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Development of an epitope-blocking-enzyme-linked immunosorbent assay to differentiate between animals infected with and vaccinated against foot-and-mouth disease virus

Jae Ku Oem [a], Byung Sik Chang [b,*], Hoo Don Joo [b], Mi Young Yang [b], Gwang Jae Kim [b], Jee Yong Park [a], Young Joon Ko [a], Yong Ju Kim [a], Jong Hyeon Park [a], Yi Seok Joo [a]

[a] *National Veterinary Research and Quarantine Service, Ministry of Agriculture, Anyang, Republic of Korea*
[b] *Department of Research and Development Laboratory, Jenobiotech Inc. Chuncheon, Gangwon, Republic of Korea*

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Abstract

An epitope-blocking ELISA (EB-ELISA) was developed to distinguish animals infected with foot-and-mouth-disease (FMDV) from those immunized with commercial vaccines. The assay used monoclonal antibodies to target the 3B core repeat motif (QKPLK) and purified recombinant 3AB proteins from the major B cell line epitopes of FMDV. Sera from uninfected and regularly vaccinated cattle, pigs, goats, and sheep (raised in FMDV free areas) were screened to evaluate the specificity of the EB-ELISA. The specificity scores of the assays were 99.8–100% and 100%, respectively. Reference sera from cattle, pigs, goats, and sheep experimentally infected with FMDV tested positive, with only a single exception. Antibodies formed in response to FMDV 3B appeared 1 week after infection and persisted at high levels for more than 8 weeks within the sera collected from serial bleeding of animals infected with FMDV O/SKR/2000. The EB-ELISA was used to differentiate between farms vaccinated against and those infected with FMDV (FMDV Asia serotype) during the 2005 epidemic in Mongolia by detecting antibodies against the FMDV Asia serotype in outbreak farms. This EB-ELISA method shows promise as an effective tool for FMDV control and eradication.

Keywords: Foot-and-mouth disease; Non-structural proteins; Epitope-blocking ELISA; 3B epitope

1. Introduction

Foot-and-mouth disease (FMD) is a serious infliction of cloven-hoofed animals. Highly contagious, infection spreads rapidly with a high rate of morbidity, causing great losses to productivity and considerable economic losses to the husbandry industry (Yang et al., 1999; Rowlands, 2003). The FMD virus (FMDV) consists of single-stranded linear RNA (8.5 kb) with an open reading frame (ORF) encoding a single polypeptide that is processed into 12 viral proteins (Forss et al., 1984). Infected animals respond by producing antibodies to both structural (SP) and non-structural proteins (NSP). In contrast, vaccinated animals only produce antibodies to SP upon FMDV infection. Although vaccination effectively reduces the spread of FMDV and prevents illness, it generally does not prevent infection, as even vaccinated individuals can become carriers due to viral latency (Cox et al., 1999).

The keys to successful eradication programs are vaccines that can serologically differentiate between infected and vaccinated animals. Vaccines derived from NSP of FMDV demonstrate this level of specificity and are used as a method of detection in several countries in combination with anti-NSP antibodies in sera (Chung et al., 2002; Mattion et al., 2004). Improving serological tests that effectively detect antibodies active against NSP is a crucial step toward eradication of FMD (De Diego et al., 1997; Mackay et al., 1998; Sorensen et al., 1998; Armstrong et al., 2005). ELISA methods that detect the anti-NSP antibody, a key serological indicator, can identify infected

* Corresponding author at: Department of Research Laboratory, Jenobiotech Inc., Chucheon, Republic of Korea. Tel.: +82 33 258 6700; fax: +82 33 258 6702.
E-mail addresses: jku0622@nvrqs.go.kr (J.K. Oem), lab-chief@jenobiotech.com (B.S. Chang), jenobio@jenobiotech.com (H.D. Joo), heimlich@jenobiotech.com (M.Y. Yang), gjkim@jenobiotech.com (G.J. Kim), parkjy@nvrqs.go.kr (J.Y. Park), koyj@nvrqs.go.kr (Y.J. Ko), kimyj@nvrqs.go.kr (Y.J. Kim), parkjh@nvrqs.go.kr (J.H. Park), jooys@nvrqs.go.kr (Y.S. Joo).

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and previously infected animals within a vaccinated population. Sero-surveillance is also a reliable tool for screening herds infected with FMDV (Sutmoller et al., 2003). Until now, only a few ELISA assays incorporating peptide or recombinant proteins in indirect or blocking ELISAs were commercially available. However, this ELISA test has two main disadvantages. Firstly, the recombinant antigens are difficult to purify and peptide synthesis is costly. Secondly, the length of the recombinant protein presents many possibilities for non-specific reactions due to the number of epitopes and potential cross-reactivity with antibodies to other picornaviruses (Neitzert et al., 1991).

