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Development of synthetic peptide ELISA based on nonstructural protein 2C of foot and mouth disease virus

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It was reported that the sera of convalescent animals contain antibodies to foot and mouth disease (FMD) virus (FMDV) 2C, highly conserved nonstructural protein (NSP), whereas the sera of vaccinated animals do not. But ELISA methods using this protein were not reported and developed until recently. In this study, NSP 2C peptides were synthesized within the amino acid sequence of the conserved 2C nonstructural region of FMDV according to the sequences from Genbank database and used for identifying antigenic determinants. One of the synthesized thirteen peptides gave strong positive reactivity with most of the sera from 13 FMD infected farms, but not with sera from vaccinated and non-infected animals. Moreover, with the sera collected through serial bleedings from four cattle and five goats infected with FMDV O/SKR/2000 experimentally, positive results were obtained in two species after 10 days post infection (DPI). Therefore, we tried to develop and evaluate this ELISA based on 2C peptides. In comparison with the commercial NSP ELISA, the 2C peptide based ELISA method showed good specificity and sensitivity. These results demonstrate that the synthetic 2C peptide ELISA can be a complementary marker to differentiate FMDV-infected from vaccinated on a herd basis.

Key words: foot and mouth disease virus, FMDV, nonstructural protein 2C, peptide ELISA

Introduction

Foot and mouth disease (FMD) is a highly contagious and economically important viral disease in cattle, pigs, sheep and goats, causing high productivity losses. As a control measure of the disease, stamping-out policy has been performed in those countries that have been free of the disease, while vaccination and movement restriction has been adopted generally in endemic areas. In such endemic areas, it is important to identify animals, whether vaccinated or not, in which replication of foot and mouth virus (FMDV) has taken place in order to eliminate potential infected animals.

Antibodies principally to the structural proteins of FMDV were induced in vaccinated animals, whereas infected animals produce antibodies to both the structural and non-structural proteins. Therefore, assays demonstrating antibodies against non-structural proteins have potential to differentiate infected animals from those that have been vaccinated [1,5,12].

With the objective to differentiate animals infected with FMDV from those vaccinated, several research groups have conducted studies to identify potential serological markers of infection among the nonstructural proteins (NSPs) of FMDV. It has been reported that NSPs such as 3A, 3AB1, 3ABC and 3C can be used as a marker of infection [2,3,14, 15,16] but the presence of antibodies against these antigens occasionally in vaccinated animals [6,13]. These NSPs have been produced primarily in expression systems using either bacteria or baculovirus and non-specific reactions can be caused by the presence of antibodies against these expression vector antigens [14,15]. Also, the number of epitopes found on such a long recombinant protein could result in unspecific reactions, because of cross-reactivities with antibodies to other picornaviruses [11].

Otherwise, it has been reported that the antibody against 2C protein is absent in the sera of vaccinated animals, and it can be explained by the association of this viral protein with cellular debris that separated from the virus harvest prior to inactivation of the supernatant for vaccine production [10]. The antibody response against this protein was examined in infected and vaccinated animals. Its result was shown that 2C is not released from infected BHK cells, and consequently

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it is not present in significant amounts in the medium from which inactivated FMDV vaccines are produced [9].

Recently, synthetic peptides containing B-cell epitopes of NSP were used as shown by Shen *et al*. [13]. They reported that the immunoreactivity to 2C peptides was primarily to those from N-terminal region of the protein. However, the study on linear B-cell epitopes of NSP 2C was partially conducted and the diagnostic method using these epitopes is not developed yet. Further recently, the overlapping synthetic peptides were used to identify FMDV-specific linear B-cell epitopes to differentiate between infected and vaccinated cattle [7].

In this paper, we also synthesized the peptide identical to pep93 of Hohich *et al.* [7] and to one of the peptides used by Shen *et al.* [13] to compare with the results. We tried to identify reliable 2C peptide marker to distinguish between infected and vaccinated animals and show that development of the ELISA based on 2C peptide can be applicable.

Table 1. Synthetic peptides used for identifying the linear FMDV-specific B-cell epitopes

Peptide ID	Amino acid sequence
Pep1 (1)*	LKARDINDIFAILKNGEC
Pep2 (54)	CQRDLNDPSKYKEA
Pep3 (92)	CVAPAPSKSRPEP
Pep4 (110)	CGKSGQGKSFLAN
Pep5 (128)	STHFTGRTDSVWYC
Pep6 (143)	CPDPDHFDGYNQQT
Pep7 (159)	CMDDLGQNPDGKDFK
Pep8 (190)	CLEDKGKPFNSKVI
Pep9 (233)	CVSAKDGYKINNKL
Pep10 (249)	CKALEDTHTNPVAM
Pep11 (275)	CEMKRMQQDMFKPQ
Pep12 (299)	CEVIDRVELHEKVSSHP
Pep13 (305)	CELHEKVSSHPIFKQ

*The amino acid position in the NSP 2C corresponding to the first amino acid of each peptide is indicated in parentheses. GenBank accession number is AY312587.

