Immunology, Tübingen, Germany

Received April 27, 2005; accepted July 6, 2005
Published online August 26, 2005

Summary. Infection of cells with classical swine fever virus (CSFV) is mediated by the interaction of envelope glycoproteins E2 and E^{rns} with receptor molecules on the cell surface. These proteins are also the major antigens for eliciting neutralizing antibodies and conferring protective immunity. Here we report the identification of multiple neutralizing epitopes on these proteins by screening a phage-displayed random peptide library with CSFV-specific neutralizing monoclonal antibodies. Two different E2-specific neutralizing mAbs (a18 and 24/10) were found to bind to a common motif SPTxL, which is similar to the sequence SPTTL of the E2 protein (aa 289–293), indicating that this is likely to be an immunodominant epitope. Similarly, an immunodominant epitope corresponding to the sequence DKN of E^{rns} (aa 117–119) was identified for two independent E^{rns}-specific neutralizing antibodies, b4-22 and 24/16, respectively. Another binding motif, CxNNxTC, was identified for mAb 24/16, but not for b4-22. Sequencing analysis of the genes coding for the light chain of these mAbs was conducted to ensure that all mAbs were derived from different hybridomas, rather than from different subclones of a common parent line. Inhibition studies using immunofluorescent antibody assay and virus neutralization test demonstrated that the mimotope peptides truly mimicked the antibody binding determinants on the viral proteins. The detailed mapping data for these neutralizing epitopes will be useful for development of improved diagnostic tests and perhaps a peptide-based vaccine for this important swine disease.

Introduction

Classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and border disease virus (BDV) are members of the genus *Pestivirus* in the family *Flaviviridae* [7, 16]. Currently there are four recognized species included in this genus, i.e., BVDV-1, BVDV-2, CSFV and BDV, plus one tentative fifth species represented by an isolate (H138) from a giraffe [3, 28]. Recently, another atypical pestivirus was isolated from a batch of fetal calf serum collected in Brazil which may represent yet another novel pestivirus species [24]. In addition, four different genotypes of BDV have been identified [2]. Pestiviruses are structurally, antigenically, and genetically related. BVDV and BDV can infect ruminants and pigs whereas CSFV infections are restricted to pigs [4]. CSFV is the causative agent for classical swine fever, a severe disease that results in high morbidity and mortality of infected swine [18]. The 12.5 kb CSFV genome consists of a single-stranded RNA of positive polarity with one long open reading frame coding for a polyprotein of approximately 4,000 amino acids (aa), which is processed into four structural proteins (C, E^{rns}, E1 and E2) and several non-structural proteins by virus-encoded and cellular proteases [19, 22, 27]

E2 and E^{rns} are major targets for antibody-mediated neutralization [13, 29–31, 34]. Monoclonal antibodies directed against E^{rns} and E2 can be used to discriminate between different pestivirus species as well as between strains of the same species [6, 12]. E2 is the major envelope glycoprotein exposed on the outer surface of the virion. E^{rns} lacks a typical membrane anchor and is secreted in considerable amounts from infected cells [23]. A highly unusual feature of the E^{rns} protein is its RNase activity, first identified by characteristic sequence motifs and then proven by enzymatic tests with the purified protein [8, 25]. In infected animals significant levels of antibodies are raised against E^{rns} and E2 [13, 35]. Entry of pestiviruses into cells is mediated by the interaction of envelope proteins E^{rns} and E2 with cellular receptor molecules.

Mapping antigenic domains on E2 of CSFV has been extensively reported in the literature [15, 29–31, 36, 39]. The protein contains four antigenic domains, A to D, located within the N-terminal half of the protein [31]. We previously reported the fine mapping of a linear epitope at the C-terminal region of E2 that is highly conserved among different pestiviruses [39]. Lin et al. [15] reported the identification of a 9-aa epitope that is conserved in all CSFV strains, but absent in BVDV and BDV. In comparison, much less is known about the location of antigenic domains on E^{rns}. It has been demonstrated that virus-neutralizing monoclonal antibodies (mAbs) were able to inhibit the RNase activity of E^{rns}, although the binding sites for these mAbs have not been determined [37]. Using a bacterial display gene-fragment library, Christmann et al. [5] found that two E^{rns}-specific mAbs recognized an overlapping region located between aa 113 to 124 of E^{rns}.

Phage-displayed random peptide libraries have proven to be a powerful technique to study protein–protein interaction, to isolate peptide mimics for a variety of biological molecules and to map antibody binding sites or epitopes

[1, 11, 20, 32]. In this study, we aimed to map mAb-defined epitopes of the E2 and E^{rns} proteins using the phage display technology. We were particularly interested in epitopes recognized by either known virus-neutralizing mAbs or those mAbs that demonstrated some involvement in virus neutralization. More specifically, we selected two E2-specific mAbs demonstrating strong neutralizing activities and three E^{rns}-specific mAbs which showed some activity in virus neutralization. Detailed epitope studies for those mAbs will be important for our understanding of the virus infection process and for development of better diagnostic and vaccine reagents to combat this significant swine disease.

Materials and methods

Viruses and cells

Classical swine fever virus strain Weybridge (CSFV-Weybridge) was used in this study, and propagated in pig kidney cells (PK-15). PK-15 cells were established in roller bottles at a seeding rate of 30×10^6 cells/roller. The cells were infected with CSFV-Weybridge at a multiplicity of 0.1 $TCID_{50}$/cell and overlaid with EMEM containing 2% FCS. The culture fluid was harvested at 48 h.p.i. by freezing and thawing twice. After clarifying the supernatant at 10,500 rpm for 10 minutes in a Beckman JA-14 rotor, the virus was concentrated 45 times by precipitation with 7% polyethylene glycerol (PEG MW 20,000–30,000) and stirred overnight at 4°C. The material was pelleted by centrifugation at 10,500 rpm for 30 minutes in a Beckman JA-14 rotor. The pellet was resuspended in 8–10 ml of TNE buffer (0.1 M Tris-HCl, 0.01 M NaCl, 0.001 M EDTA, pH7.4). The suspension was layered onto a 20–40% sucrose gradient in TNE buffer and centrifuged for 18 h at 26,000 rpm in a Beckman SW-28 rotor at 4°C. Approximately 1 ml fractions were collected and assayed for infectivity by peroxidase-linked assay [26]. Peak fractions having a titer around 10^6–10^7 $TCID_{50}\,ml^{-1}$ were pooled, diluted with TNE and centrifuged for 2 h at 35,000 rpm in a Beckman 55.2 Ti rotor. The resulting pellet was resuspended in 1 ml of PBS at a concentration of 10^7 $TCID_{50}\,ml^{-1}$. Aliquots of 200 µl were stored at –80°C until further use.