Recently, an FMDV specific line of B cell epitopes was reported that differentiates between infected and vaccinated animals. The three 3B proteins (3B1, 3B2, 3B3) share a high amino acid sequence homology and a common amino acid core motif [QKPL(M)K] which helps distinguish between infected and vaccinated animals (Höhlich et al., 2003).

This principal aim was to develop an epitope-blocking ELISA (EB-ELISA) to detect serum antibodies active against the FMDV 3B repeat core motif. It was investigated whether this assay could be used to screen for FMDV infection in a broad range of domestic animals without major modifications to the protocol. This study focuses on the 3B repeat motif, highlighting its promise toward the development of a sensitive and reliable ELISA for differentiating FMDV-infected and vaccinated animals.

2. Materials and methods

2.1. Cloning and expression of recombinant non-structural proteins of FMDV (3AB, 3A, and 3B)

The genes for the non-structural proteins 3AB, 3A, and 3B of FMDV O/SKR/2002 (AY312588) were expressed in *E. coli* as fusion proteins using a p-BAD102/D-Topo vector (Invitrogen, USA) with both thioredoxine and 6xHis tags. Viral RNA fragments were purified by RNeasy Mini Kit (Qiagen, USA). Reverse transcriptase PCR (RT-PCR) was used to generate cDNA corresponding to the 3AB, 3A, and 3B proteins, using pfx DNA polymerase (Invitrogen, USA) and viral template RNA. The following oligonucleotides were used: J103 (5′-CACC ATC TCA ATT CCT TCC CAA AAG GC-3′) and J104 (5′-CTC AGT GAC AAT CAA ATT CTT AGC-3′), for amplification of the 3AB gene, nucleotides 4988–5659; J103 and J168 (5′-TTC AGT TTG TGG TTG TTC CAC-3′) for amplification of the 3A gene, nucleotides 4988–5446; and J169 (5′-GGA CCC TVC ACC GGT CCA CTC GA-3′) and J104 for amplification of the 3B gene, nucleotides 5447 to 5659. The PCR comprised 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. DNA fragments were ligated into the expression vectors. The plasmids were transformed into *E. coli* (DH5a) competent cells, and the resultant clone was identified by restriction enzyme analysis and the orientation of the insert was confirmed. The identity and reading frame of the recombinant clone were confirmed by sequencing. The recombinant plasmids selected for protein expression were designated p-BT3AB, p-BT 3A, and p-BT 3B.

Expression and purification of p-BT3AB, p-BT 3A, and p-BT 3B proteins were each carried out according to the same protocol. The three recombinant strains were inoculated in Luria Bertani (LB) medium (Difco, USA) with 100 μg/ml ampicillin (Sigma, USA) and incubated for 2 h at 37 °C with shaking until A_{600} reached 0.5–1.0. Protein expression was induced by adding 0.02% arabinose (Sigma, USA) followed by incubation for an additional 4 h at 37 °C with shaking. The cells were pelleted by centrifugation at 8000 × *g* for 20 min at 4 °C. The pellets were resuspended in buffer A (0.02 M NaH_2PO_4, 0.3 M NaCl, 0.01 M Imidazole, pH 8.0), and egg white lysozyme (100 μg/ml) was added. The cells were incubated on ice for 30 min and then sonicated on ice for 5 min using an UltraSonic processor (Ultrasonic, USA). Following cell disruption, the cell supernatant was passed over Ni-NTA agarose resin (Qiagen, USA) and the protein was eluted over a linear gradient with buffer B (0.02 M NaH_2PO_4, 0.3 M NaCl, 0.25 M Imidazole, pH 8.0). The three purified proteins were identified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the 3AB protein was selected for use as a diagnostic antigen. The FMDV 3B protein was injected into mice, and generation of monoclonal antibodies (MAbs) for the three recombinant proteins was monitored by MAbs profiling.

