Journal of Virological Methods 159 (2009) 112–118

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A recombinant protein-based ELISA for detecting antibodies to foot-and-mouth disease virus serotype Asia 1

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Article history:
Received 24 December 2008
Received in revised form 3 March 2009
Accepted 9 March 2009
Available online 20 March 2009

Keywords:
Recombinant protein
ELISA
Foot-and-mouth disease virus
Serotype Asia 1
Serology

A B S T R A C T

A recombinant protein-based ELISA was evaluated for detecting antibodies to foot-and-mouth disease virus (FMDV) serotype Asia 1. The recombinant protein (rP13C) was derived from the P1 precursor and 3C protease genes that were cloned into a single expression vector and expressed in insect cells. This protein elicited a low titer of FMDV neutralizing antibodies in pigs. Its utility as a diagnostic antigen was explored in a blocking ELISA using monoclonal antibodies. The rP13C ELISA yielded higher endpoint titers than the liquid phase blocking (LPB) ELISA and virus neutralization test performed on sera from goats challenged with FMDV post-vaccination. The rP13C ELISA correctly scored the FMD international reference weak positive serum. The relative sensitivity between the rP13C ELISA and LPB ELISA was equivalent for vaccinated sera. With this comparable sensitivity, the rP13C ELISA exhibited a specificity of 99.7% for domestic naive swine, bovine and caprine sera. This report demonstrates that an ELISA using recombinant proteins has the potential to replace the LPB ELISA using an inactivated FMDV antigen as a simple and robust serological tool for screening antibodies to FMDV serotype Asia 1.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed animals such as cattle, pigs, sheep and goats. It causes economical loss due to the dairy production loss and constraint imposed on international trade in live animals and their products (Kitching et al., 2007). Foot-and-mouth disease virus (FMDV), the etiological agent, belongs to the genus *Aphthovirus* in the family *Picornaviridae*. FMDV consists of a single-stranded, plus-sense RNA genome of approximately 8500 bases surrounded by an icosahedral capsid that comprises 60 copies of four structural proteins: VP1, VP2, VP3 and VP4 (Grubman and Baxt, 2004). FMDV exists in the form of seven different serotypes: O, Asia 1, A, C, South African territories 1 (SAT 1), SAT 2 and SAT 3, and a large number of subtypes have evolved within each serotype (Domingo et al., 2003; Knowles and Samuel, 2003; Mason et al., 2003).

Should animals show clinical signs, they can be simply diagnosed through clinical surveillance or direct detection of the virus in clinical samples. However, the diagnosis of FMD in animals infected subclinically can only be achieved through serological surveillance. The virus neutralization test is recognized as the standard method for detecting antibodies to FMDV structural proteins (OIE, 2008). However, it is a laborious procedure, takes 2–3 days to complete and requires a containment facility such as a biosafety level 3 laboratory. These aspects make the virus neutralization test inappropriate for mass serological surveillance. The other prescribed test in the OIE manual is the liquid phase blocking (LPB) ELISA (Hamblin et al., 1986). Although it has been applied worldwide, it also has several drawbacks. First, it requires training and experience to yield reproducible results and so is not robust (Mackay et al., 2001). Secondly, false positive reactions can occur at a rate of 4% in normal, non-vaccinated cattle and can be as high as 18% in those which are stressed (Haas, 1994). To improve this complicated process, the solid-phase competitive (SPC) ELISA has been developed and evaluated extensively, especially for FMDV serotype O (Mackay et al., 2001; Paiba et al., 2004). However, the use of an inactivated FMDV still requires the antigen to be produced only in a containment laboratory. Despite the biosecurity requirements, the risk of escape of the virus from laboratories and vaccine plants cannot be excluded and certain countries discourage or prohibit institutes from manipulating FMDV in their territory (Valarcher et al., 2008). Examples of outbreaks caused by laboratory escapes include those that occurred in Germany in 1987 and 1988, and in Russia in 1993 (Valarcher et al., 2008). To address this limitation, recombinant

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doi:10.1016/j.jviromet.2009.03.011

proteins derived from FMDV P1 precursor and 3C protease genes have been developed for use as an alternative to the inactivated virus antigen. Previously, a pentamer-like structure for FMDV serotype O was reported to be generated when the P1 and 3C genes were expressed in insect cells (Oem et al., 2007). However, any effort to produce this kind of recombinant protein as a diagnostic antigen for other serotypes has not yet been reported. The spread of FMD virus isolates throughout the Asian continent, especially the outbreaks in North Korea in 2007 and in China over several years led to the development of a recombinant protein-based ELISA as a simple and robust tool to replace the LPB ELISA for detecting antibodies to FMDV Asia 1 with the aim of identifying infected animals in unvaccinated areas and assessing the vaccine titers in vaccinated animals.

