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A simple and rapid assay to evaluate purity of foot-and-mouth disease vaccine before animal experimentation

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ABSTRACT

Currently, foot-and-mouth disease (FMD) vaccine purity is tested in cattle to detect antibodies against the non-structural protein (NSP) after repeated immunization with the final vaccine product. In case of vaccine failure, the manufacturing company would suffer significant economic loss. To prevent such unfortunate losses with the final vaccine product, *in vitro* testing is required to quantitate an NSP antigen during the manufacturing process prior to animal experiments. A novel lateral-flow assay device was developed using a monoclonal antibody (MAb) against the 3B NSP. To determine the minimal amount of NSP required to elicit antibodies in livestock, goats were immunized several times with various concentrations of either the recombinant 3AB (rec.3AB) protein or FMD virus culture supernatant. Antibodies against 3AB were elicited after a second immunization with 10.6 ng to 42.5 ng of rec.3AB and a third immunization with a 10-fold diluted FMD virus culture supernatant in goats. The lateral-flow assay device detected the minimal amount of rec.3AB and native NSP in FMD virus culture supernatant required to induce NSP antibodies in goats. The *in vitro* assay device is simple and economical, provides rapid results, and should be useful for FMD vaccine-manufacturing companies prior to conducting animal experiments to test the vaccine purity.

1. Introduction

Foot-and-mouth disease (FMD), which infects cloven-hoofed animals, is economically important due to its high contagiousness [1]. FMD virus (FMDV), the causative agent of FMD, belongs to the *Aphthovirus* genus of the *Picornaviridae* family [2]. FMDV has a positive-sense, single-stranded RNA genome that is translated into a polyprotein, which is further cleaved into 4 structural proteins (SPs; VP1, VP2, VP3, and VP4) and 10 nonstructural proteins (NSPs; L, 2A, 2B, 2C, 3A, 3B, 3C, 3D, 3AB, and 3ABC) [1,3,4].

In South Korea, eleven FMD outbreaks have occurred since 2000 (March 2000, May 2002, January 2010, April 2010, November 2010, July 2014, December 2014, January 2016, February 2017, March 2018, and January 2019) [5]. To control annual outbreaks, the Korean government implemented a nationwide vaccination policy after the catastrophic FMD outbreak occurring in 2010 and 2011.

During serological surveillance in vaccinated areas, NSP antibodies were detectable not only in FMDV-infected animals, but also in some uninfected animals, especially those that were vaccinated several times (because current FMD vaccines inevitably contain small amounts of NSP even though purification steps are involved in FMD vaccine-manufacturing processes). To differentiate between infected and vaccinated animals, the World Organization for Animal Health (OIE) set up guidelines for determining vaccine purity; after at least eight cattle are immunized twice with an

Abbreviations: TMB, 3,3′,5,5′-tetramethylbenzidine; BHK-21, Baby hamster kidney-21; BEI, Binary ethylenimine; BSA, Bovine serum albumin; ELISAs, Enzyme-linked immunosorbent immunoassays; FAL-ELISA, Filtration-assisted chemiluminometric enzyme-linked immunosorbent assay; FMDV, FMD virus; FMD, Foot-and-mouth disease; HRP, Horseradish peroxidase; LFD, Lateral-flow assay device; MAb, Monoclonal antibody; MOI, Multiplicity of infection; NSP, Non-structural protein; PI, Percent inhibition; PBS, Phosphate-buffered saline; PBS, Phosphate-buffered saline; PBS-T, containing 0.05% Tween 20; POCT, Point-of-care testing; rec., Recombinant; RT, Room temperature; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SP, Structural protein; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 0.1% of Tween 20; VPg, Viral genome-linked protein; OIE, World Organization for Animal Health.

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FMD vaccine, the presence of NSP antibodies in up to one animal is acceptable [6]. However, this animal experiment is time consuming and costly. Moreover, if a vaccine fails to meet the requirements of purity, manufacturers are required to discard the entire batch.