2.2. Production of Mab against 3B epitope

Hybridomas producing MAb to FMDV 3B were prepared by fusion of splenic lymphoid cells of immunized Balb/c mice with the sp2/0 myeloma cell line (Galfre and Milstein, 1981). The 3B protein (200 μg) was intra-muscularly (IM) injected into Balb/c mice (Orient, Charles River Laboratory, Korea). Hybridoma cells were screened using an indirect ELISA with p-BT3AB, 3A, 3B, and using an indirect immuno-fluorescence assay (IFA) with FMDV O/SKR/2002, swine vesicular disease virus, and vesicular stomatitis virus infected IB-RS cell (Indiana and New Jersey strain). Clones with both a positive/negative (P/N) ratio greater than 5 in the indirect ELISA and a positive reaction to IFA were selected for further propagation. The MAbs were isotyped using MonoAB TM ID/SP kits (Zymed, USA). Indirect ELISA was performed with ovabumin (OVA)-conjugated synthetic peptides to analyze epitopes of MAbs (Table 1). Western blots of recombinant p-BT 3AB, 3A, and 3B were also performed. The

Table 1
Synthetic peptides used to identify FMDV-specific B cell epitopes active against MAbs

Peptide	Amino acid sequence	Location	Protein
2C1	VSAKDGYKINNKL	1399–1412	2C
3A1	TDDKTLDEAEKNPL	1525–1538	3A
3B1F	GPYTGPLERQKPLK	1579–1592	3B1
3B2F	GPYAGPMERQKPLK	1602–1615	3B2
3B3F	GPYEGPVKKPVALK	1626–1639	3B3
3B3B	PVALKVKAKNLIVTE	1635–1649	3B3
3C1	LHRGNRVRDITKHF	1739–1752	3C

Peptides were synthesized based on the sequences from FMDV O/SKR/2002 (AY312588) and conjugated ovalbumin. Purity was determined to be >80% by HPLC. The peptides were stored at −20 °C in a stock of 2 mg/ml in distilled water.

positive hybridomas (5×10^6/ml) were injected into Balb/c mice pretreated with pristain (Sigma, USA). Antibodies were purified from ascitic fluid using Affi-Gel protein A (Bio-Rad, USA) conjugated with peroxidase (POD) according to the instruction manual (Roche, Germany).

2.3. EB-ELISA procedure

The EB-ELISA was used to detect 3B specific antibodies in the serum. Checkerboard titration was used to determine the optimal conditions for dilution of p-BT3AB and blocking MAb (5B27) conjugated to POD. The p-BT3AB construct in 0.1 M bicarbonate buffer, pH 9.6, was added to each well of a 600 binding ELISA plate (Greiner, Germany) and incubated at 4 °C overnight. Once sensitized, plates were washed three times with 0.01 M PBS, pH 7.4, 0.05% (v/v) Tween-20. Non-specific binding was obviated by incubating the plates with blocking buffer (250 μl, 3% BSA in PBS-T) at room temperature (RT) for 2 h. Test serum (100 μl) diluted 1:4 with serum dilution buffer (0.015 M Tris-Cl, 0.02 M EDTA, 1% BSA, pH 7.4) was added to the plate and incubated at RT for 1 h. The plates were washed, and diluted MAb conjugated to POD was added to each well, followed by incubation at RT for 60 min. Each well was washed three times to remove unbound conjugate and developed by addition of 3,3′,5,5′-tetramethylbenzidine substrate (TMB, Moss, USA). The color fully developed within 15 min at RT and the reaction was stopped by addition of 50 μl of 0.5 M H_2SO_4. The optical densities of the resulting samples were measured at 450 nm, and ratios were calculated relative to a negative control (S/N value). The cut-off value was determined by comparing the data to the two-graph receiver-operating characteristic (TG-ROC) curve with a known reference serum panel.

2.4. Test sera

The reference serum panel consisted of positive ($n = 19$), vaccinated ($n = 171$), and negative sera ($n = 48$). Positive sera were collected from animals experimentally infected with FMDV O/SKR/2000 and obtained from animals 56 days post infection (DPI). Virus neutralizing tests (VNT) were carried out using FMDV O/SKR/2000 according to the Office International des Epizootics (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2004). The sera from animals vaccinated regularly with commercial vaccine were collected. Antibodies for the structural proteins were screened by liquid-phase blocking ELISA (LPB-ELISA) or VNT. Negative sera from FMD free areas were also collected and screened.

Animal experiments were conducted to identify seroconversion of FMDV NSP antibodies at the USDA Plum Island Animal Disease Center (PIADC), Greenport, NY. A 1-year-old Holstein cow was inoculated with homogenized tissue from native Korean cattle, with FMDV serotype O, designated the Chungju strain (Kweon et al., 2003). The inoculated cow was housed among uninfected susceptible cows. The experiment was also performed with directly or indirectly infected pigs, sheep, and goats. Sera were collected over various time periods until 56 DPI. All sera were tested with LPB ELISA to confirm viral infection.