2. Materials and methods

2.1. Cells and viruses

IBRS-2 cells were grown in alpha minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA, USA) in a 37 °C incubator. *Spodoptera frugiperda* (Sf9) cells (Invitrogen) for the propagation of recombinant baculovirus were grown in Grace's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, antibiotic–antimycotic solution, and lactalbumin hydrolysate (Invitrogen) at 27 °C. A Mongolian strain of FMDV Asia 1 (As1/MOG/05) which was isolated at our institute from samples provided by the State Central Veterinary Laboratory in Mongolia, was amplified in IBRS-2 cells and used for RNA extraction. The complete genomic sequence is recorded in GenBank under Accession No. EF614458 (Lee et al., 2009). The Cambodian strain of FMDV Asia 1 (CAM 9/80) was obtained from the Institute for Animal Health (Pirbright Laboratory, Surrey, UK). Virus manipulation was conducted in a biosafety level 3 containment laboratory in accordance with the regulation of the National Veterinary Research and Quarantine Service in the Republic of Korea.

2.2. Generation of recombinant baculovirus containing the P1 and 3C genes

The viral RNA was extracted from FMD-infected IBRS-2 cells using the RNeasy extraction mini kit (Qiagen, Valencia, CA, USA). Complementary DNA for P1 and 3C RNA was produced using AccuPower RT premix (Bioneer, Daejeon, Korea). The genes were amplified from cDNA using nPfu DNA polymerase (Enzynomics, Seoul, Korea). Primers were as follows. P1F, 5′-GAA G*GG ATC* CAT GGG AGC CGG GCA ATC AGT CCG-3′; P1R, 5′-TAG G*AC TAG T*TA CAA AGT CTG TTT CTC AGG TGC-3′; 3CF, 5′-GAT T*CT CGA G*AT GAG CGG TGC CCC CCC GAC CGA C-3′; and 3CR, 5′-AAC A*GC ATG C*TA CTC ATG GTG TGG TTC AGG GTC-3′. PCR amplification of the P1 gene was carried out in a thermal cycler, with an initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min 30 s, and a final extension at 72 °C for 5 min. PCR amplification of the 3C gene was conducted as above except for the elongation step at 72 °C for 1 min. Each of the amplified P1 and 3C genes was cloned separately into a pFastBacDual vector. The P1 gene was inserted under the polyhedrin promoter using BamHI and SpeI. The 3C gene was inserted under the P10 promoter using XhoI and SphI. Recombinant baculovirus DNA was generated by the recombination of pFastBac/P1/3C into a baculovirus shuttle vector (bacmid) propagated in competent DH10Bac *Escherichia coli* (Invitrogen) using the bac-to-bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. The recombinant baculovirus containing the P1 and 3C genes was produced following transfection of the recombinant bacmid DNA into sf9 cells and then stored at 4 °C until use.

2.3. Expression of recombinant proteins

Sf9 cells grown in flasks were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 5 and harvested daily to determine the optimal expression time to yield maximal recombinant proteins. Cells were frozen and thawed three times and clarified by centrifugation at 10,000 × *g* for 30 min. The supernatant (rP13C) was used as the diagnostic antigen in this study.

An immunofluorescence assay (IFA) was used to confirm the expression of recombinant proteins. Sf9 cells grown in chamber slides were infected with the recombinant baculovirus at 5 MOI. The infected cells were fixed with a mixture of cold acetone and methanol and then probed with rabbit anti-VP1 peptide serum diluted 1:100 in phosphate-buffered saline (PBS) for 1 h at 37 °C. After washing with PBS, FITC-conjugated goat anti-rabbit antibodies [Kirkegaard–Perry Laboratories Inc. (KPL), MD, USA] diluted 1:100 in PBS were added and incubated for 1 h at 37 °C. Following washing, reactivity was observed using a fluorescence microscope.

For Western blot analysis, protein samples were separated using NuPAGE Novex Bis–Tris gels with an Xcell SureLock mini-cell (Invitrogen) according to the manufacturer's instructions. Separated proteins were transferred from the gels onto nitrocellulose membranes. After blocking with diluent [Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skim milk] for 1 h, rabbit anti-VP1 peptide serum diluted 1:100 in diluent was added and incubated for 1 h at 37 °C. Following washing with TBST, the secondary antibody, goat anti-rabbit conjugated with alkaline phosphatase (KPL), was added at 1:1000 in diluent and incubated for 1 h at 37 °C. After washing, the colorimetric reaction was developed by adding the one-component solution of 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (KPL).

2.4. Sera and monoclonal antibody

Rabbit serum against a VP1 peptide was prepared by inoculating rabbit subcutaneously with keyhole limpet hemocyanin (KLH)-coupled synthetic peptide (^{139}TGPTRRGDLALQRVSNRLP^{157}, based on the sequence of AY304994; Asia 1/Ind/63/72) mixed with Freund's complete adjuvant at first and then incomplete adjuvant at three boosters. These sera were obtained from Peptron Inc. (Daejeon, Korea).

Bovine sera enclosed in the LPB ELISA kits purchased from the Institute for Animal Health (Pirbright Laboratory) were used for a preliminary comparison of the relative sensitivity and cross-reactivity of the rP13C ELISA relative to the LPB ELISA. These are control strong-positive and moderate-positive bovine sera for serotype Asia 1 (Shamir strain), and strong-positive bovine sera for serotypes O, A, and C.