Antibodies and protein G-coated beads

Hybridomas for E2-specific mAbs a18 and 24/10 and E^{rns}-specific mAbs 1B5, b4-22 and 24/16 were derived from spleens of two mice (No. 1 and No. 4) that had been immunized with CSFV strain Alfort Tübingen before fusion with SP2/0 myeloma cells in the presence of polyethylene glycol [12]. Mouse anti-M13 antibody conjugated with horseradish peroxidase (HRP) was purchased from Amersham Biosciences (Sydney, Australia). Sheep anti-mouse IgG conjugated with alkaline phosphatase (AP) was purchased from Chemicon (Melbourne, Australia). Goat anti-mouse IgG conjugated with FITC was obtained from Silenus (Melbourne, Australia). Protein G-coated magnetic beads were purchased from Dynal Pty Ltd. (Melbourne, Australia).

Panning of phage-displayed random peptide library

The 12mer-random peptide library was purchased from New England Biolabs (Beverly, USA). The peptides are displayed on the surface of M13 phage fused to the N-terminus of the pIII protein via a 4-aa spacer, GGGS. The library had a complexity of 2×10^9 and was amplified once in our laboratory to give a final titer of 3×10^{12} plaque-forming units (pfu) ml^{-1}.

Before panning, DNA inserts from 20 randomly picked individual phage clones were sequenced to make sure that all codons for the 20 aa were represented in the random sequences close to their expected frequencies based on the genetic code.

Library screening was conducted by panning in solution at room temperature using a protocol developed by combining the method provided with the kit and that described previously in [21, 38]. For each panning, 50 µl of hybridoma supernatant was incubated with 30 µl of phage solution containing approximately 0.9×10^{11} pfu in a total volume of 250 µl of TBS (10 mM Tris-HCl, 150 mM NaCl, pH7.5) containing 2% skim milk for 30 min. The antibody-phage complex was then captured onto protein G-coated magnetic beads by incubation for 30 min. After washing ten times with PBST buffer (PBS plus 0.05%Tween20, pH 7.4) and two times with PBS buffer (no Tween 20), bound phage was eluted by incubating with 100 µl of elution buffer (1 mg/ml BSA, 0.2 M glycine, pH 2.2) for 10 min. The eluate was removed from the beads and neutralized with 15 µl of 1 M Tris-HCl (pH9.5), and immediately used to infect *E. coli.* strain ER2738 for phage titration and amplification following protocols provided with the kit. Three rounds of panning were conducted using identical conditions.

Phage ELISA

At the end of third round panning, different dilutions were plated to obtain individual phage clones for amplification. Each clone was inoculated into 400 µl of LB broth containing 20 µg/ml of tetracycline in a QSP 96-micro-titertube (Quantum Scientific, Brisbane, Australia), and *E. coli* strain ER2738 at the logarithmic phase of growth, and tubes were incubated overnight at 37°C with vigorous shaking. Bacterial cells were removed by centrifugation at 2000 × g for 10 min and the culture supernatant containing phage particles was transferred to a clean tube. Phage ELISA was conducted using our previously published method [38]. For each ELISA, two plates were used: one coated with 50 µl of the target mAb and the other with 50 µl of a control mAb which does not react with CSFV. The target and control plates were always processed in parallel at the same time under identical conditions. The binding specificity of each phage clone was determined by the ratio of readings from the target plate over those from the control plates. A ratio of at least 2 to 3 is set as the minimum for defining a specific binding.

DNA sequencing of selected phage inserts

Sequence determination of the phage DNA insert coding for the 12-aa peptide sequence was achieved by direct PCR amplification of phage plaque DNA after boiling using the following vector-specific primers: FUSE-U, 5′-GCA AGC TGA TAA ACC GAT AC and FUSE-D, 5′-CCA TGT ACC GTA ACA CTG AG. The amplified PCR fragments were sequenced using the FUSE-U primer and the Big-Dye Dideoxy Termination Cycle Sequencing Kit (Applied Biosystem, Foster City, USA), run on an ABI 377 automatic sequencer (Applied Biosystems). Sequence management and analysis were done using the Clone Manager 7 suite of programs (S&E Software, Durham, USA).

SDS-PAGE and Western blot analysis

Phage proteins, containing mimotope peptides fused to the pIII coat protein, were analyzed by SDS-PAGE under reducing conditions as described [14], followed either by direct staining with Commassie blue or by Western blotting. The blots were then probed with target monoclonal antibodies, followed by detection using AP-conjugated anti-mouse secondary antibodies and the BCIP/NBT substrates (Promega, Wisconsin, USA) as previously described [33].

Inhibition studies by immunofluorescent antibody assay (IFAA)

Approximately 1×10^6 PK-15 cells were mixed with 100–200 $TCID_{50}$ of CSFV- Weybridge in 2 ml of growth medium and seeded onto cover slips or chamber slides (Nalge Nunc International, USA), followed by incubation at 37°C in a humidified CO_2incubator for 24 to 48 hours. Slides were then fixed in acetone, air dried and stored at –20°C if not processed immediately. Different concentrations of phage solution were mixed with mAb and incubated at 37°C for 1 h, before the mixture was added to the infected cells on the slide. After incubation at 37°C for 1 hour in a CO_2 incubator, slides were washed three times with PBS. Anti-mouse FITC conjugate (100 µl at 1:100 dilution) was added to the slide and the incubation-washing cycle was repeated as above. Examination and photography was conducted using a Leica Polyvar fluorescence microscope. To reduce variation, positive sample, negative and blank controls were all processed in parallel on the same slide.