Blood samples were taken from cattle ($n = 2$) and pigs ($n = 4$) vaccinated IM with two doses of commercial grade monovalent and trivalent vaccine over a 2-week interval and screened for antibodies 0, 14, 28, 35, and 42 days post vaccination (DPV). Pigs were inoculated with the vaccine in four doses/head at 28 DPV in the same method as above to make highly immunized animal sera. All sera were screened with LPB ELISA.

In total 3098 negative sera samples were obtained from unvaccinated animals in FMD-free regions collected between November 2003 and March 2005. Another 313 samples of vaccinated animal sera were obtained from different farms after an emergency vaccination forced by the 2000 FMDV outbreak in Korea.

The FMDV Asia strain elicited an outbreak in Mongolia in 2005. A total of 57 sera samples from 13 farms were kindly supplied by the State Central Veterinary Laboratory, Ulaanbaatar, Mongolia. Several sera samples were taken from herds that tested positive for antibodies against the FMDV Asia strain as shown by LPB ELISA; there were 19 positive sera samples from four outbreak farms. Sera testing negative were also confirmed by the method above, and there were 38 negative sera and 8 FMD-free farms.

3. Results

3.1. Characteristics and analysis of recombinant NSPs and MAb 5B27

The genes encoding 3AB (672 bps), 3A (459 bps), and 3B (213 bps) were amplified, sequenced, and inserted into pBAD D/102 TOPO expression vectors. The proteins were expressed in large quantities, were highly soluble in supernatant after lysis, and were purified using Ni-NTA agarose with an imidazole gradient. The molecular masses of the purified proteins pBT3AB (48 kDa), pBT3A (39 kDa), and pBT3B (29 kDa) were evaluated by SDS-PAGE (Fig. 1A). The hybridoma cell line producing MAb (5B27) corresponding to FMDV 3B (5B27) was isolated from Balb/c mice immunized with pBT3B. The isotype of 5B27 was classified as IgG1 using a MonoAb TM ID/SP kit. An indirect ELISA revealed that MAb 5B27 was reactive with recombinant pBT3AB and pBT3B, but not pBT3A. MAb 5B27 reacted strongly with the peptide 3B1F and weakly with peptide 3B2F (Table 2). Only the 3B region containing the core repeat motif of QKPLK proved to be immunoreactive when the epitopes were analyzed with OVA-conjugated synthetic peptides and recombinant proteins. Western blotting of NSPs from the 3B region revealed that MAb 5B27 reacted specifically with the 3B protein, and not with the 3A protein (Fig. 1B). MAb 5B27 was also reactive with IB-RS2 cells infected with FMDV O/SKR/2002, but not with those infected by SVDV and VSVs (Fig. 1C).

3.2. Determination of S/N ratio in EB-ELISA

The reference serum panel ($n = 238$) were tested to plot a distribution of S/N ratios. The results indicated that 18/19 sera