The international reference sera (strong, weak, cut-off and negative) for serotype Asia 1 were generated for the FAO standardization study phase XVIII and obtained from the Institute for Animal Health (Pirbright Laboratory).

Caprine sera ($n = 4$) were collected from four goats at 40 days after intradermal challenge in the neck with the Mongolian strain (As1/MOG/05) at 20 days post-single immunization with a trivalent (O, A, and Asia 1) vaccine from Merial (Surrey, UK). All subjects exhibited no symptoms of FMD throughout the experimental period.

Two 60-day-old pigs were immunized twice intramuscularly with the rP13C protein in a mixture with IMS1313 adjuvant (Seppic, Paris, France) in a final volume of 3 ml. They were bled at 14 days post the first immunization and at 13 and 20 days post the second immunization.

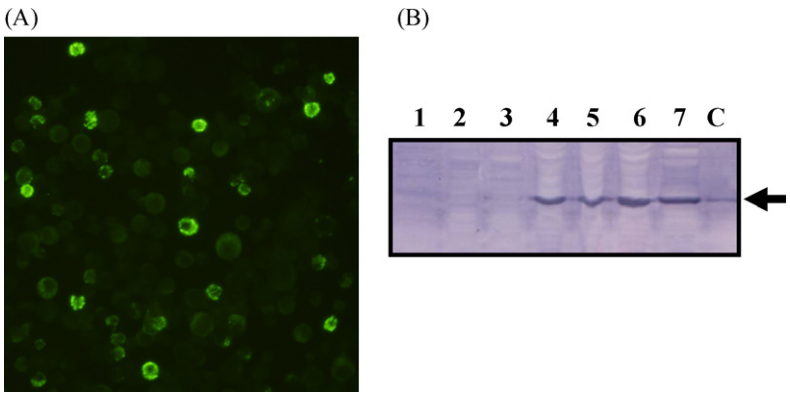

Fig. 1. Identification of the rP13C expressed in sf9 cells. (A) Immunofluorescence assay for the rP13C expressed in sf9 cells. The cells were probed with rabbit serum against the FMDV VP1 peptide. (B) Time course profile of the rP13C expression in sf9 cells. The recombinant proteins were collected daily post-recombinant baculovirus infection and Western blots were conducted with a rabbit anti-VP1 serum to detect the VP1 band derived from the P1 precursor via cleavage by 3C protease. Numbers 1–7 indicate 1 day post-infection (dpi) to 7 dpi. (C) A control positive protein, an inactivated FMDV antigen enclosed in the LPB ELISA kit.

Vaccinated bovine sera ($n = 68$) were obtained in the field during the 2000 FMD outbreak in the Republic of Korea. They were vaccinated with two doses of trivalent (O, A, and Asia) vaccine.

Sera ($n = 1760$) from animals with no history of exposure to FMD virus were obtained from cattle ($n = 640$), pigs ($n = 560$) and goats ($n = 560$) in domestic farms that had been diagnosed as negative by nonstructural protein (NSP) ELISA (Jenobiotech, Gangwon, Korea).

A monoclonal antibody (Mab) to be used as a capture antibody (70-17) was produced by the cell fusion method as described previously (Oem et al., 2007). To obtain Mab to be used as a detector antibody to compete with serum antibodies, mice (BALB/c) were immunized four times with KLP-conjugated peptide corresponding to the VP1 epitope, ^{138}EESSRRGDLAALARRVNNRLP^{158}, based on the sequence of As1/MOG/05, which was synthesized at Peptron Inc. and received intravenous injection as a final booster 3 days before cell fusion with SP2/O myeloma cells. Hybridoma cells were screened by peptide ELISA, IFA and virus neutralization test. Finally, a selected Mab (1A31) was purified using the ImunoPure IgG purification Kit (Pierce, IL, USA) and labeled using perodixase labeling kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

2.5. Recombinant protein-based ELISA (rP13C ELISA)

MaxiSorp ELISA plates (Nunc, Glostrup, Denmark) were coated with purified Mab 70-17 (2 μg/ml) in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then incubated with 50 μl of rP13C diluted 1:8 in diluent (PBST containing 5% skim milk) at 37 °C for 1 h. Following washing, 50 μl of test and control sera diluted 1:10 in diluent was distributed in each well and incubated at 37 °C for 1 h. After washing with PBST, 50 μl of 0.1 μg/ml Mab (1A31) conjugated with horseradish peroxidase was added and the plates were incubated at 37 °C for 1 h. After the plates were washed five times, the colorimetric reaction was developed for 15 min by adding 0.6 mg/ml of O-phenylenediamine in 0.05 M citrate phosphate buffer (pH 5.0) containing 0.015% hydrogen peroxide. The reaction was stopped by adding 50 μl of 1.25 M sulphuric acid. Optical density (OD) was measured at 492 nm. The OD value was converted to percentage inhibition (PI) value using the formula: $PI\ (\%) = 100 \times [1 - (OD_{test\ serum}/OD_{control})]$, where $OD_{control}$ is the mean OD of wells containing Mab alone.