Inhibition of virus neutralization by phage-displayed mimotopes

Serial dilutions of mAbs were carried out in growth medium and 50 µl of diluted mAb was mixed in wells of a 96-well plate well (Nunclon^{TM} Delta Surface) with 50 µl of phage solution containing approximately 10^{10} pfu phage particles in the same growth medium. Negative phage, no phage, and cells only controls were included on each plate. The plate was incubated at 37°C for 1 hr in a humidified CO_2 incubator before the addition of viruses at 1.5 to 2.5 $\log_{10}TCID_{50}$ per well with the exception of the cells only control wells. After incubation for a further hour under the same conditions, 100 µl of freshly prepared PK-15 cells was added to each well at 2×10^5 cells/well. The incubation was continued for four days before further processing. Growth medium was taken out by aspiration, and the wells were washed once with PBS (200 µl per well). The cells were fixed for 20 min with 10% formaldehyde in PBS containing 0.1% NP40 followed by three washings with PBS. To each well, 50 µl of primary antibody diluted 1:300 in PBS/0.1% skim milk was added and the plate was incubated at 37°C for one hour followed by the washings. The same incubation-washing cycle was then carried out with the HRP-goat anti-mouse antibody (at 1:1000 dilution) before colour development with the addition of 100 µl of freshly prepared substrate solution, 2 mM amino ethylcarbazole in N, N-Dimethyl formamide.

Determination of cDNA sequences of mouse IgG light chain variable regions

Isolation of total RNA from hybridoma cells ($3–4 \times 10^7$) was performed following the protocol provided with the Qiagen RNeasy Midi Kit (Clifton Hill, Australia). Quantities of purified RNA extracted were measured by spectrophotometry using the GeneQuant II RNA/DNA Calculator (Pharmacia, USA). Synthesis of first strand cDNA was carried out using the Qiagen Omniscript^{TM} RT kit with $pd(N)_6$ random primer. PCR amplification was carried out using the PCR Supermix (Gibco BRL) with murine light chain variable region 5′ primers and murine k light chain 3′ primer (see Table 1). Sequencing of the PCR product was performed as described above using the reverse primer (mIgGLcVr) derived from a constant region of the light chain, 5′-GCA CCT CCA GAT GTT AAC TGC-3′.

Results

Identification and sequence analysis of mAb-selected mimotope peptides

A total of five mAbs (a18 and 24/10 for E2, b4-22, 24/16 and 1B5 for E^{rns}) were chosen for this study based on the following criteria: they are known to neutralize CSFV infection *in vitro*; their target antigen (E2 or E^{rns}) has been

Table 1. List of primers used to amplify cDNA fragments coding for the variable regions of mouse IgG light chains

Primer	Sequence
	Murine light chain 5′ primers*
LC1	5′-CCA-GTT-CCG-AGC-TCG-TTG-TGA-CTC-AGG-AAT-CT-3′
LC2	5′-CCA-GTT-CCG-AGC-TCG-TGT-TGA-CGC-AGC-CGC-CC-3′
LC3	5′-CCA-GTT-CCG-AGC-TCG-TGC-TCA-CCC-AGT-CTC-CA-3′
LC4	5′-CCA-GTT-CCG-AGC-TCC-AGA-TGA-CCC-AGT-CTC-CA-3′
LC5	5′-CCA-GAT-GTG-AGC-TCG-TGA-TGA-CCC-AGA-CTC-CA-3′
LC6	5′-CCA-GAT-GTG-AGC-TCG-TCA-TGA-CCC-AGT-CTC-CA-3′
LC7	5′-CCA-GTT-CCG-AGC-TCG-TGA-TGA-CAC-AGT-CTC-CA-3′
	Murine k light chain 3′ primer
MLK-3	5′-CCA-GTT-CCG-AGC-TCG-TGA-TGA-CAC-AGT-CTC-CA-3′

*The variable residues in the primer sequences are highlighted

identified; and most of them react with their target antigens under denaturing conditions in Western blotting. The production and characterization of these mAbs has previously been reported in several publications [12, 34, 35].

Following each round of panning, enrichment of specific phage clones binding to the target mAb was confirmed by monitoring the phage recovery ratio (data not shown). At the end of the third round, 96 clones were randomly picked for each target antibody and propagated for phage production individually. Specific binding to their target antibodies was determined by phage ELISA, and positive clones were defined as those giving at least two-fold higher OD readings to target antibody over control antibody. Phage clones showing the highest OD readings were sequenced to determine the phage-encoded mimotope peptide sequence. The results are summarized in Table 2.

Selection by the two E2-specific mAbs identified a common binding motif from the alignment of all the deduced mimotope sequences. For mAb a18, 17 out of the 20 clones sequenced contained peptide sequences which had significant matches to the consensus motif SPTxL (x represents non-conserved aa residues). A very similar peptide sequence to this motif, SPTTL, could be identified in the E2 protein (aa 289–293) of all CSFV strains sequenced to date, but not in the E2 protein of other pestiviruses (Fig. 1). Two observations are worth noting here: first, none of the mimotope sequences had an exact match to the native epitope sequence, i.e., the x residue at the 4th aa was never a T (see Table 2); second, none of the 20 clones had identical aa sequences suggesting that each represented an independent phage clone in the original library. For mAb 24/10, a total of 22 clones were sequenced, and all but one contained the same SPTxL motif. The majority of the clones also had an R residue next to the L, thus making up the consensus motif SPTxLR, which matched the SPTTLR sequence of the E2 protein (see Fig. 1).

Table 2. Peptide sequences consensus motifs derived from mAb-selected phage-displayed mimotopes