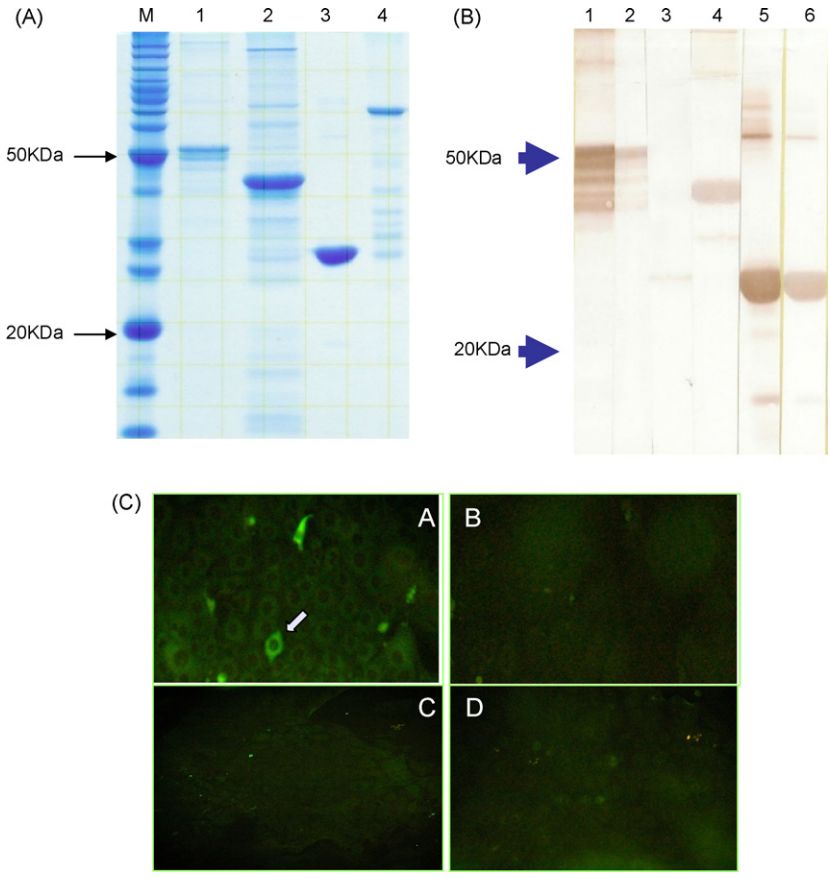

Fig. 1. Analysis of the expression profiles for thioredoxin tagged recombinant proteins from FMDV. (A) SDS-PAGE (12%) of purified thioredoxin tagged recombinant proteins: lane 1, 3AB; lane 2, 3A; lane 3, 3B; lane 4, vector control. (B) Western blot analysis of purified thioredoxin tagged proteins (100 μg/ml) reacted with MAb (5B27) resolved on SDS-PAGE (12%): lanes 1 and 2, pBT3AB (48 kDa); lanes 3 and 4, pBT3A (39 kDa); lanes 5 and 6, pBT3B (29 kDa). The gel was transferred to a nitrocellulose membrane and probed with MAb (lanes 1, 3, 5) and anti-6xHis antibodies (lanes 2, 4, 6). The MAb 5B27 reacted with pBT3AB and pBT3B proteins. A control antibody active against histidine was reactive with all of the recombinant proteins. A molecular weight marker was also used in the test (Invitrogen, USA). (C) MAb reactivity to FMDV (a), VSV Indiana strain (b), SVD (c) infected IB-RS cell line in IFA. The open arrow indicates FMDV within the cell.

tested from FMDV-infected animals had an S/N of 0.60 or lower: the negative sera ($n = 48$) and vaccinated animal sera ($n = 171$) tested above 0.60 (Table 3). One positive serum reference sample had a negative S/N value of 0.64. The serum was tested again with a different commercial kit, and the results were also negative (data not shown). With this S/N value, the specificity based on both negative and vaccinated sera was estimated to be 100% (219/219). An S/N of 0.60 was selected as the cut-off value to secure maximum specificity based on the results from the negative and vaccinated sera tested.

Table 2
Reactivity of MAb 5B27 to FMDV synthetic peptides and recombinant proteins

Protein	Name	Property	ELISA (A450)
Recombinant protein	pBT3AB	48 kDa	3.863
	pBT3A	39 kDa	0.161
	pBT3B	29 kDa	3.843
Synthetic peptide	2C1	OVA conjugated	0.156
	3A1	OVA conjugated	0.107
	3B1F	OVA conjugated	2.686
	3B2F	OVA conjugated	1.365
	3B3F	OVA conjugated	0.043
	3B3B	OVA conjugated	0.047
	3C1	OVA conjugated	0.083
Control antigen	OVA		0.055
	E. coli	DH5a	0.044

Recombinant proteins were coated to a final concentration of 1.0 μg/ml and synthetic OVA-conjugated peptides (2 μg/ml) were dissolved in bicarbonate coating buffer, pH 9.6.

3.3. EB-ELISA

Using this cut-off value, a specificity of 99.96% was obtained for serum samples ($n = 3098$) collected from FMD-free herds in Korea between 2004 and 2005. Only one sample tested positive out of the 683 samples of bovine serum screened; a net specificity of 99.8% in bovine species and 100% for the others species were recorded. No positive samples were found in the population of emergency vaccinated animal sera ($n = 313$), yielding a zero sample rate and a specificity of 100% (Table 4). A frequency distribution of S/N ratios for sera from experimentally vaccinated animals was obtained by an EB-ELISA. Animal sera collected from cattle and pigs vaccinated over a 2-week period was screened for FMDV antibodies 42 DPV, resulting in an S/N ratio above 0.80. The sera from pigs immunized several times (three times, eight doses) tested negative relative to the S/N cut-

Table 3
S/N value distribution of the serum panel ($n = 238$) determine by EB-ELISA