2.6. Other tests

IFA was carried out to examine the reactivity of the selected Mab (1A31) for FMDV Asia 1. The IBRS-2 cells grown in 96-well microplates were infected with As1/MOG/05 and CAM 9/80 for 48 h before being fixed with a mixture of cold acetone and methanol. The cells were treated with 1A31 (of which the epitope should be located on VP1) for 1 h at 37 °C. After washing with PBS, FITC-conjugated goat anti-rabbit antibodies (KPL) diluted 1:100 in PBS were added and incubated for 1 h at 37 °C. Following washing, the fluorescent cells were observed using a fluorescence microscope.

The virus neutralization test was carried out with As1/MOG/05 and CAM 9/80 using IBRS-2 cells as described previously (OIE, 2008). Titers were expressed as the reciprocal of the final dilution of serum at which 50% of the wells are protected. A virus neutralization test titer of less than or equal to 11 was considered negative, that between 16 and 32 was inconclusive, and a titer equal to or greater than 45 was considered positive.

The LPB ELISA was also performed as described previously (OIE, 2008). The titer was expressed as the reciprocal of the final dilution at which reaction of the test sera resulted in OD value equal to 50% inhibition compared to the control OD. A serum titer above 45 was considered positive with the cut-off of PI 50.

3. Results

3.1. Expression of recombinant proteins in insect cells

A recombinant baculovirus containing the P1 and 3C genes under two distinct promoters was generated by transfecting a recombinant bacmid DNA into insect cells. The rP13C expressed in insect cells by the recombinant baculovirus was identified by IFA with rabbit serum raised against a FMDV VP1 peptide (Fig. 1A). To investigate whether the reactivity was derived from the P1 precursor or VP1 subunit cleaved by 3C, Western blot analysis was conducted with serum against the VP1 peptide (Fig. 1B). A prominent band corresponding to VP1 was observed, indicating that the above fluorescence reactivity was due mainly to the VP1 protein cleaved by 3C protease as expected. In contrast, the VP1 band was not apparent in insect cells that were infected with the recombinant baculovirus containing only P1 (data not shown). As a control protein, inactivated FMDV antigen enclosed in the LPB ELISA kit (Asia 1) also showed the same VP1 band (Fig. 1B, lane C).

To examine the time course expression profile, the rP13C protein was collected daily after insect cells were infected with the recombinant baculovirus and analyzed by Western bloting with rabbit serum raised against FMDV VP1 (Fig. 1B). The VP1 band was observed starting from 4 days post-infection (dpi) up to the last day of the experiment (7 dpi).

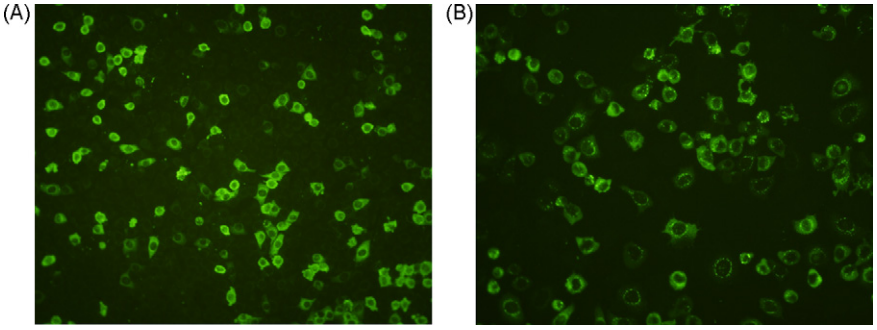

Fig. 2. Immunofluorescence assay of 1A31 for FMDV serotype Asia 1. IBRS-2 cells were infected with (A) the Mongolian strain and (B) the Cambodian strain of FMDV Asia 1 and probed with 1A31 Mab specific to FMDV VP1.

3.2. Immunogenicity of recombinant proteins in pigs

To investigate whether the rP13C protein could elicit neutralizing antibodies in susceptible animals, two pigs were immunized twice with the rP13C protein at an interval of 2 weeks. As shown in Table 1, the sera derived from one pig (#51) exhibited a virus neutralization test titer of 45 at 13 days post the second immunization and the titer declined to 22 at 20 days post the second immunization. The sera from the other pig (#71) exhibited a virus neutralization test titer of 16 at 13 days post the second immunization, followed by a decline at 20 days post the second immunization. The LPB ELISA also showed a similar pattern of titers for all swine sera, indicating that the rP13C protein preserved the neutralizing epitopes.

3.3. Production and characterization of Mab

The neutralizing Mab was produced from mice immunized with the FMDV VP1 peptide based on As1/MOG/05. The selected antibody, 1A31, is an IgG1 isotype and contains kappa light chains. It displayed positive reactivity for the Mongolian strain by IFA (Fig. 2A). To investigate whether the Mab could also bind to other strains of FMDV serotype Asia 1, IFA was conducted with Cambodian strain (CAM 9/80) and resulted in the same positive reactivity as the Mongolian strain (Fig. 2B). The Mab possesses FMDV neutralizing activity: the supernatant of the hybridoma culture showed virus neutralization test titer of 128 for the Mongolian and Cambodian strains altogether, indicating that the use of Mab is feasible as a detector antibody to compete with serum antibodies in the rP13C ELISA described in this study.