Antibody		Phage clone	Peptide sequence*	Frequency
E2	**a18**	18/pg05	MCSPTALRCQAH	1
		18/pg14	YMSPTGRVMTTQ	1
		18/pg15	ELSPTNLRRSFV	1
		18/pg16	KSDYDSPTQTFAL	1
		18/pg24	GANYKSPTGFLRG	1
		18/pg51	QPPQWLWPQKEV	1
		18/pg54	SPTFPTLTSSAL	1
		18/pg55	ISPTQLYAQVRA	1
		18/pg57	DMQYRSPTGLST	1
		18/pg61	HDSPTIRYVEAY	1
		18/pg62	YTSPTFPNFFLS	1
		18/pg72	IYTSPTFPPTAH	1
		18/pg73	ATISPTGLVYQT	1
		18/pg75	VVSPTGLSKHAP	1
		18/pg76	EWSPTKLLWFHS	1
		18/pg79	LQSPTGLTNYFL	1
		18/pg80	LPTMLSPTGLSR	1
		18/pg85	QESTRLTYAVWM	1
		18/pg86	FGTSPTGLKLAP	1
		18/pg88	CLAAPTGLKLAP	1
			SPTxL	
	24/10	24/pg01	FLPTSPTGLRIE	1
		24/pg02	HLSPTMLRWEQF	1
		24/pg03	HELSPTGLKTSW	1
		24/pg07	YTLPSPTRLWMQ	2
		24/pg10	GFKETSPTGLRW	1
		24/pg11	TLRVSPTQEVMA	1
		24/pg14	FGINCSPTYLRC	1
		24/pg19	MCSPTALRCQAH	6
		24/pg21	HTSPTGLRFQVQ	1
		24/pg31	HYSPTGLRWTTM	1
		24/pg32	TAQWLSRTQMDS	1
		24/pg33	VPLSPTMEFMRK	1
		24/pg38	TALSPTGLRLQF	1
		24/pg51	NAFQLSPTRLLM	1
		24/pg56	AYSPTGLVWHPG	1
		24/pg81	WWSPTMLTQSKE	1
			SPTxLR	
E^{rns}	**b4-22**	b4/pg50	SVLFDKNRGQEI	17
		b4/pg52	QTCCDKNQGIFM	2
		b4/pg73	VLDWKTRGKDLP	3
			VLxDKNRG	
	24/16	16/pg23	AMGYNKNNDSLL	1
		16/pg24	YYNKNNASYHSS	1
		16/pg31	TWWDKNSATVAW	1
		16/pg38	HSWDKNSSIWWP	1
		16/pg39	QPYDKNRGPTLN	1
		16/pg50	SLYDKNRGQFGS	1
		16/pg72	SFFDKNSAHWSP	1
		16/pg77	DLPRWPGWYKNR	1
			YDKN	
		16/pg60	KACSNNSTCFNP	1
		16/pg74	QACQNNDTCPRL	15
		16/pg84	TCRNNDTCRAPD	1
			CxNNxTC	
	1B5	b5/pg15	YPYSMWKNLWPS	5
		b5/pg93	WIEEWMFIPVMS	1
		b5/pg21	ANSFWVNHPTIT	1
		b5/pg84	IPPFLYKNFWWP	1
			WxNxW	

*Consensus sequence motif (in bold) is provided at the bottom of each alignment. The following grouping of conserved residues was used in the alignment: (Y, F, W);(D, N, E, Q); (R, K)

		281	299
CSFV	**Alfort**	GVIECTAV	**SPTTLR** TEVVK
	Brescia	GVIECTAV	**SPTTLR** TEVVK
	Weybridge	GVIECTAV	**SPTTLR** TEVVK
	China-C	GVIECTAV	**SPTTLR** TEVVK
BVDV	**NADL**	GTVSCTSF	NMD**TL**A TTVVR
	SD1	GTVSCMLA	NRD**TL**D TAVVR
	Oregon	GTVSCMLA	NRD**TL**D TAVVR
BDV	**X818**	GRVECTTV	**S**KS**TL**A TEVVK
	BD78	GRIECTLA	NQD**TL**D TTVVR

a

		111	126
CSFV	**Alfort**	AVTCR	**YDKD** TDVNVVT
	Brescia	AVTCR	**YDKN** ADINVVT
	Weybridge	AVTCR	**YDKD** ADINVVT
	China-C	AVTCR	**YDKD** ADINVVT
BVDV	**NADL**	AVTCR	**YD**RA SDLNVVT
	SD1	AVTCR	**YDR**D SDLNVVT
	Oregon	AVTCR	**YDR**D SDLNVVT
BDV	**X818**	AVTCR	F**DK**E ADINIVT
	BD78	AVTCR	**YDK**E AELNIVT

b

Fig. 1. Comparison of pestivirus E2 and E^{rns} protein sequences covering the immunodominant epitopes identified in this study. **a** E2 sequence covering the SPTTLR motif. **b** E^{rns} sequences covering the YDKN motif. Identical residues within the motifs are highlighted in bold. The aa numbering for the first and last residue for each protein is indicated above the sequence. GenBank accession number for each virus strain (given in parenthesis): Alfort (X87939), Brescia (M31768), Weybridage (X71789, AY94006), China-C (Z46258), NADL (AJ133739), SD1 (M96751), Oregon (AF091605), X818 (AF037405), and BD78 (U18330)

Again, none of the residues at the 4^{th} position was a T. In contrast to the a18-selected clones, 6 of the 22 clones selected by 24/10, had the identical peptide sequence MCSPTALRCQAH. This same sequence was also picked up by mAb a18 (clone 18/pg05, Table 2), indicating that this mimotope was recognized by both mAbs. Another clone with mimotope sequence YTLPSPTRLWMQ appeared twice.

The consensus sequences derived from E^{rns}-specific mAbs were as follows. Out of the 22 clones sequenced for mAb b4-22, 17 of them were identical with the sequence of SVLFDKNRGQEI, 3 of VLDWKTRGKDLP and 2 of QTCCDKN QGIFM. The strong selection bias towards one clone made it difficult to deduce a truly representative consensus motif, but a putative motif of VLxDKNRG could still be identified. For mAb 24/16, a total of 25 clones were sequenced. Seven individual clones displayed a motif YDKN, which shares the aa sequence DKN

with the motif derived from mAb b4-22. The YDKN sequence was found in E^{rns} of different CSFV strains at aa 116–119 (Fig. 1b). It is interesting to note that the Y residue seems to be interchangeable with either a W or F residue in some mimotopes. When this happens, the immediate residue following the DKN is occupied mostly by an S residue, a conservative change to the T residue in the original YDKNT sequence as shown in Fig. 1. The change of Y to W (or F) seems to be compensated by the presence of the S residue on the other side of the binding site. In addition to the YDKN motif, the remaining 24/16-selected clones share a common motif CxNNxTC. However, 15 of the 17 clones were identical containing the sequence QACQNNDTCPRL. Extensive searching of CSFV E^{rns} sequences failed to identify the CxNNxTC motif, including Erns from CSFV strain Alfort from which the original mAb was derived [12]. Selection by mAb 1B5 only resulted in 8 positive clones as determined by phage ELISA. Five of them had an identical peptide sequence of YPYSMWKNLWPS and the other three all had different sequences (Table 2). When these four classes of sequences were aligned, the consensus motif WxNxW was identified. However, we were unable to identify this motif in the native protein sequence of E^{rns}. To confirm that the binding detected by phage ELISA is genuine, we conducted Western blot analysis of the mimotope-pIII fusion protein using mAb 1B5, and found that only peptides YPYSMWKNLWPS and IPPFLYKNFWWP were positive (see below) whereas two other peptides, WIEEWMFIPVMS and ANSFWVNHPTIT, failed to react in Western blots (data not show). Peptides from the two positive clones share the motif PxxxΦKNxW (Φ = W or Y), which again was not identifiable in the E^{rns} sequence.