Materials and Methods

Peptide design and synthesis

The antigenic sites of FMDV NSP 2C were analyzed and the peptides were designed by Protean program (Dnastar, USA), and then the peptides based on the sequence of FMDV O/SKR/2000 and those of FMDV from Genbank database were synthesized (Table 1). The thirteen peptide antigens were produced by synthesis on a solid-phase support using an automated peptide synthesizer (Model 430A; Applied Biosystem, USA), and Fmoc protection for the a-NH_2 terminus and side chain protection groups of tri-functional amino acids. The synthesized peptides were cleaved from the solid support and side chain protecting groups removed by 90% trifluoroacetic acid. Synthetic peptides for immunoassay were prepared by matrix-assisted laser desorption time-of-flight mass spectrometric methods using a Perseptive Biosystems/VesTec Benchtop 11 Mass Spectrometer (Applied Biosystem, USA), and by Reverse Phase HPLC.

Test sera

To identify bovine FMDV-specific linear B-cell epitopes, three reference sera were obtained from animal experiment conducted at the Plum Island Animal Disease Center (PIADC; USDA). Also, one vaccinated reference serum

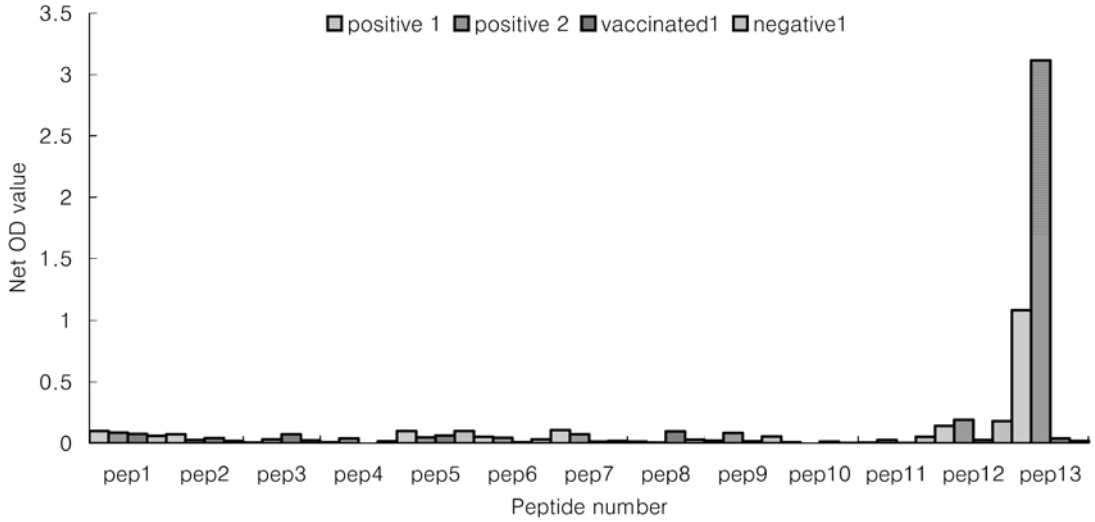

Fig. 1. Identification of FMDV-specific linear B-cell epitopes against 2C synthetic peptides in cattle. Positive 1 and 2: positive sera taken from the same animal experimentally infected with FMDV (O/SKR/2000) at 28 DPI and 56 DPI, respectively. Negative 1: negative sera from the same animal prior to infection. Vaccinated 1: vaccinated serum obtained from cattle vaccinated by O1 Manisa at the emergency vaccination in Korea in 2000. All tests were performed with at least two replicates.

obtained from cattle vaccinated by O1 Manisa at the emergency vaccination in Korea, 2000 was used. The three reference sera were composed of two positive sera obtained from cattle at 28 days post infection (DPI) and 56 DPI and one negative serum from the same animal prior to infection (Fig. 1).

Forty-eight bovine sera were prepared for evaluation and these sera were classified as field positive, experimentally positive, field negative, experimentally vaccinated, and field vaccinated groups (Table 2). All field positive sera were collected from naturally FMDV-infected cattle showed obvious vesicular lesions at various stages during the FMD outbreak in Korea , 2000 and verified as FMD positive by liquid phase blocking-ELISA (LPB-ELISA; Pirbright, UK).