Table 1
Endpoint titers obtained by the virus neutralization test, liquid phase blocking ELISA, and rP13C ELISA.

Pig	Serum	Virus neutralization test	LPB ELISA	rP13C ELISA[a]
#51	Pre-immune	<16	<45	<10
	1st, 14 dpv[b]	<16	<45	<10
	2nd, 13 dpv	45 (1.0)[c]	90 (2.0)	60 (6.0)
	2nd, 20 dpv	22 (0.5)	45 (1.0)	40 (4.0)
#71	Pre-immune	<16	<45	<10
	1st, 14 dpv	<16	<45	<10
	2nd, 13 dpv	22 (0.5)	64 (1.5)	20 (2.0)
	2nd, 20 dpv	<16	45 (1.0)	10 (1.0)

[a] The endpoint titers by the rP13C ELISA indicate the final dilution of sera above the cut-off (PI 50) through serial dilution of sera.
[b] dpv: days post-vaccination.
[c] Brackets indicate the fold titers relative to the cut-off (titer 45) of the virus neutralization test and LPB ELISA, and standard serum dilution (1:10) of the rP13C ELISA.

Table 2
Specificity of the rP13C ELISA for naive sera ($n = 1760$) collected from domestic livestock.

Species	No. tested	No. positive	No. negative	Specificity (%)
Pigs	560	4	556	99.3
Cattle	640	2	638	99.7
Goats	560	0	560	100.0
Total	1760	6	1754	99.7

3.4. Establishment of rP13C ELISA

The rP13C ELISA was established using the rP13C and the neutralizing Mab (1A31). An initial standardization of all components was conducted to determine the optimal concentrations of rP13C antigen, peroxidase-conjugated Mab and serum dilution (data not shown).

To determine the cut-off level of PI for the rP13C ELISA, the naive sera ($n = 1760$) derived from domestic pigs, cattle and goats, were examined by the rP13C ELISA (Table 2). The cut-off level of PI was established at 50% to secure high specificity regardless of species, by calculating the mean value plus three times the standard deviation. With this cut-off level, the rP13C ELISA exhibited more than 99% specificity for each species sera, and overall, only six were interpreted as positive, the final specificity being 99.7%. In addition, as shown in Fig. 3, more than half of the total sera tested fell within PI 10.

On the basis of the preliminary conditions, the rP13C ELISA was evaluated in comparison with the LPB ELISA and virus neutralization test. These three tests were compared for caprine sera collected at 40 days after challenge with As1/MOG/05 FMDV following single vaccination with a trivalent vaccine (Fig. 4). The endpoint titers by the rP13C ELISA and LPB ELISA were equivalent for one caprine serum (#1) and approximately 3-fold higher than that by the virus neutralization test on the basis of each assay's cut-off level. For the other two caprine sera (#2 and #3), the endpoint titers by rP13C ELISA were 1.5-fold and 2-fold higher than those by the LPB ELISA

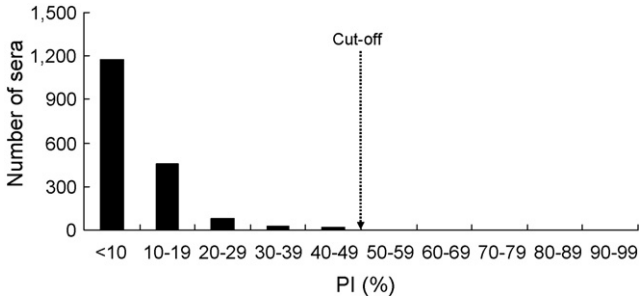

Fig. 3. Frequency distribution of the percentage inhibition recorded from naive sera ($n = 1760$) by the rP13C ELISA.

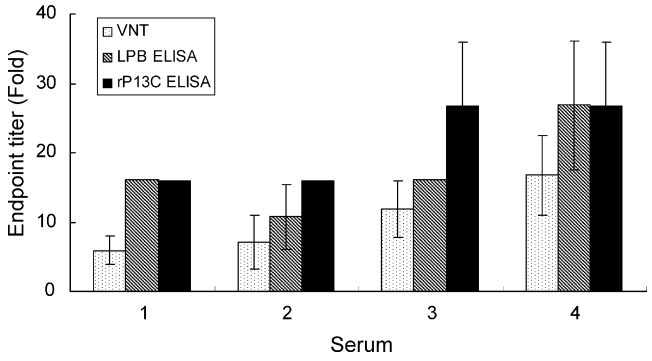

Fig. 4. Comparison of endpoint titers by three serological tests of sera derived from goats challenged with FMDV serotype Asia 1 post-vaccination. The endpoint titers in terms of fold are defined as the fold titers relative to the cut-off (titer 45) of the virus neutralization test and LPB ELISA, and standard serum dilution (1:10) of the rP13C ELISA.

and virus neutralization test, respectively. The rP13C ELISA and LPB ELISA indicated equivalent endpoint titers which were 1.5-fold higher than that by the virus neutralization test for one serum (#4).