Characterization of phage-displayed mimotopes by Western blot

To further investigate the binding specificity of the mimotopes, Western blot analysis was conducted on selected clones for each mAb. Mimotopes displayed on the phage surface in fusion with the pIII protein were purified from 30 ml of culture supernatant by PEG/NaCl precipitation (see Methods). For E^{rns} clones, each phage was tested against all three mAbs to examine specificity and cross reactivity. If the sequence motifs were correct, it was expected that b4-22 and 24/16 clones would cross react, but not the 1B5 clones. For E2 clones, mAb 1B5 was also included as a negative control in addition to a18 and 24/10. The results are summarized in Table 3. As predicted from the sequence motifs, 1B5-selected mimotopes reacted with mAb 1B5 only. On the other hand, mimotopes selected by b4-22 and 24/16 showed some cross reactivity between the two mAbs. But the two mAbs displayed different reactivity patterns towards different mimotopes. For example, mimotopes with the VLxDKNRG motif had stronger reactivity with mAb b4-22 than with 24/16 whereas mimotopes containing the CxNNxTC motif did not react with b4-22 at all (Table 3A).

The mimotopes selected by 24/10 seemed to be equally reactive with both mAbs. On the other hand, mimotopes selected by mAb a18 only had weak reactivity with mAb 24/10 (Table 3B). No mimotopes from the two groups showed

Table 3. Binding specificity of representative mimotopes analyzed by Western blot*

A.	Phage no.	Peptide sequence	mAb used for detection		
			b4-22	24/16	1B5
	b4/pg50	SVLFDKNRGQEI	S	S	-
	b4/pg52	QTCCDKNQGIFM	S	W	-
	b4/pg73	VLDWKTRGKDLP	S+	W-	-
	16/pg50	SLYDKNRGQFGS	S	S	-
	16/pg38	HSWDKNSSIWWP	W	S	-
	16/pg23	AMGYNKNNDSLL	W-	S+	-
	16/pg24	YYNKNNASYHSS	W	S	-
	16/pg60	KACSNNSTCFNP	-	S+	-
	16/pg74	QACQNNDTCPRL	-	S+	-
	16/pg84	TCRNNDTCRAPD	-	S+	-
	B5/pg13	YPYSMWKNLWPS	-	-	S
	B5/pg84	IPPFLYKNFWWP	-	-	S
	10C6/07	AQSYPDPPQTLK	-	-	-

B.	Phage no.	Peptide sequence	mAb used for detection	
			a18	24/10
	18/pg15	ELSPTNLRRSFV	S+	W
	18/pg51	QPPQWLWPQKEV	S	-
	18/pg55	ISPTQLYAQVRA	S	S
	18/pg72	IYTSPTFPPTAH	S	-
	18/pg85	QESTRLTYAVWM	S	W
	24/pg14	FGINCSPTYLRC	S+	S+
	24/pg19	MCSPTALRCQAH	S+	S+
	24/pg07	YTLPSPTRLWMQ	W	W
	24/pg56	AYSPTGLVWHPG	S+	S+
	24/pg81	WWSPTMLTQSKE	W	S
	10C6/07	AQSYPDPPQTLK	-	-

*Strength of reactivity on Western blots is indicated as follows: –, negative; W−, very weak; W, weak; S, strong; S+, very strong

any reactivity with mAb 1B5, indicating that the reactivity observed for each mimotope was specific for the E2 mAbs. The negative control phage 10C6/07, which was derived from panning using a mAb unrelated to CSFV, showed no

reactivity to any of the five mAbs used in this study, again confirming the specificity of the Western blot approach employed in this analysis.

Sequence comparison of mouse IgG light chain variable regions

From the mimotope sequence analysis presented above, it was evident that both motifs, SPTxL in E2 and DKN motif in E^{rns}, were recognized by two mAbs, respectively. In order to make sure that these mAbs were derived from genuine independent hybridoma cells, rather than from different subclones of the same parental hybridoma line, the gene fragments coding for the IgG light chain variable region of the four mAbs were isolated and sequenced. Isotyping of mAbs indicated that they all contain the kappa light chains (data not shown). So we used the kappa light chain 3′ primer and a set of seven light chain 5′ primers (see Table 1) to amplify the variable region. The deduced aa sequences are presented in Fig. 2. None of the light chain variable regions had identical sequences, indicating that they were all derived from independent hybridoma clones. It is interesting to note that the sequence variation between the pair of E^{rns}-specific mAbs was much greater than that between the pair of the E2-specific mAbs, especially in the CDR2 and CDR3 regions.

Inhibition of antigen binding and virus neutralization by phage-displayed mimotopes

It has been reported that not all peptides selected by panning from random peptide libraries are true mimics of viral epitopes that react with the antigen-binding sites

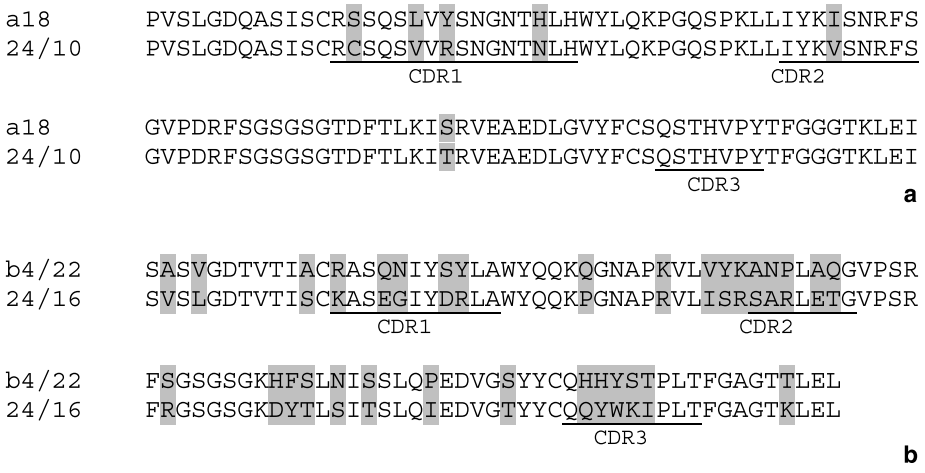

Fig. 2. Deduced amino acid sequences of IgG light chain variable regions. **a** Alignment of sequences derived from two E2-specific mAbs. **b** Alignment of sequences derived from two E^{rns}-specific mAbs. Variable residues between each pair of mAbs are highlighted by shading. The antigen complementarity determining regions (CDR) are assigned beneath the sequences according to the Kabat scheme [17]. These sequences have been deposited into the GenBank with the following accession numbers: AY942921 for mAb a18; AY942922 for 24/10; AY942923 for b4-22 and AY942924 for 24/16