Previously, the filtration-assisted chemiluminometric enzyme-linked immunosorbent assay (FAL-ELISA) method was reported to quantitate an NSP antigen in vaccines [7]. However, the FAL-ELISA method is expensive to conduct regularly, demands experimental proficiency, and takes several hours. Therefore, we aimed to develop a novel simple, economic, and rapid NSP-detection assay to perform during the FMD vaccine-manufacturing process prior to conducting animal experiments to test the vaccine purity.

2. Materials and methods

2.1. Cells and viruses

A type-O FMDV strain (Jincheon/SKR/2014), which was isolated in Jincheon County (South Korea) in 2014, was used to inoculate a suspension of baby hamster kidney-21 (BHK-21) cells (6×10^6 cells/ml) a multiplicity of infection (MOI) of 0.01 in a shaking incubator at 37 °C with 5% CO_2. Viruses were harvested at 8, 12, 16, or 20 h post-infection and clarified by centrifugation at 3,134 × *g* for 20 min at 4 °C to remove cell debris. Binary ethylenimine (BEI) was added at 3 mM to the cell culture supernatants to inactivate FMDV, followed by incubation at 65 rpm for 24 h at 26 °C. Subsequently, the BEI was neutralized by adding 2% sodium thiosulfate (Daejung Chemicals & Metals, Siheung, Korea).

2.2. Production of synthetic peptides and recombinant proteins

Recombinant NSP proteins were expressed in *Escherichia coli*, and the synthetic peptides were produced in Bioneer, Inc. (Daejeon, Korea) as described previously [8].

2.3. Selection of monoclonal antibody (MAb) against the 3B epitope

Hybridomas producing MAbs against 3B were prepared by fusing splenic lymphoid cells from immunized BALB/c mice with the SP2/0 myeloma cell line. For this procedure, the 3B protein (0.2 mg), which was emulsified with adjuvants, was intraperitoneally injected twice into BALB/c mice at two-week intervals (Orient Bio, Seongnam, Korea). Hybridoma cells were screened via indirect enzyme-linked immunosorbent immunoassays (ELISAs) using recombinant 3A (rec.3A), rec.3B, and rec.3AB. Clones specific to the 3B epitope with a positive/negative ratio greater than 5 in the indirect ELISA were selected for further propagation. The final selected MAb (4G24) was isotyped using mouse MonoAB-ID/SP kits (Zymed, San Francisco, USA).

2.4. ELISA for measuring reactivity of MAb 4G24 with recombinant proteins and peptides

Indirect ELISAs were conducted to evaluate the 4G24 reaction specificity for the 3B epitope. Briefly, 96-well plates were coated overnight at 4 °C with 100 μl (1.0 μg/ml) recombinant proteins (3A, 3B, or 3AB) or a synthetic peptide conjugated to ovalbumin (2C, 3A, 3B, or 3C) in 0.1 M bicarbonate buffer (pH 9.6). After washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), the plates were incubated with 250 μl of blocking buffer (3% bovine serum albumin [BSA] in PBS-T) at room temperature (RT) for 2 h. After washing with PBS-T, 50 μl MAb 4G24 (1.0 μg/ml) was added to the plates and the plates were incubated at RT for 1 h. The plates were washed with PBS-T, and 50 μl (0.5 μg/ml) of a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) was added to each well, followed by incubation at RT for 1 h. After washing the plates, the 3,3′,5,5′-tetra methylbenzidine (TMB) substrate was added. The color was developed over a 15-min incubation at RT, and the reaction was stopped by adding 50 μl of 0.5 M sulfuric acid. The optical densities of the samples were measured at 450 nm.