To examine the utility of the rP13C ELISA for other FMD susceptible species, sera from two pigs immunized with the recombinant proteins (rP13C) were employed (Table 1). Three serological tests showed similar titer patterns, reaching a peak at 13 days post the second immunization and declining at 20 dpi. For one swine serum (#51) collected at 13 days post the second immunization, the endpoint titer by rP13C ELISA was about 6-fold and 3-fold higher than those by the virus neutralization test and LPB ELISA, respectively. For the serum (#51) collected at 20 days post the second immunization, the endpoint titer by rP13C ELISA was 8-fold and 4-fold higher than those by the virus neutralization test and LPB ELISA, respectively. The endpoint titer by the rP13C ELISA was equivalent to that by the LPB ELISA and higher than that by the virus neutralization test for the other swine sera (#71) collected at 13 and 20 days post the second immunization.

Since the rP13C protein and 1A31 Mab were produced on the basis of the Mongolian strain sequence, it was necessary to ascertain whether the present rP13C ELISA could also detect antibodies against other strains of FMDV Asia 1. As a preliminary test for this broad spectrum of reactivity, positive control sera enclosed in the LPB ELISA kit were employed. As shown in Table 3, the rP13C ELISA gave an endpoint titer of 4-fold higher than that by the LPB ELISA for Asia 1 strong-positive serum (C++) which was derived from cattle infected with Israeli strain of FMDV. For the Asia 1 moderate-positive serum (C+), the rP13C ELISA also resulted in an endpoint titer that was 2-fold higher than that by the LPB ELISA. To test cross-reactivity with other serotypes, control positive sera (also enclosed in the LPB ELISA kits) were applied. While the rP13C ELISA reacted

Table 3
Examination of various serotypes of bovine sera by the rP13C ELISA and LPB ELISA.

Serum	rP13C ELISA[a]	LPB ELISA
Asia 1 (C++)[b]	160 (16)[c]	180 (4)
Asia 1 (C+)[b]	40 (4)	90 (2)
O[d]	10 (1)	45 (1)
A[d]	<10	45 (1)
C[d]	20 (2)	180 (4)

[a] The endpoint titers of the rP13C ELISA indicate the final dilution of sera above the cut-off (PI 50) through serial dilution of sera.

[b] Asia 1 (C++) and Asia 1(C+) are the control strong- and moderate-positive sera enclosed in the LPB ELISA (serotype Asia 1) kit, respectively.

[c] Brackets indicate the fold titers relative to the cut-off (titer 45) of the LPB ELISA and standard serum dilution (1:10) of the rP13C ELISA.

[d] O, A, and C are the control strong-positive sera enclosed in the LPB ELISA (serotypes O, A, and C) kits, respectively.

Table 4
Examination of the international reference sera for FMD Asia 1 from by the rP13C ELISA and LPB ELISA.

Serum[a]	rP13C ELISA[b]	LPB ELISA[b]
Strong positive	91 ± 0.0	94 ± 0.4
Weak positive	60 ± 0.6	93 ± 0.5
Cut-off	26 ± 3.9	50 ± 4.2
Negative	6 ± 1.2	18 ± 4.8

[a] International reference sera were generated for the FAO standardization study phase XVIII and obtained from the Institute for Animal Health (Pirbright Laboratory, Surrey, UK).

[b] Values are expressed as the mean percentage inhibition of the serum ± standard deviation ($n = 6$).

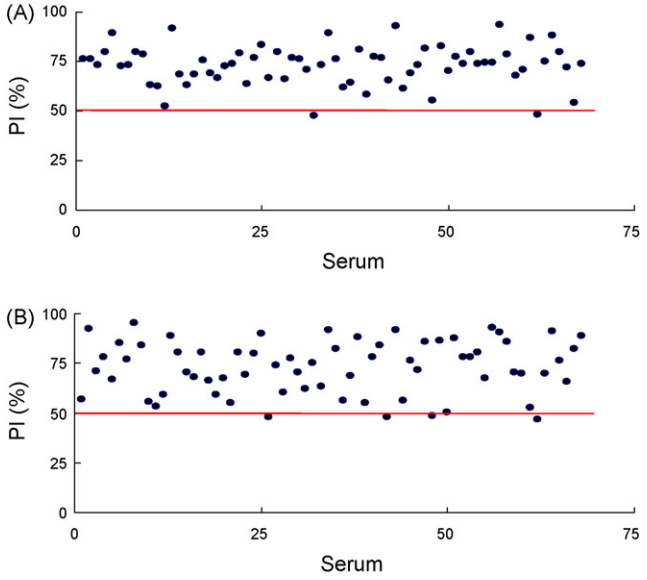

Fig. 5. Comparison of (A) the LPB ELISA and (B) the rP13C ELISA for bovine sera ($n = 68$) collected post the second vaccination with a FMDV trivalent (O, A, and Asia 1) vaccine during the 2000 FMD outbreak in the Republic of Korea.

with serotype O serum at a level comparable to the LPB ELISA, it showed less cross-reactivity than the LPB ELISA for serotype A and C sera.