To compare the test results of the 2C peptide ELISA with other kits, forty-six bovine sera were divided into four groups. Group 1 was collected from 23 cattle during the FMD outbreak in Korea , 2000 and Group 2 was collected sequentially at 10,14 and 21 DPI from cattle (n = 4) exposed experimentally to the O/SKR/2000 virus. Group 3 was collected at 30 days post vaccination from 3 cattle inoculated with divalent oil-adjuvant FMD vaccine including Type O, A, Asia1 and group 4 was collected from cattle never infected nor vaccinated were also included (Table 3).

To examine the kinetics and duration of FMDV NSP 2C antibody, positive sera from four cattle and five goats experimentally infected with the O/SKR/2000 virus at the PIADC were collected. These serum panels consisted of sequential bleeds from 0 DPI to 56 DPI.

To determine the cut-off value and specificity of this ELISA method, a total of 467 negative sera were collected from FMD free cattle. These sera were collected in 2001 from the southern part of Korea where FMD had never occurred and vaccination had never been practiced. A total of 786 bovine vaccinated sera were collected from cattle farms which were subject to emergency vaccination implemented during FMD outbreak in Korea , 2000. These sera were collected from cattle inoculated with divalent oil-adjuvant FMD vaccine including type O. All samples were verified and classified by LPB-ELISA and CHEKIT-FMD 3ABC kit (Intervet, Netherland).

Finally, The ninety-eight sera were collected from several herds in Korea to apply into this peptide ELISA and compare with other kits.

Table 2. Bovine sera samples for serological validation of 2C peptide ELISA

Sera samples	LPB-EILSA*	Description	Absorbance at 405 nm	Absorbance at 450 nm
			CHEKIT FMD 3ABC kit*	2C pep13
1	+	FPS	0.88 (+)	2.69
2	+	FPS	1.12 (+)	2.37
3	+	FPS	1.06 (+)	0.19
4	+	FPS	0.58 (+)	0.94
5	+	FPS	1.13 (+)	2.66
6	+	FPS	0.56 (+)	0.42
7	+	FPS	0.10 (-)	0.00
8	+	FPS	0.11 (-)	0.00
9	+	FPS	0.46 (+)	0.20
10	+	FPS	0.11 (-)	2.33
11	+	FPS	0.11 (-)	0.00
12	+	FPS	0.12 (-)	0.97
13	+	FPS	0.09 (-)	0.00
14	+	FPS	0.10 (-)	0.00
15	+	FPS	0.11 (-)	0.00
16	+	FPS	0.12 (-)	2.97
17	+	FPS	0.10 (-)	0.02
18	+	FPS	0.10 (-)	0.19
19	+	FPS	0.10 (-)	0.83
20	+	FPS	1.02 (+)	3.02
21	+	FPS	0.11 (-)	0.06
22	+	FPS	0.10 (-)	0.13
23	+	FPS	NT	0.16
24	+	FPS	0.21 (+)	0.32
25	+	FPS	NT	2.99
26	+	EPS	0.41 (+)	1.58
27	+	EPS	0.49 (+)	1.86
28	+	EPS	0.20 (+)	0.43
29	+	EPS	0.39 (+)	1.03
30	+	EPS	0.09 (-)	0.41
31	+	EPS	0.13 (-)	0.96
32	+	EPS	1.10 (+)	3.27
33	+	EPS	1.11 (+)	3.38
34	+	EPS	1.13 (+)	3.41
35	+	FVS	0.10(-)	0.10
36	+	EVS	0.14(-)	0.10
37	+	EVS	0.12(-)	0.02
38	-	FNS	0.09(-)	0.00
39	-	FNS	0.09(-)	0.00
40	-	FNS	0.13(-)	0.01
41	-	FNS	0.10(-)	0.00
42	-	FNS	0.10(-)	0.02
43	-	FNS	0.09(-)	0.02
44	-	FNS	0.09(-)	0.01
45	-	FNS	0.16(-)	0.03
46	-	FNS	0.09(-)	0.02
47	-	FNS	0.16(-)	0.01
48	-	FNS	0.09(-)	0.04

*+: positive; -: negative

FPS, field positive serum; EPS, experimental positive serum; FNS, field negative serum; FVS, field vaccinated serum; EVS, experimentally vaccinated serum. In experimental ELISA, sera displaying Net OD value of >0.20 were scored as reactive.