2.5. Western blot analysis of MAb 4G24

Purified recombinant proteins (3A, 3B, and 3AB) or FMDV culture supernatant were mixed with 4 × LDS sample buffer (Invitrogen, Carlsbad, USA). The pretreated samples were heated at 99 °C for 10 min. Samples were separated on 12% Bis-Tris gels (Invitrogen, Carlsbad, USA). The resolved samples were electro-transferred to a polyvinylidene difluoride membrane using the iBlot™ gel-transfer device (Invitrogen, Carlsbad, USA). The membranes were blocked with 2% (w/v) skim milk in TBS-T buffer (Tris-buffered saline containing 0.1% of Tween 20) and incubated with MAb 4G24 (1.9 mg/ml) diluted 1:2,000, followed by incubation with a goat anti-mouse HRP conjugated secondary antibody (Invitrogen, Rockford, USA) diluted 1:5,000. Proteins were visualized with the Pierce ECL Western Blotting Substrate (Invitrogen, Rockford, USA) using an Azure C600 device (Azure Biosystem, Dublin, USA).

2.6. Animal experiments

To determine the minimal amount of NSP to induce NSP antibodies in goats, serially diluted rec.3AB (170, 42.5, 10.6, or 2.6 ng/dose) or inactivated FMDV culture supernatant (diluted 10^0, 10^{-1}, 10^{-2}, or 10^{-3}) were mixed 1:1 with ISA 206VG adjuvant (Seppic, Paris, France). The mixture was stirred at 300 rpm for 5 min at 30 in a water bath.

Four-month-old male goats, which were not previously FMD-vaccinated, were tested by ELISA after selecting animals without antibodies for SP and NSP. Goats in the first set (n = 40) were immunized with in-house vaccines containing different concentration of rec.3AB. Goats in the second set (n = 40) were immunized with the in-house vaccine comprised of virus culture supernatant at different dilutions. All goats were immunized three times at 4-week intervals and bled at the ages of 16, 20, 24, and 28 weeks. Animal experiments in this study were approved (APQA 2017-375) and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.7. Elisas for detecting SP and NSP antibodies

PrioCHECK FMDV type O (Prionics, Lelystad, Netherlands) and PrioCHECK FMDV NS (Prionics, Lelystad, Netherlands) ELISA kits were used according to the manufacturer's instructions. The ELISA results were expressed as percent inhibition (PI) values. Samples showing a PI value of ≥ 50% were considered positive, and those with a PI value of < 50% were considered negative.

2.8. Construction of lateral-flow assay device (LFD) and application

The LFD was prepared as previously described [9], with some modifications. The LFD strip consisted of a sample pad, a nitrocellulose membrane, and an absorbance pad mounted on a plastic support. MAb 4G24 and anti-mouse IgG (Arista Biologicals, Allentown, USA) were diluted in 2.5 mM PBS containing 0.5% sucrose (Sigma–Aldrich, St. Louis, USA) and 0.1% sodium azide, to obtain final antibody concentrations of 2 mg/ml. Anti-mouse IgG and MAb 4G24 were immobilized onto the control line and test line, respectively, on a nitrocellulose membrane (Millipore, Darmstadt, Germany) attached to a backing card (PJ, Bucheon, Korea), using an automatic distributer (Kinematic automation, Twain Harte,

USA). After the membrane was dried in a dehumidification chamber for over 24 h, it was cut into 4 mm wide strips using a cutting machine (Zeta Corporation, Gunpo, Korea). Each membrane piece was then assembled on a membrane cassette, with a sample pad and an absorbance pad. The strips were then stored under dry conditions at RT until used.

Sample buffer was prepared as PBS containing 0.05% Tween 20 and 0.01% sodium azide. Colloidal gold with a mean diameter of 40 nm (BBI Solutions, Crumlin, UK) and MAb 4G24 were mixed at 37 °C for 30 min (final concentrations of 5 μg/ml for both materials), and then 0.3% casein (Sigma–Aldrich, St. Louis, USA) was added as a blocking agent. The mixture was incubated with stirring at RT for 30 min. After the mixture was centrifuged at 14,000 × *g* for 30 min, the supernatant was discarded, and the pellet was resuspended in 1/10 of the original volume in 2 mM borax buffer containing 1% BSA. The concentration of the conjugate was adjusted to OD_{525} = 1.5.