(the paratopes) of an antibody. Some could be selected from binding to other parts of an antibody molecule which does not involve the direct interaction with the paratope [20, 32]. Inhibition studies were therefore conducted to examine

Table 4. Inhibition of viral antigen detection by phage mimotopes in IFAA

Phage clone	Mimotope sequence	Target mAb(s)	Phage (pfu)	mAb used in IFAA				
				a18	24/10	b4-22	24/16	1B5
Buffer	n/a	n/a	n/a	−	−	−	−	−
10C6/07	AQSYPDPPQTLK	10C6	1.50×10^{11}	−	−	−	−	−
b4/pg50	SVLFDKNRGQEI	b4-22	1.50×10^{11}	n/d	n/d	+	+	n/d
16/pg74	QACQNNDTCPRL	24/16	9.75×10^{10}	n/d	n/d	−	+	n/d
B5/pg15	YPYSMWKNLWPS	1B5	5.25×10^{11}	n/d	n/d	n/d	n/d	+
24/pg19* (18/pg05)	MCSPTALRCQAH	24/10 a18	1.75×10^{10}	+	+	n/d	n/d	n/d

n/a, not applicable; n/d, not determined; +, positive inhibition, i.e., significant reduction of immunofluorescence observed in IFAA; −, no inhibition

*The two phage mimotopes have identical sequence although selected by two different mAbs (see Table 2)

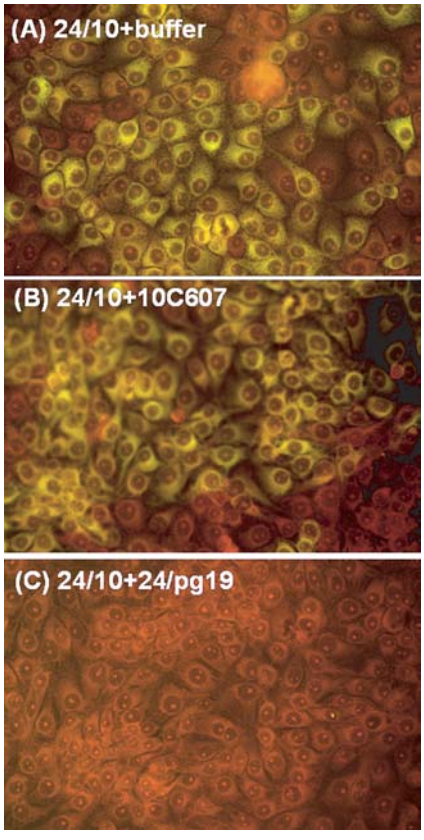

Fig. 3. Inhibition of mAb-virus binding by phage-displayed mimotope. PK-15 cells infected with CSFV-Weybridge were stained with mAb 24/10 in the absence of any phage (**A**) or in the presence of the control phage 10C6/07 (**B**) and 24/10-selected phage 24/pg19 (**C**) respectively. Photograph was taken at 400x magnification (using Evans blue as a counter stain)

Table 5. Inhibition of mAb 24/10-mediated virus neutralization by phage mimotope*

mAb dilution	Phage dilution**			Blank control
	24/pg19		10C6/07	
	1:10	1:20	1:10	
1:50				
1:100				
1:200				
1:400				
1:800				
1:1600				
1:3200				
1:6400				

*The non-shaded region indicates no virus growth, i.e., effective neutralization by mAb 24/10. The shaded region indicates the inhibition of mAb-mediated virus neutralization, i.e., virus was able to grow in the presence of the mAb at this particular dilution

**Phage stock solution was at 1×10^{12}pfu/ml

whether the mimotopes selected in this study were true mimic of the original epitopes. Inhibition of mAb binding to native viral antigens in CSFV infected cells was studied using an immunofluorescent antibody assay (IFAA). Four phage-displayed mimotopes representing each of the four consensus motifs previously identified (Table 2) were included in this study together with the negative control clone as used in the Western blot study. These four clones were selected based on their highest OD readings in phage ELISA from each group. As shown in Table 4, all of them were able to specifically inhibit the binding of their target mAbs in IFAA. Among the five mAbs used in this study, the E2-specific mAb 24/10 showed the strongest staining of CSFV-infected cells in IFAA. This strong staining was completely inhibited by phage mimotope 24/pg19, but not by the control phage 10C6/07 (Fig. 3), demonstrating that the inhibition is both effective and specific. This was further confirmed by virus neutralization tests and the results are shown in Table 5. Virus neutralization activity of mAb 24/10 was inhibited by mimotope 24/pg19, but not by the control phage 10C6/07.

Discussion

Detailed analysis of antibody-defined epitopes of viral proteins is important not only for understanding antibody-virus interaction, but also for development of better diagnostic tools and possible vaccine candidates. This is especially true when the antibodies in question are capable of neutralizing their target viruses. Among the various approaches available for mapping epitopes, phage-displayed random peptide libraries are emerging as one of the most favored methods of

choice due to their simplicity and the universality in the sense that the same library can be used for mapping an epitope for any antibody. Although there is no guarantee that this approach will work for every antibody, when it does work this technique has the added advantage of being able to differentiate subtle differences in binding patterns of similar antibodies, as demonstrated in this study.