Peptide-based ELISA

Ninety six-well microtitre plate (Nunc, USA) was coated with 2C peptide (2 ug /ml) in the volume of 100 µl per well in 10 mM $NaHCO_3$ buffer (pH 9.6) and kept at 4°C overnight. The plate was washed three times with 0.05% (W/V) Tween-20 in PBS (PBS-T). The plate was incubated with 250 µl of blocking buffer (10% gelatine, 1% BSA in PBS-T) per well at 37°C for at least 1 hour to block

Table 3. Comparison of the relative results of the 3ABC, 2C and LPB ELISA

Description	LPB-ELISA*	CHECKIT FMD-3ABC kit†	FMDV 2C peptide ELISA‡
	No. of positive/ no. of tested		
FMDV infected sera	17/23	7/23	10/23
Experimentally Infected sera	10/10	9/10	10/10
Vaccinated sera	3/3	0/3	0/3
FMDV free sera	0/10	0/10	0/10

* LPB ELISA was carried out as prescribed in the OIE Manual of Standards (2000).
†The results were expressed as a percentage of the positive control and test sera were considered unequivocally positive if >30%.
‡The results were considered as positive if net OD value is >0.2.

nonspecific protein binding sites and washed three times with PBS-T. Test samples were diluted with serum dilution buffer (5% horse serum, 1% BSA in PBS-T) at a dilution of 1 : 100. One hundred microliter of the diluted sera was added to each well and allowed to react for 1 hour at 37°C. The wells were then washed three times with PBS-T. The standardized preparation of anti-species specific IgG antibody labeled with horseradish peroxidase (HRP) for the sera was added to each well and incubated at 37°C for 40 minutes. The wells were again washed three times with PBS-T to remove unbound labeled conjugate and reacted with 100 µl of 3, 3', 5, 5'-tetramethyl-benzidine substrate (KPL, USA) for 10 minutes. Reactions were stopped by the addition of 100 µl of 0.5M H_2SO_4 and were measured at the absorbance at 450 nm (OD_{450}). Blank wells were included as negative control in this assay. The concentrations of the polyclonal anti-bovine conjugates were adjusted for optimum sensitivity and specificity of the test serum panels.

Other ELISA tests

All of the serum samples were examined with LPB-ELISA and CHECKIT FMD-3ABC kit. The LPB-ELISA was carried out using the test kit produced by Pirbright Lab, Institute for Animal Health, USA. CHECKIT FMD-3ABC kit based on *E. coli*-expressed recombinant 3ABC antigen was used and all kits were used according to the manufactures' instructions. FMDV 3D Ab ELISA method developed by National Veterinary Research and Quarantine Service, Korea for 3D antibody detection was also used [4].

Results

Identification of bovine FMDV specific antigenic sites

Each peptide was used to coat the microtitre plates at the concentration of 2 µg/ml. Reactivities were determined with 4 sera consisting of two positive sera derived from one FMDV-infected animal at 28 DPI and 56 DPI, one negative serum from the same animal prior to infection and one serum from the vaccinated cattle. For the initial screening, each serum was added to the peptide-coated plates at 1 : 100 dilutions.

Figure 1 shows the reactivity of the test sera against a series of synthetic peptides. No peptide except for the pep13 provided an absorbance above 0.2 with either positive or negative. Thus, the Pep13 was selected as a candidate antigen and used to develop peptide ELISA. The pep1 from N-terminal region of 2C protein, containing a region known to be B-cell epitope [13], did not react with the two bovine positive sera in this study.

Determination of cut-off value

The frequency distribution of absorbance obtained in the 2C peptide ELISA of the test sera from FMDV naïve, vaccinated, and infected cattle are shown in Fig. 2. The reactivity of sera from infected animals was compared to those of both naïve sera and vaccinated sera to establish cut-off value for absorbance that clearly distinguish reactive from non-reactive samples. The 467 sera from naïve cattle had mean OD_{450} of 0.024 ± 0.102 and the 786 sera from vaccinated cattle had mean OD_{450} of 0.014 ± 0.040. The entire sera from naive and vaccinated cattle showed mean OD_{450} of 0.018 ± 0.060. The 38 sera collected from infected cattle (n = 5) above 10 DPI and naturally FMDV- infected cattle mentioned above had mean OD_{450} of 1.40 + 1.09. Thus, cut-off value for this provisional differential peptide ELISA could be determined at 0.198 by adding three standard deviations to the mean for non-reactive cattle sera of 0.018. The cut-off value is shown as a vertical line in frequency distribution of Fig. 2.