A brief description of the test procedure is as follows. The colloidal gold–MAb conjugate was mixed 1:4 with sample buffer, and then the diluted gold–MAb conjugate was reacted with the same volume of antigen samples for 5 min at RT. After these components were mixed well, 100 μl of each sample was added to the strip sample-application area. Following a 20-min incubation at RT, the line in the strip was examined by the naked eye or with a point-of-care testing (POCT) reader (Medisensor, Inc., Daegu, Korea). To quantitate an NSP antigen in each sample, the LFD was applied to the POCT reader to digitalize the result. Briefly, the POCT reader detected the densities of each band, indicating positivity in the LFD, and the analog signal was converted to a digital signal through an analog/digital converter in the device. First, a standard curve equation was deduced from two-fold serial dilutions of rec.3AB. Second, the putative amount of 3B-containing NSP in the virus culture supernatant was estimated by interpolating the POCT reader value with the standard curve equation.

2.9. Evaluating the repeatability of LFD

Five rec.3AB samples were prepared by diluting a stock solution (1 mg/ml) with PBS to respective designated concentration (50, 25, 12.5, 6.25, and 1 ng/ml) and tested in triplicate to evaluate the repeatability of LFD using the POCT reader. The coefficients of variation (CV) of intra- and inter-assay were also calculated.

3. Results

3.1. Characterization of MAbs against NSP

The reactivity of MAb 4G24 was examined by indirect ELISA and western blot analysis using purified rec. proteins (3A, 3B, and 3AB) and FMD virus culture supernatant. In indirect ELISA, the MAb 4G24 reacted strongly with rec.3B, rec.3AB, and peptide 3B1, but not peptides 2C, 3A, 3C, and rec.3A (Table 1). MAb 4G24 also reacted with rec.3B (29 kDa) and rec.3AB (48 kDa), but not rec.3A (39 kDa) in western blots (Fig. 1A). The MAb reacted with 3AB proteins in virus culture supernatants derived from BHK-21 cells infected with FMDV. The virus culture supernatants were collected from 8 h to 20 h post-infection (p.i.) (Fig. 1B). Among the NSPs in the FMDV-infected cell lysate supernatant, 3AB consisting of three split bands ($3AB_1$, $3AB_{12}$, and $3AB_{123}$) was the major band, and its expression level gradually increased over time as viral infection continued.

3.2. The minimal amount of rec.3AB required to induce NSP antibodies in goats

Experimental oil vaccines containing various concentrations (170, 42.5, 10.6, and 2.6 ng/dose) of rec.3AB were administered to goats three times at 4-week intervals (Fig. 2). We found that 4/8 goats inoculated with 170 ng rec.3AB produced NSP antibodies following the first immunization, while 2/8 goats inoculated with 42.5 or 10.6 ng rec.3AB produced NSP antibodies following second immunization. Sera from goats inoculated with 2.6 ng rec.3AB were all negative for NSP antibodies (Fig. 2A). Before immunization, all goats showed a PI value of approximately 20% (Fig. 2B). The group inoculated with 170 ng rec.3AB showed 48.4%, 62.5%, and 91.1% positivity for NSP antibodies after the first, second, and third immunization, respectively.

Whereas goats inoculated with 42.5 ng or 10.6 ng rec.3AB showed 56.8% or 50.7% positivity for NSP antibodies, respectively, after the third immunization, goats inoculated with 2.6 ng rec.3AB remained negative for NSP antibodies (even after the third immunization), and the PI values did not increase compared to the pre-immunization values (Fig. 2B).

Table 1
Reactivity of MAb 4G24 with recombinant proteins and synthetic peptides.

Antigen	Name	ELISA (A_{450nm})
Recombinant protein	3A	0.183
	3B	3.852
	3AB	3.879
Synthetic peptide	2C[1]	0.139
	3A[2]	0.157
	3B[3]	2.267
	3C[4]	0.126
Control protein	OVA	0.073

[1] 2C: N-VSAKDGYKINNKL-C.
[2] 3A: N-TDDKTLDEAEKNPL-C.
[3] 3B: N-GPYTGPLERQKPLKY-C.
[4] 3C: N-LHRGNRVRDITKHF-C.