The elucidation of the SPTxLR motif as the binding site of two E2-specific neutralizing mAbs is significant in several aspects. As shown in Fig. 1, the SPTTLR motif is highly conserved among CSFV strains, yet not present in other pestiviruses. So this is a CSFV-specific neutralizing epitope. This information will be of use in developing differentiation diagnosis and virus-specific subunit vaccines. The fact that two independent mAbs bind to the same site would suggest that this is an immunodominant site involved in virus neutralization. This notion is further supported by a parallel study conducted by Lin et al. [15] in which a deletion approach was used to define the 9-aa sequence TAVSPTTLR as the binding site of mAb WH303, which reacted with all 56 CSFV strains but none of the BVDV or BDV strains. Furthermore, the same study also reported that sera from CSFV-infected pigs were able to block the binding of WH303, indicating that the region containing the SPTTLR sequence is an immunodominant epitope in pigs as well. Finally, it is important to note that this immunodominant neutralizing epitope of the E2 protein is a linear epitope with the SPTTLR as the major binding motif, which will increase the chance of success for development of peptide-based vaccine.

Although the E2 antigen was the first to be identified as the major target of virus neutralization immune responses, it is now well established that E^{rns} is also involved in virus neutralization [9, 10, 34, 35]. For this reason, neutralizing mAbs against E^{rns} were also included in this study. Out of the three mAbs included in this study, two of them (b4-22 and 24/16) were shown to bind to a shared motif containing the DKN sequence. This was very similar to the E2 mAbs in that two independent mAbs seemed to bind to precisely the same site. While this work was being conducted, an independent study was carried out by Christmann et al. [5] to map the epitopes for these two mAbs using a totally different approach. They used an E^{rns}-derived gene-fragment library displayed on bacterial surface, and concluded that these mAbs bound to linear epitopes covering the common sequence YDKNTDVNV. So the DKN motif defined in this study was independently confirmed by a different approach. However, our study revealed further information for these two mAbs which could not have been obtained using other methods. Firstly, it was evident from the binding specificity analysis (Table 3) using mimotopes selected by the two mAbs that b4-22 and 24/16 were not identical in their binding patterns, indicating that they are two independent mAbs rather than two subclones of a common parental line. Secondly, an additional mimotope motif CxNNxTC was identified for mAb 24/16 which was not reactive to b4-22. The inhibition studies carried out using different mimotopes (Table 4) indicated that the CxNNxTC is specific to mAb 24/16 and that this mimotope is able to specifically compete with viral antigen for mAb-binding. The fact that we were unable to identify the CxNNxTC motif in the E^{rns} sequence would suggest that it might mimic a conformational contact point for mAb 24/16 which would not

have been identified using the gene-fragment library approach used by Christmann et al. [5].

The third E^{rns} mAb (1B5) failed to identify a strong consensus motif from mimotopes, and the only motif we could identify, WxNxW, did not match any part of the E^{rns} protein. Western blot analysis indicated that while 1B5 was able to react with a recombinant E^{rns} expressed in *E. coli*, its reactivity was not as strong as that exhibited by mAb b4-22 (data not shown). Our results would suggest that the motif WxNxW represents a conformational mimic of the 1B5 binding site since the binding of 1B5 with CSFV can be specifically inhibited by mimotopes containing this motif (Table 4).

The findings of a CxNNxTC motif for mAb 24/16 and WxNxW for mAb 1B5 demonstrate that antibody-binding sites on a protein antigen can not and should not be simply defined as linear or conformational based on their reactivity in Western blots. In fact, most if not all antibody-antigen interaction is expected to have some component of a conformational nature [20, 32]. While the information derived from these mimotopes has limited use in terms of defining binding sites in the primary protein sequence, it can be of use in drug development.

Binding of different mAbs to the same antigenic site is not unusual and is often used as an indicator of the immunodominant nature of the epitope. For example, we have previously demonstrated that two independent mAbs were able to bind to the same epitope on the VP7 protein of bluetongue viruses [33]. However, it is important to differentiate between two mAbs of separate origin versus those derived from two subclones of the same origin as this can occur due to the random selection nature of the hybridoma technology. Considering the antibody-antigen interaction, it is also of interest to compare the sequences of the antibody variable regions for those antibodies which bind to the same or similar epitope. For these reasons, we decided to sequence the light chain variable regions for the two pairs of mAbs which recognize a very similar motif in each case. The results led us to two conclusions. Firstly, it is obvious that all of the mAbs were truly independent from each other and represent separate hybridoma clones. Secondly, the light chains of the E^{rns}-specific pair were much more divergent than the E2-specific pair. This is consistent with the results obtained from the mimotope sequence analysis. The sequence homology derived from the two E2-specific mAbs were higher than those derived from the E^{rns}-specific pair. One particular mimotope, MCSPTALRCQAH, was identified by both E2 mAbs. On the other hand, the CxNNxTC motif identified by the 24/16 mAb was non-reactive with mAb b4-22, demonstrating the significant difference in binding patterns between the two E^{rns} mAbs.

In summary, using a phage-displayed random peptide library we have identified two epitope motifs for the CSFV E2 and E^{rns} proteins, respectively. These are important epitopes which could be used in the development of better diagnostic tools and potential vaccine or drug targets. Our results also demonstrated that although other approaches have been successfully used for mapping similar epitopes on CSFV proteins, the phage display method employed in this study is more able to reveal subtle differences in antibody-antigen interactions and in

defining contact points of a conformational nature which have been impossible or extremely difficult to discover by other approaches.

Acknowledgments

We wish to acknowledge Eric Hansson, Kaylene Selleck, Greer Meehan and Winsome Goff for technical assistance and Tony Pye for help with DNA sequencing. The work was supported by a grant from the Australian Council for International Agriculture Research (ACIAR), and F. Zhang is the recipient of a PhD Scholarship from ACIAR.