Serological validation of peptide ELISA

Peptide ELISA was performed with various serum samples to validate immunoreactivities of the selected peptide. The test serum samples were classified into four groups depending on its origin, and absorbances in this ELISA was compared with the results of commercial ELISA (Table 2). Experimentally infected sera showed clear reactivity in this peptide ELISA. However, field positive sera showed only thirteen out of twenty five sera. When compared with the CHECKIT FMD-3ABC kit, the sensitivity of 2C peptide ELISA was slightly higher. Both the vaccinated and the naïve sera did not show any reactivity with the peptide used in the assays.

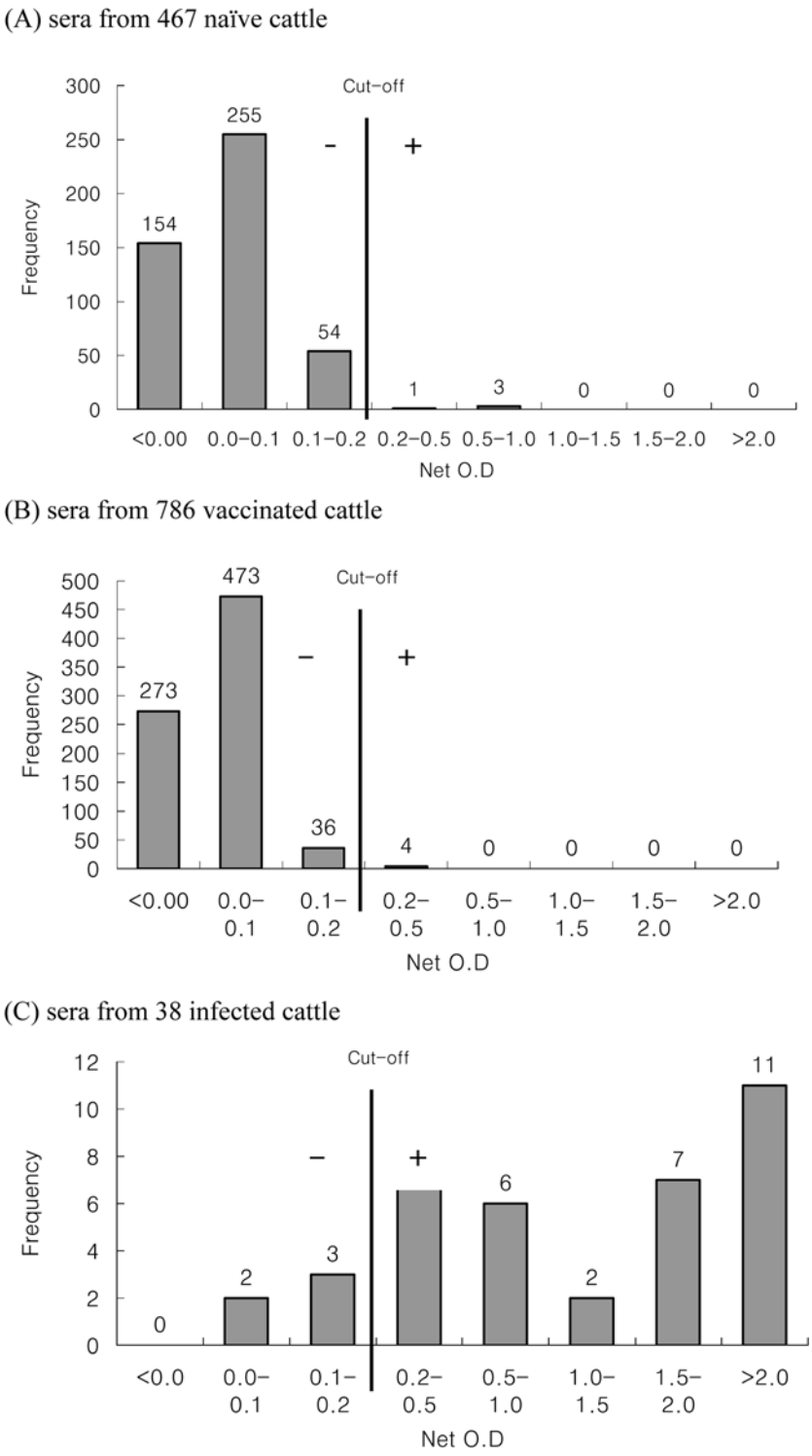

Fig. 2. Frequency distribution of the Net O.D values tested by FMDV 2C peptide ELISA. Interpretation of the assay was simplified by adopting cut-off value of at NET O.D $\geq$ 0.20, which gave the optimum sensitivity and specificity.