S/N value range	Serum panel				Frequency of POS sera (%)	Frequency of VAC and NEG sera (%)
	POS[a]	VAC[b]	NEG[c]	Total		
0.0–0.1	4			4	21.1	0.0
0.1–0.2	5			5	26.3	0.0
0.2–0.3	1			1	5.3	0.0
0.3–0.4	4			4	21.1	0.0
0.4–0.5	2			2	10.5	0.0
0.5–0.6	2			2	10.5	0.0
0.6–0.7	1	2		3	5.3	0.9
0.7–0.8		17		17	0.0	7.8
0.8–0.9		48	4	52	0.0	23.7
0.9–1.0		65	27	92	0.0	42.0
1.0–1.1		33	15	48	0.0	21.9
1.1–1.2		5	2	7	0.0	3.2
>1.2		1		1	0.0	0.5
Total	19	171	48	238		

The sensitivity (94.7%) and specificity (100%) of the assays were optimized by setting the S/N ratio threshold value to 0.60. MAb against 3B protein was used as a positive control; normal goat serum was used as the negative control.

[a] POS: Cattle ($n = 6$), pigs ($n = 6$), goats ($n = 4$), and sheep ($n = 3$) were experimentally infected with FMDV O/SKR/2000. The sera were harvested and screened 56 DPI.

[b] VAC: Sera of cattle ($n = 112$), pigs ($n = 25$), and goats ($n = 34$) were collected from animals vaccinated with commercial vaccines from O1 Manisa, Asia 1 Shamir, and A22 Iraq strains. The FMDV antibodies were screened by LPB ELISA or VNT.

[c] NEG: Sera from cattle ($n = 15$), pigs ($n = 12$), goats ($n = 15$), and sheep ($n = 6$) were collected from an FMD-free area.

off of 0.6 based on the reactivity of the negative control (Table 5). Samples of FMD-vaccinated sera all tested negative for antibodies with the EB-ELISA, regardless of the SP antibody population screened.

3.4. Kinetics of the antibody response to 3B epitope

The conditions for sero-conversion and the duration of sero-reactivity were identified using different sera from experimentally FMDV-infected animals. Antibody responses of infected animals are shown in Fig. 2. Sero-conversion was detected 7–8 DPI among directly infected animals (#4, #1135, #717, and #814). Positive responses against NSP between 7 and 12 DPI were shown indirectly infected animals. Although slight variations were observed in the antibody response to sera from infected animals, NSP antibodies proved positive up to 56 DPI. Although only one serotype virus was used, there was no difference in the response to the 3B epitopes of 3AB recombinant protein among cattle, sheep, goats, or pigs.

3.5. Application of EB-ELISA in Mongolia

Out of the samples testing positive for FMDV ($n = 19$) from infected farms ($n = 4$), 14 (73.6%) positive cases came from 3 (75%) of the farms tested by EB-ELISA. The 100% specificity was calculated for the cases of sera testing negative ($n = 38$) by LPB ELISA from negative farms ($n = 8$) as shown in Table 6.

4. Discussion

In the past, there was no general method available for many countries to screen for animals infected with or vaccinated

Table 4
Specificity of the EB-ELISA for sera from vaccinated animals ($n = 313$) and negative sera from FMD-free areas ($n = 3098$)

Status	Animal	No. samples	EB-ELISA		Specificity (%)
			Positive	Negative	
Vaccinated	Cattle	227	0	227	100
	Pigs	86	0	86	100
	Sub-total	313	0	313	100
FMD free	Cattle	684	1	683	99.86
	Pigs	2225	0	2225	100
	Goats	124	0	124	100
	Sheep	54	0	54	100
	Deer	11	0	11	100
	Sub-total	3098	1	3097	99.96
Total		3411	1	3410	99.97

Table 5
Comparison of the EB-ELISA for sera from sequential bleeding of experimentally vaccinated cattle and pigs

Group	Animal	Test	Days post vaccination (DPV)						
			0	7	14	21	28	35	42
BM01[a]	Cattle	S/N[b]	1.02	0.92	1.02	1.01	1.02	0.97	1.00
		PI (%)[c]	40.4	84.0	87.4	95.5	95.7	96.1	97.0
BT02[d]	Cattle	S/N	1.03	1.03	0.93	0.88	0.86	0.89	1.00
		PI (%)	36.7	42.5	83.4	95.1	94.9	95.0	97.0
SM03[e]	Pigs	S/N	0.94	1.02	0.87	0.94	0.82	NT[f]	0.85
		PI (%)	32.8	51.4	74.0	88.1	91.6	NT	88.4
ST04[g]	Pigs	S/N	0.96	0.86	0.99	0.92	0.92	NT	0.98
		PI (%)	31.5	9.3	49.7	79.0	84.2	NT	84.2