In addition, the rP13C ELISA was evaluated for the FAO Phase XVIII international reference sera comprising strong, weak and cut-off levels (Table 4). Whereas the LPB ELISA scored the cut-off serum as bordering positive, the rP13C ELISA scored the weak positive serum as positive and the cut-off serum as negative.

3.5. Validation of the rP13C ELISA

To examine whether the rP13C ELISA could replace the LPB ELISA for serological surveillance in the field, 68 bovine sera collected post-vaccination during the 2000 FMD outbreak in Korea were employed. While the rP13C ELISA scored four sera as negative, the LPB ELISA scored only two sera as negative (Fig. 5). Of the four negative sera by the rP13C ELISA, only one serum was also negative by the LPB ELISA. The other three sera, however, were distributed around the cut-off level, PI 50, suggesting that overall, the rP13C ELISA is equivalent to the LPB ELISA in detecting antibodies to FMDV Asia 1 in vaccinated bovine sera.

4. Discussion

In this study, recombinant proteins were produced in insect cells to replace the inactivated FMDV used for detecting antibodies to FMDV serotype Asia 1.

Previously, a VP1 peptide-based ELISA (United Biomedical Inc., NY, USA) was commercially available to measure the serological response to FMDV serotype O structural proteins. However, it was reported that sera collected from animals immunized with the commercial vaccine failed to react with the VP1 peptide antigen in the ELISA, indicating that the VP1 peptide lacks the antigenic structure recognized by neutralizing antibodies elicited by inactivated FMDV in the commercial vaccine (Rodriguez et al., 2003). Hence, it was necessary to overcome the limitation of the peptide antigen including only B cell epitope by constructing virus-like particle with P1 and 3C genes in accordance with the natural FMDV assembly mechanism.

Several studies have reported that the P1 precursor and 3C or 3CD protease genes in the family *Picornaviridae* make virus-like particles by *E. coli*, baculovirus, and vaccinia virus vectors (Abrams et al., 1995; Brautigam et al., 1993; Hu et al., 2003; Lewis et al., 1991; Roosien et al., 1990). However, these studies did not yield a sufficient amount of particles to be analyzed further. In contrast to poliovirus, the inclusion of 3CD instead of 3C alone did not affect the efficiency of protein processing in FMDV (Roosien et al., 1990; Ypma-Wong et al., 1988). In addition, when individual P1 and 3CD baculoviruses were co-infected into insect cells, the yield of virus-like particle was low, suggesting that a single baculovirus containing both P1 and 3C genes might be more productive because P1 and 3C could be expressed at similar levels (Hu et al., 2003). Based on these results, an effort to produce FMDV-like particles were made using a single baculovirus vector containing both P1 and 3C genes in insect cells to replace the current inactivated FMDV used for detecting antibodies to serotype Asia 1.

The expression and cleavage of the P1 precursor by 3C protease was confirmed using Western blot analysis by identification of VP1 band which did not appear when P1 alone was expressed. Even though it was not confirmed by electron microscopy that the subunits derived from P1 cleavage by 3C could assemble into pentamer-like particles as the O serotype (Oem et al., 2007), it was apparent that the rP13C protein shares common structures with the authentic FMDV because it competed with FMDV neutralizing antibodies elicited by vaccination or by FMDV infection, as demonstrated in this study.

The rP13C expressed in insect cells could elicit neutralizing antibodies in pigs, although, as expected, the virus neutralization test titers were low because the immunogen, rP13C, was not either concentrated or purified, but the lysate supernatant. To assess the rP13C as a recombinant protein vaccine, further immunogenicity study remains to be done with serial concentrations of purified rP13C protein in comparison with the current inactivated FMDV vaccine.

The Mab (1A31) used as a diagnostic detector in this study was generated by immunizing a VP1 peptide corresponding to the GH loop which is known to be immunologically important as it contains the cell receptor site (Acharya et al., 1989; Bittle et al., 1982; Cooke and Westover, 2008; Pfaff et al., 1982; Strohmaier et al., 1982; Wang et al., 2002). A previous study reported of a Mab recognizing continuous antigenic site I (residues 144–159) that was used for solid-phase ELISA to detect O serotype FMDV antibodies (Chenard et al., 2003). The use of a Mab in the present ELISA also assures consistent quality control compared to a polyclonal serum by minimizing batch to batch variation. Since most of the FMDV variation leading to codon changes and subsequent new viral phenotypes occurs within VP1 (Bittle et al., 1982; Cooke and Westover, 2008; DiMarchi et al., 1986; Grubman and Baxt, 2004), it was necessary to ascertain that the 1A31 Mab also binds to FMDV Asia 1 strains other than the Mongolian strain from which the Mab was derived. In this regard, 1A31 Mab was analyzed against the Cambodian FMDV strain. More extensive study needs to be done for other strains of FMDV in the future to ensure that 1A31 Mab can bind to a broad spectrum of Asia 1 strains.