The results of the 2C peptide ELISA were also compared with those of commercial ELISA methods from IZSLE (Table 3). Both vaccinated sera and FMDV-free sera showed all negative reaction when tested with the two non-structural protein based ELISA methods, 2C peptide ELISA and CHECKIT FMD-3ABC kit. All positive reaction was also shown by LPB-ELISA in both experimentally infected and vaccinated sera. Out of twenty-three sera from FMDV-

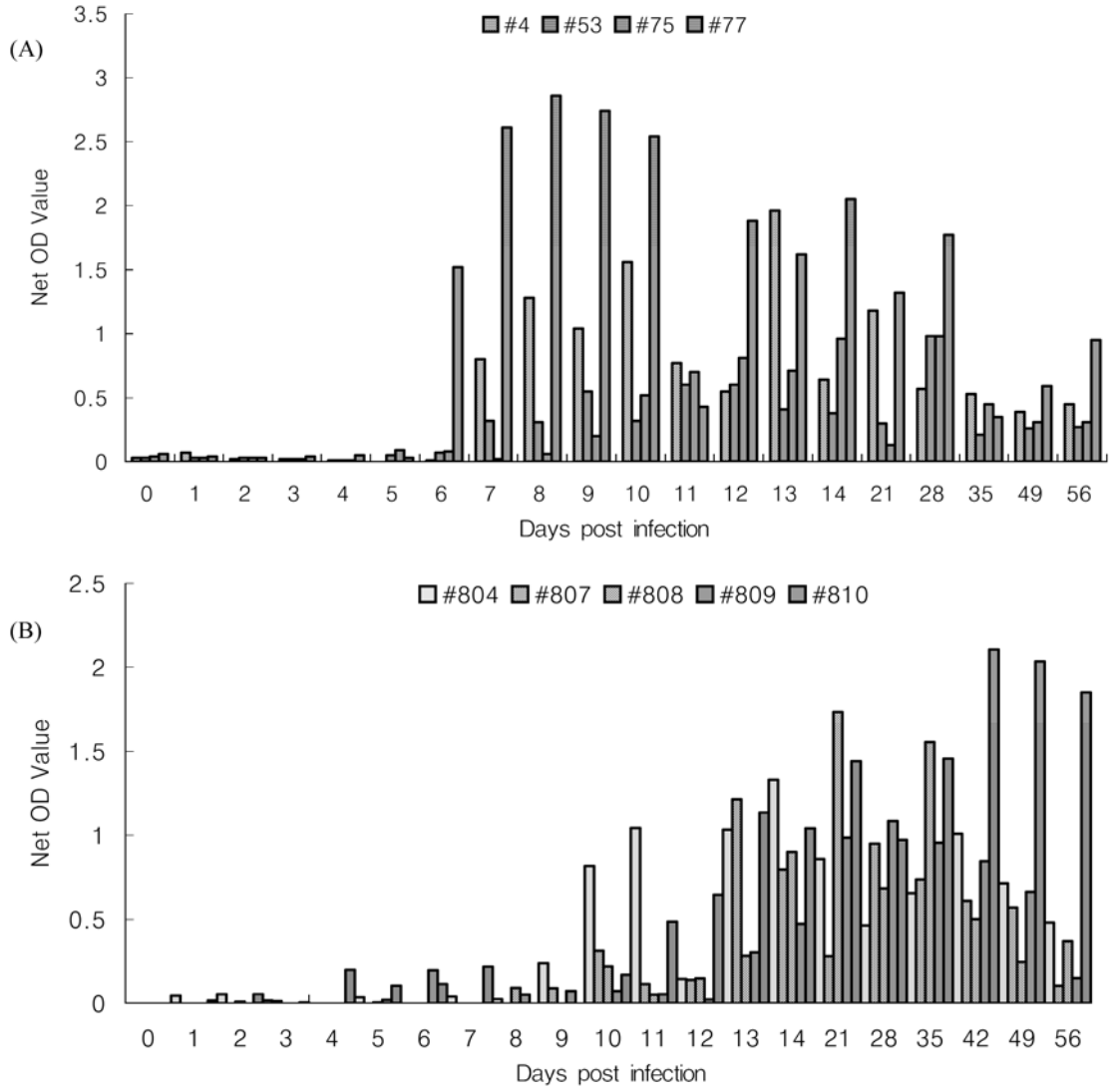

Fig. 3. Time course profiles of 2C antibodies from experimentally infected cattle and goats. Four cattle (A) and five goats (B) infected with O/SKR/2000 and the results were expressed as Net OD value up to 56 days post infection (DPI). The serum at 42 DPI was not tested in this experiment.

infected farms, positive reactivity showed in seventeen sera when tested LPB-ELISA while the number of sera identified as positive in the 2C peptide ELISA and CHECKIT FMD-3ABC kit were ten and seven, respectively. Also, the number of sera identified as positive in the experimentally infected sera were nearly identical for two NSP ELISA methods.