[a] BM01 was immunized with vaccine from the O1 Manisa strain, two times over a 2-week interval.
[b] S/N value of EB-ELISA.
[c] PI of LPB-ELISA O1 Manisa type (Pirbright, UK).
[d] BT02 was immunized with vaccine from the O1 Manisa, Asia 1, and A22 Iraq strains, two times over a 2-week interval.
[e] SM03 ($n=2$), were immunized with a vaccine from the O1 Manisa strain, two times over a 2-week interval and boosted with four additional doses at 28 DPV resulting in multiple vaccinations of the animals.
[f] NT: not tested.
[g] ST04 ($n=2$), were immunized with vaccines from the O1 Manisa, Asia 1, and A22 Iraq strains, two times over a 2-week interval and boosted with four additional doses at 28 DPV resulting in multiple vaccinations of the animals.

against FMDV. Reports of antibodies active against FMDV NSP antigens suggested they could be used to distinguish infected animals from vaccinated animals in the field (De Diego et al., 1997; Meyer et al., 1997; Silberstein et al., 1997; Sorensen et al., 1998). An ELISA detecting3ABC was evaluated and compared to commercial ELISA products. This method proved limited, since non-specific reactions of the NSP antibody were detected in vaccinated animals within FMDV-free regions. An EB-ELISA with