3.3. The minimal amount of NSP in virus culture supernatant required to induce NSP antibodies in goats

FMDV culture supernatant contains a variety of NSP forms, including 3AB. To determine the minimal amount of NSP in FMDV culture supernatant needed to induce NSP antibodies, experimental oil vaccines containing virus culture supernatant (diluted 10^0, 10^{-1}, 10^{-2}, or 10^{-3}) were administered to goats three times at 4-week intervals. Before immunization, all goats were negative for both SP and NSP antibodies (Fig. 3). While the SP antibody was elicited after the first inoculation with crude FMDV culture supernatant (n = 7/10, PI = 63%, Fig. 3A), the goats did not show NSP antibody responses at all. Only after the second immunization did two goats inoculated with the crude FMDV culture supernatant produce NSP antibody (n = 2/10, PI = 24.5%, Fig. 3B), and the mean PI value also increased through the third immunization. For the group inoculated with 10-fold-diluted virus culture supernatant, one out of ten goats showed seroconversion for NSP antibodies after the third immunization, with a mean PI value of 23.2% (Fig. 3B). Even though the group immunized with virus culture supernatant diluted 10^3-fold exhibited seroconversion for SP antibodies (n = 1/10), FMDV culture supernatants diluted 10^2-fold or more neither induced positive responses in terms of NSP antibodies, nor showed elevated mean PI values, even after the third immunization.

3.4. Development and evaluation of the LFD

To quantify the LFD results, we generated a standard curve using 2-fold serial dilutions of rec.3AB (85–2.6 ng/ml) and calculated the amount of 3AB in the tested samples using the POCT reader. In the repeatability test using five different concentrations

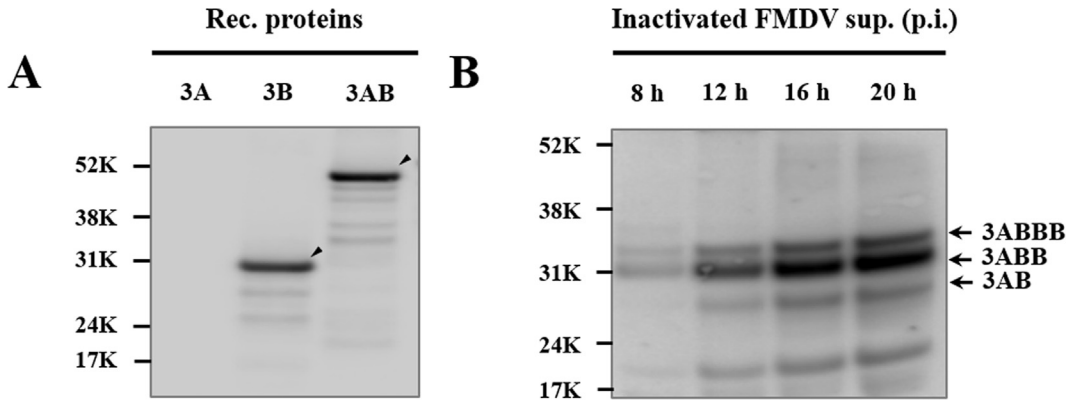


Fig. 1. Characteristics of MAb 4G24. (A) Purified rec. proteins (3A, 39 kDa; 3B, 29 kDa; 3AB, 48 kDa) or (B) 3B-containing elements in FMDV culture supernatants (sup.), collected at different time post-inoculation (p.i.) were separated by SDS-PAGE. Western blotting was performed using MAb 4G24 against the FMDV 3B epitope.