Since the rP13C ELISA is a sandwich ELISA with the 70-17 Mab being coated to capture the rP13C proteins, it was not necessary to purify rP13C to remove the other proteins derived from insect cells. In addition, since the rP13C ELISA is performed in a blocking ELISA format, it could be used to test sera in the same manner regardless of animal species.

It may seem contradictory that while the VP1 peptide epitope did not display sufficient antigenicity to match the whole FMDV structure (Rodriguez et al., 2003), the Mab generated using the VP1 peptide could be used successfully in this study. However, it is well known that epitopes on the antigen surface may be recognized directly by the competitor Mab or blocked by steric hindrance or conformational changes induced by antibodies binding to other epitopes (Sugiyama et al., 1997).

The higher endpoint titer derived by the rP13C ELISA compared to those by the LPB ELISA and virus neutralization test for sera collected from goats challenged with FMDV Asia 1 post-vaccination (Fig. 4) may have been due to the strain (As 1/MOG/05) being homologous to that from which the rP13C and Mab were derived. The 1A31 Mab used in the rP13C ELISA could also neutralize the Cambodian strain of FMDV Asia 1 to the same extent as the Mongolian strain. These results suggest that although it remains to be examined with many sera, the rP13C ELISA has the potential to replace the LPB ELISA and virus neutralization test for FMDV Asia 1 serological surveillance in Southeast Asian countries where FMD is endemic.

The rP13C ELISA resulted in a higher endpoint titer than those by the virus neutralization test and LPB ELISA for sera derived from pigs immunized with rP13C protein. This result may also have been due to the homologous immunogen. However, it is notable that the rP13C ELISA exhibited the same titer pattern as the virus neutralization test and LPB ELISA for all swine sera, of which titer values bordered the cut-off level of each assay.

Since Middle Eastern areas are at high risk for FMD through trade in live ruminants from Asia and Africa, and some countries are considered to have endemic FMD (Rweyemamu et al., 2008), it was necessary to test sera derived from FMDV strains having originated in this area. Hence, additional control positive sera, enclosed in the LPB ELISA kit, which were derived from cattle infected with the Israeli strain of FMDV Asia 1, were employed to assess the rP13C ELISA in relation to the LPB ELISA. Although the control positive serum was derived from a FMDV strain homologous to the antigen enclosed in the LPB ELISA kit, the endpoint titer by the rP13C ELISA was higher than that by the LPB ELISA. In the future, this comparison needs to be made with sera derived from many other strains of FMDV Asia 1 to validate the rP13C ELISA more thoroughly.

As additional evidence of the rP13C ELISA utility, international reference sera (derived from Shamir strain of FMDV Asia 1) from the FAO standardization study phase XVIII were examined. The rP13C ELISA scored weak positive serum correctly. The weak positive serum has been suggested to be used in representing the minimum standard for detecting antibodies in any test applied for herd-based serosurveillance (Niedbalski, 2004). The result that the rP13C ELISA scored the cut-off serum as negative while the LPB ELISA scored it as borderline positive does not devaluate the utility of the rP13C ELISA, considering the previous report that the OIE cut-off reference sera for FMDV serotype O antibody assays were negative by the SPC ELISA (Niedbalski, 2004).

Serological tests to detect antibodies against FMDV structural proteins can also be useful to assess vaccination status, as well as FMDV infection status. To this end, the rP13C ELISA was compared to the LPB ELISA for bovine sera vaccinated with a trivalent vaccine (O, A, and Asia 1) in the field, revealing that overall, the two tests were equivalent in detecting antibodies to FMDV structural proteins in vaccinated bovine sera. With the trivalent vaccine, antibodies

would be raised against serotype O, A, and Asia 1. This may explain why the LPB ELISA scored more sera as positive compared to the rP13C ELISA, since there was a slightly higher cross-reactivity for serotype A by the LPB ELISA (Table 3).

Currently, the potency of FMD vaccines is assessed in vivo by challenging fully susceptible vaccinated cattle. Many studies have reported on alternative FMD vaccine potency tests that are more amenable from an animal welfare perspective and show better statistical reliability (Barnett et al., 2003; Goris et al., 2008; Pay and Hingley, 1992; Robiolo et al., 1995; Van Maanen and Terpstra, 1989). In this regard, the rP13C ELISA could be employed as a simple and reliable method to replace the LPB ELISA or virus neutralization test. Most of all, the recombinant protein antigen, rP13C, could be easily produced in general laboratory without any limitation, which is an advantage of the rP13C ELISA over the other current serological tests that require inactivated FMDV as the diagnostic antigen.

In conclusion, this is the first report to demonstrate that a recombinant protein-based ELISA could be used as an alternative to the LPB ELISA or virus neutralization test for detecting antibodies to FMDV serotype Asia 1.

The recent spread of FMD virus throughout the Asian continent demonstrates the continuing need for active surveillance and further development of regional control programs (Valarcher et al., 2005). The present rP13C ELISA would be a useful tool for these purposes.

Acknowledgements

We would like to thank Hyo-Rim Ko for her excellent technical assistance. This study was supported by a grant from the National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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