References

1. Adda CG, Anders RF, Tilley L, Foley M (2002) Random sequence libraries displayed on phage: identification of biologically important molecules. Comb Chem High Throughput Screen 5: 1–14
2. Arnal M, Fernandez-de-Luco D, Riba L, Maley M, Gilray J, Willoughby K, Vilcek S, Nettleton PF (2004) A novel pestivirus associated with deaths in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). J Gen Virol 85: 3653–3657
3. Becher P, Ramirez RA, Orlich M, Rosales SC, König M, Schweizer M, Stalder H, Schirrmeier H, Thiel HJ (2003) Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. Virology 311: 96–104
4. Carbrey EA, Stewart WC, Kresse JL, Snijder M (1976). Natural infection of pigs with bovine diarrhoea virus and its differential diagnosis from hog cholera. J Am Vet Med Assoc 169: 1217–1219
5. Christmann A, Wentzel A, Meyer C, Meyers G, Kolmar H (2001) Epitope mapping and affinity purification of monospecific antibodies by Escherichia coli cell surface display of gene-derived random peptide libraries. J Immunol Methods 257: 163–173
6. Edwards S, Moennig V, Wensvoort G (1991) The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet Microbiol 29: 101–108
7. Francki RIB, Fauquet CM, Knudson DL, Brown F (1991) Flaviviridae. Arch Virol [Suppl] 2: 223–233
8. Hulst MM, Himes G, Newbigin E, Moormann RJM (1994) Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. Virology 200: 558–565
9. Hulst MM, Moormann RJM (1997) Inhibition of pestivirus infection in cell culture by envelope proteins E^{rns} and E2 of classical swine fever virus: E^{rns} and E2 interact with different receptors. J Gen Virol 78: 2779–2787
10. Hulst MM, Van Gennip HGP, Moormann RJM (2000) Passage of classical swine fever virus in cultured swine kidney cells selects virus variants that bind to heparan sulfate due to a single amino acid change in envelope protein E^{rns}. J Virol 74: 9553–9561
11. Irving MB, Pan O, Scott JK (2001) Random-peptide libraries and antigen-fragment libraries for epitope mapping and the development of vaccines and diagnostics. Curr Opin Chem Biol 5: 314–324
12. Kosmidou A, Ahl R, Thiel HJ, Weiland E (1995) Differentiation of classical swine fever virus (CSFV) strains using monoclonal antibodies against structural glycoproteins. Vet Microbiol 47: 111–118
13. König M, Lengsfeld T, Pauly T, Stark R, Thiel HJ (1995) Classical swine fever virus: independent induction of protective immunity by two structural proteins. J Virol 69: 6479–6486

14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
15. Lin M, Lin F, Mallory M, Clavijo A (2000) Deletions of structural glycoprotein E2 of classical swine fever virus strain Alfort/187 resolve a linear epitope of monoclonal antibody WH303 and the minimal N-terminal domain essential for binding immunoglobulin G antibodies of a pig hyperimmune serum. J Virol 74: 11619–11625
16. Lindenbach BD, Rice CM (2001) *Flaviviridae*: the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 4th edn. Lippincott-Raven, Philadelphia, pp 991–1041
17. Messmer BT, Sullivan JJ, Chiorazzi N, Rodman TC, Thaler DS (1999) Two human neonatal IgM antibodies encoded by different variable-region genes bind the same linear peptide: evidence for a stereotyped repertoire of epitope recognition. J Immunol 162: 2184–2192
18. Moennig V, Plagemann J (1992) The pestiviruses. Adv Virus Res 41: 53–91
19. Meyers G, Thiel H-J (1996) Molecular characterization of pestiviruses. Adv Virus Res 47: 53–118
20. Rodi DJ, Makowski L (1999) Phage-display technology-finding a needle in a vast molecular haystack. Curr Opin Biotechnol 10: 87–93
21. Rowley MJ, Scealy M, Whisstock JC, Jois JA, Wijeyewickrema LC, Mackay IR (2000) Prediction of the immunodominant epitope of the pyruvate dehydrogenase complex E2 in primary biliary cirrhosis using phage display. J Immunol 164: 3413–3419
22. Rümenapf T, Unger G, Stark R, Thiel HJ (1991) Molecualr characterization of hog cholera virus. Arch Virol [Suppl] 3: 7–18
23. Rümenapf T, Unger G, Strauss JH, Thiel HJ (1993) Processing of the envelope glycoproteins of pestiviruses. J Virol 67: 3288–3294
24. Schirrmeier H, Strebelow G, Depner K, Hoffmann G, Beer M (2004) Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. J Gen Virol 85: 3647–3652
25. Schneider R, Unger G, Stark R, Schneider-Scherzer E, Thiel HJ (1993) Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science 261: 1169–1171
26. Terpstra C, Bloemraad M, Gielkens AJL (1984) The neutralizing peroxidase linked assay for detection of antibody against swine fever virus. Vet Microbiol 9: 113–120
27. Thiel HJ, Stark R, Weiland E, Rumenapf T, Meyers G (1991) Hog cholera virus: molecular composition of virions from a pestivirus. J Virol 65: 4705–4712
28. van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (2000) Virus taxonomy. Classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego
29. Van Rijn PA, van Gennip RGP, de Meijer EJ, Moormann RJM (1992) A preliminary map of epitopes on envelope glycoprotein E1 of HCV strain Brescia. Vet Microbiol 33: 221–230
30. Van Rijn PA, van Gennip HGP, de Meijer EJ, Moormann RJM (1993) Epitope mapping of envelope glycoprotein E1 of hog cholera virus strain Brescia. J Gen Virol 74: 2053–2060
31. Van Rijn PA, Miedema GKW, Wensvoort G, van Gennip HGP, Moormann RJM (1994) Antigenic structure of envelope glycoprotein E1 of hog cholera virus. J Virol 68: 3934–3942
32. Wang L-F, Yu M (2004) Epitope identification using phage display libraries. Curr Drug Targets 5: 1–15
33. Wang L-F, Yu M, White J, Eaton BT (1996) BTag: a novel six-residue epitope tag for surveillance and purification of recombinant proteins. Gene 169: 53–58

34. Weiland E, Ahl R, Stark R, Weiland F, Thiel HJ (1992) A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. J Virol 66: 3677–3682
35. Weiland E, Stark R, Haas B, Rumenapf T, Meyers G, Thiel HJ (1990) Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J Virol 64: 3563–3569
36. Wensvoort G (1989) Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. J Gen Virol 70: 2865–2876
37. Windisch JM, Schneider R, Stark R, Weiland E, Meyers G, Thiel HJ (1996) RNase of classical swine fever virus: biochemical characterization and inhibition by virus-neutralizing monoclonal antibodies. J Virol 70: 352–358
38. Yu M, Than K, Colegate S, Shiell B, Michalski WP, Prowse S, Wang L-F (2005) Peptide mimotopes of phomopsins: identification, characterization and application in an immunoassay. Mol Diversity 9: 233–240
39. Yu M, Wang L-F, Shiell BJ, Morrissy CJ, Westbury HA (1996) Fine mapping of a C-terminal linear epitope highly conserved among the major envelope glycoprotein E2 (gp51 to gp54) of different pestiviruses. Virology 222: 289–292

Author's address: Meng Yu, CSIRO Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria 3220, Australia; e-mail: meng.yu@csiro.au