Sensitivity on time-course sera from FMDV-infected animals

To evaluate the synthetic 2C peptide based assay for sensitivity and specificity with respect to early detection of seroconversion and duration of seroreactivity, the animals were infected with the O/SKR/2000 virus and the sera were taken up to 56 DPI. Fig. 3 shows the antibody responses of infected cattle and goats with 2C peptide. Although there were a few variations in antibody responses with the sera from the infected cattle and goats, positive results were clearly observed from 7 DPI and lasted until 56 DPI.

Application of field samples and its comparison

Field samples from 6 outbreak herds in Korea were applied to compare the results of this peptide ELISA with three other ELISA methods (Table 4). When ninth-eight sera from FMDV outbreak herds were tested with LPB-ELISA, the number of positive signals was sixty and FMDV-specific antibodies was detected in all outbreak herds. Four sera from herd 4 showed negative reaction in all three NSP ELISA, but the number of positive sera was similar when tested with 3ABC ELISA, 3D ELISA and 2C peptide ELISA. The results indicate that 2C peptide ELISA is comparable to other assay based NSP when applied to field and used on a herd basis.

Table 4. The comparison of 2C peptide ELISA with other detection methods

FMDV Outbreak Herd	No of samples tested*	LPB (type O) ELISA	CHECKIT FMD-3ABC kit	FMDV 3D Ab ELISA†	FMDV NSP 2C Ab ELISA
Herd 1	15	11/15*,‡	5/15‡	4/15‡	5/15‡
Herd 2	51	28/51	10/51	9/51	11/51
Herd 3	11	11/11	2/11	1/11	1/11
Herd 4	4	2/4	0/4	0/4	0/4
Herd 5	7	3/7	2/7	0/7	2/7
Herd 6	10	5/10	4/10	2/10	3/10
Total	98	60/98	23/98	21/98	22/98

*Field sera samples from cattle in FMD outbreaks of Korea, 2000.
†The ELISA method developed by NVRQS for 3D antibody detection [4].
‡No. of positives/No. of tested.

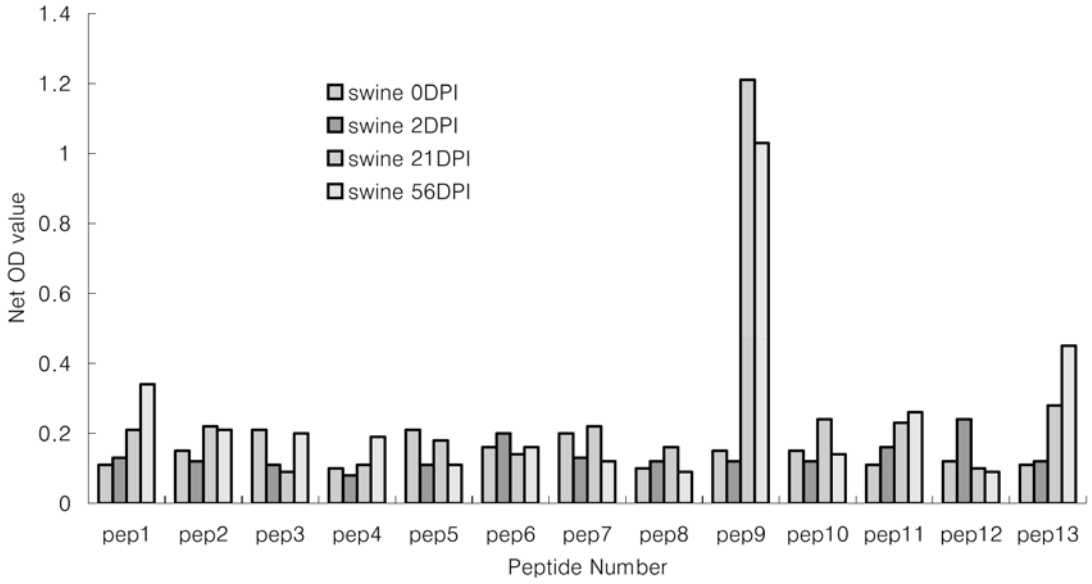

Fig. 4. Identification of FMDV-specific linear B-cell epitopes against 2C synthetic peptide in pig. Swine 0 day post infection (DPI): sera from an FMDV O/SKR/2000-infected pig were collected prior to infection. Swine 2, 21, 56 DPI: sera from an FMDV O/SKR/2000-infected pig.