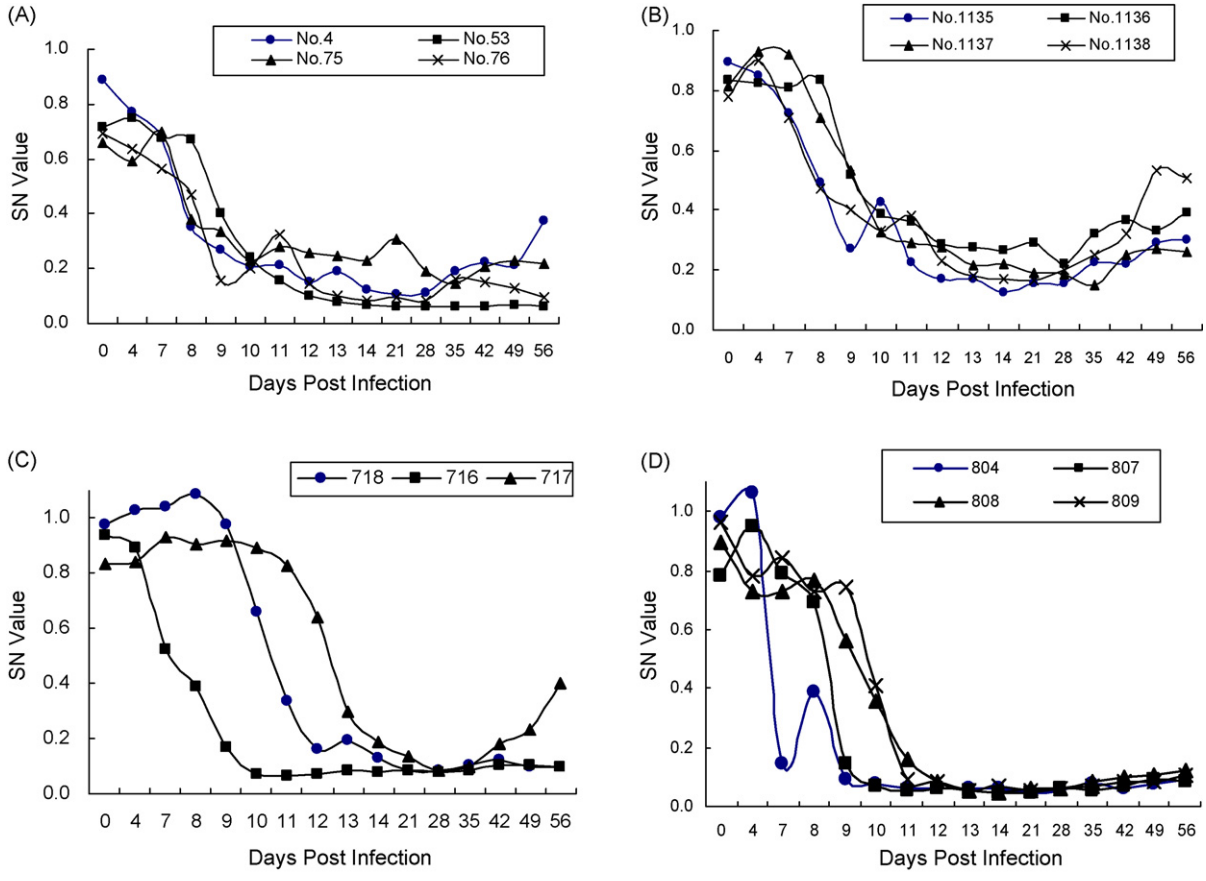

Fig. 2. Time course study of experimental sera from FMDV O/SKR/2000 infected animals. (A) Cattle ($n=4$); (B) pigs ($n=4$); (C) sheep ($n=3$); (D) goats ($n=4$). The numbers of animals directly infected with FMDV were 4, 1135, 716, 804, respectively. The other animals contacted the infection in house.

Table 6
Field application of the EB-ELISA for sera from FMDV outbreak herds ($n = 19$) and negative sera ($n = 38$) in FMDV-free provinces

Status	Herd	No. samples	EB-ELISA	
			Positive	Negative
FMD outbreak	A1-1	10	8	2
	A1-2	6	5	1
	A1-3	1	0	1
	A1-4	2	1	1
	Total	19	14	5
FMD free	A2-1	1	0	1
	A2-2	3	0	3
	A2-3	2	0	2
	A2-4	1	0	1
	A2-5	1	0	1
	A2-6	3	0	3
	A2-7	5	0	5
	A3-1	10	0	10
	A3-2	12	0	12
	Total	38	0	38

MAb was chosen instead due to enhanced specificity regardless of the animal species being tested. Monoclonal antibodies for epitopes of the immuno-reactive domain of the 3B core repeat and recombinant 3AB protein were used to develop an EB-ELISA. Analysis of the core QKPLK repeat motif, found in B cell line epitopes of the 3B protein revealed several differences between infected and vaccinated animals, depending on the type of animal. The 3AB protein was chosen among FMDV NSP, since the homology of this region is highly conserved within FMDV genomes of all serotypes (Clavijo et al., 2004). The 3B core repeat of the pBT3AB recombinant NSPs was selected as the ELISA antigen for its excellent reactivity with MAb clone 5B27. The EB-ELISA was able to distinguish between FMD-infected, vaccinated, or negative animals when the reference serum panel was screened. The sensitivity and specificity of the reference panel were determined to be 95% and 100% on the basis of an S/N cut-off value of 0.60.

Time course studies revealed early detection (7–8 DPI) in experimentally infected animals and animals infected by contact with other infected animals were sero-conversed 8–12 DPI, depending on the species susceptibility to FMDV. As mentioned by Sutmoller et al. (2003), this study revealed an unavoidable time lag in detection of NSP antibodies in sheep (#717, 718) which were infected by other infected animals. It was due to the small quantity of FMDV present in the air that the animals were exposed to. The EB-ELISA successfully identified the Asia strain of FMDV in herds from three out of four FMDV positive farms during a recent outbreak in Mongolia, with fourteen out of nineteen samples testing positive (sensitivity of 74.5%). The EB-ELISA not only detects the Asia strain in infected animals, but also the O type. The main advantage of the EB-ELISA is its reduced rate of false positives for vaccinated animals. The experimentally vaccinated animal sera were all negative as determined by the EB-ELISA. Sera from animals that underwent emergency vaccination were assessed to a specificity of 100%. The S/N value was found to be greater than 0.80 in sera from pigs vaccinated multiple times (maximally vaccinated three times in eight doses with commercial vaccines). These results suggest the EB-ELISA has non-specific reactivity in vaccinated animals. Negative sera samples ($n = 3098$) from an FMD-free region in Korea were analyzed to evaluate the specificity of the EB-ELISA. The specificity was assessed as 99.96%.

The EB-ELISA still needs refinement against a large number of known positive sera (e.g., FMDV C type-infected animals), carrier animals (i.e., FMDV-infected animals post vaccination or animals testing positive within vaccinated areas), maternal antibodies derived from an immune dam, and sera from outbreak farms. The current results suggest the EB-ELISA shows promise for: (1) differentiating infected animals from vaccinated animals, (2) rapid detection of infected animals irrespective of the vaccination status, (3) serological surveillance to identify animals exposed to the virus and which have developed antibodies, (4) monitoring the progress of FMDV eradication and emergency programs, and (5) detecting NSP antibodies against all serotypes of the virus. This EB-ELISA is a useful serological test for situations where the serotype of the virus is unknown.

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