Discussion

For many years, economic losses caused by vaccination and the difficulty of differentiating vaccinated from infected animals have kept many countries from considering FMDV vaccination as primary control measure. However, when the FMD occurred in Korea, emergency vaccination was implemented and proven to be successful for the eradication of FMD. In addition, after the enormous economic losses caused by the recent outbreaks of Great Britain, many countries have taken vaccination policies into consideration.

For these vaccination policies, it is important to determine whether animals have been infected with FMDV or vaccinated and also to develop effective diagnostic method to differentiate vaccinated from infected animals. In our experiment, thirteen peptides based on the NSP 2C region were synthesized and screened to search for linear B-cell eptitope in FMDV-infected cattle. The pep13 of thirteen peptides was used for effective discrimination between FMD-infected and vaccinated cattle.

The pep1 corresponding to N-terminal region of 2C protein described by Shen *et al.* [13] as a B-cell epitope did not show any reactivity to our serum panels. It was supposed that the size of synthesized peptide or amino acid sequence similarity of synthetic peptides has an influence on the reactivity to specific antibody. Another research group published in 2003 that the reaction with the 2C peptide was FMDV strain depended [16].

They also mentioned that individual differences in the reaction of the peptide-specific antibodies that could be detected in the serum became obvious. It could be suggested from data analyses that the reaction with the 2C peptide would be rather individual animal difference than difference in FMDV strain used.

Table 5. Comparison of the immunogenic sequences derived from different FMDV strains

No. of Genbank accession	Types and subtypes	Sequences	Homology (%)
AF428245	O	CELHEKVSSHPIFKQ*	
AAD38188	O	CELHEKVSSHPIFKQ	100
AAM91949	O	CELHEKVSSHPIFKQ	100
CAB60266	C	CELHEKVSSHPIFKQ	100
CAA52812	A	CELHEKVSSH-IFKQ†	92
AAQ90285	Asia 1	CELHEKVSSHPIFKQ	100
NP740463	O	CELHEKVSSH-IFKQ†	92
NP740341	SAT 2	CELHEKVSS-PIFKQ†	92
AAF09193	O1	CELHEKVSSHPIFKQ	100
AA36718	Asia 1	CELHEKVSSHPIFKQ	100
NP740508	C	CELHEKVSSHPIFKQ	100

*The sequence of the pep13 was used as a basis for other sequences. Sequences homologies are indicated as white fields and changes between the types and subtypes marked by the respective amino acids.
†Amino acid deletion

It was known that, in contrast to the structural proteins, the amino acid sequences of NSPs of FMDV are highly conserved irrespective of different subtypes and serotypes. A comparison of the sequences of reactive peptide (Pep13) derived from the NSP 2C region revealed that in all cases high similarity of above 92% at the amino acid level. Only one amino acid deletion was shown when compared with the pep13 sequence. Because ELISA based on 2C peptide has been performed only with the sera from animals infected with FMD O type virus, some substitutions may affect the reactivity of peptides against sera from animals infected by other virus strains. Thus, further experiments are needed to investigate the relationship between the amino acid sequence substitution of synthetic peptide and its reactivity.

The identification of linear B-cell epitopes on NSP 2C and its diagnostic application has been done only with sera of cattle and goats. To elucidate the reactivity of sera from other species with the identified peptide, all peptides synthesized were screened using small number of pig sera. Unexpectedly, all pig sera did not react with the identified pep13 but had reacted with pep9. Further experiments will help elucidate the exact reactivity of sera from several species with the specific regions of 2C.

Recently, the ELISA based on primarily 3ABC protein among NSP regions were developed and used [8]. However, it has been reported that anti-3ABC antibodies were present in some of the sera from vaccinated animals [8,9]. Therefore, development of ELISA based on other NSP regions such as 2C will give support to diagnose and differentiate infected from vaccinated animals more exactly. In addition, this kind of methodology is simple and specific one that can be applied for the elucidation of nonspecific reactions in other NSP ELISA. Therefore, the use of this peptide ELISA in combination with other NSP ELISAs will certainly contribute to FMD eradication programs.

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