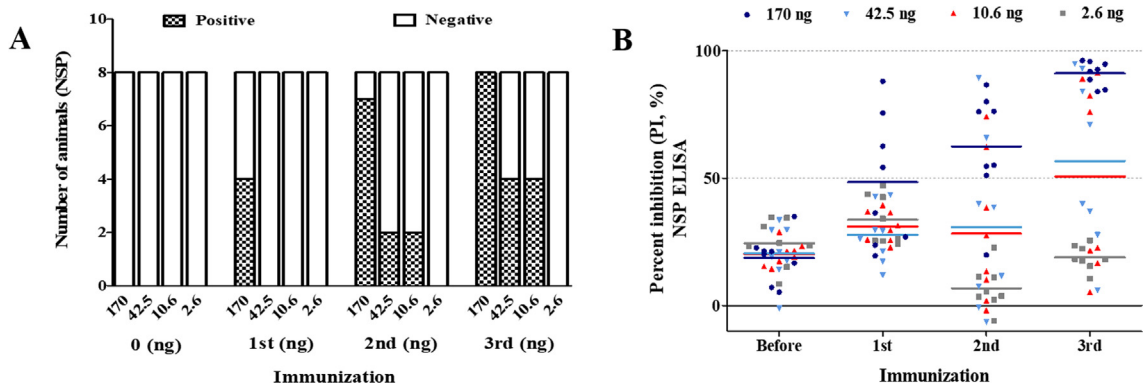


Fig. 2. Determination of the minimal amount of rec.3AB required to induce NSP antibodies in goats. The results are expressed as (A) the number of animals or (B) percent-inhibition values (PI < 50%, negative; PI ≥ 50%, positive).

of rec.3AB, the CV% of intra-assay ranged from 2.76 to 8.75, while that of inter-assay was between 2.6 and 12.93 (Table 2).

When virus culture supernatants were applied (Table 3), the undiluted ($1\ 0^{0}$) virus culture supernatant showed a standardized value of 45.73 ± 1.95 ng/ml, and NSP antibodies were elicited in goats after the second immunization. This finding was reasonable based on the result showing an NSP antibody response in goats after the second immunization with 42.5 ng rec.3AB. The supernatant diluted 10^{-1} contained 15.03 ± 0.24 ng/ml of 3AB protein, which triggered NSP antibody responses after the third immunization in goats. The quantity of 3AB protein present in the virus culture supernatants after the 10^{-2} or 10^{-3} dilutions was not detectable using the LFD. In goat experiments, those two dilutions did not induce NSP antibodies, as was the case after inoculating goats with 2.6 ng of rec.3AB.

4. Discussion

To examine the FMD vaccine purity, manufacturers have to conduct animal experiments with final vaccine products according to the guidelines of the OIE Terrestrial Manual. Before 2017, cattle needed to be vaccinated three times and then tested for the presence of NSP antibodies. The vaccine purity was acceptable only if all cattle showed negative results in NSP assays [10]. Since 2017, the OIE guidelines for FMD vaccine purity changed so that FMD vaccines were considered acceptable if less than two cattle were positive for NSP antibodies after being vaccinated twice [6]. However, this *in vivo* test imposes an economic burden and labor-intensive tasks to FMD vaccine manufacturers, since it is to be conducted with a final vaccine product. In cases where the vaccine induced NSP antibodies in cattle after repeated vaccination, those batches should be discarded.

In this regard, we sought to devise an *in vitro* assay to quantitate NSPs during the vaccine antigen-production process prior to animal experiments. Although a FAL-ELISA kit (Prionics AG, Zurich, Switzerland) is commercially available, it is expensive to use on a regular basis, requires expertise, and takes several hours. Therefore, the aim of this study was to develop a novel simple, economical, and rapid NSP-detection assay for use during the manufacturing process as a vaccine-purity test. The present LFD overcame some drawbacks of the commercial FAL-ELISA, suggesting that it can be utilized more routinely. To obtain robust test results by LFD, test band intensities were quantified by connecting the LFD to an automatic POCT reader. After a standard curve was generated based on the POCT reader results with serially diluted rec.3AB, the amount of NSP in unknown samples was determined by interpolation.

In this study, we used rec.3AB as a standard material not only for applications with the POCT reader, but also for determining the minimal amount of NSP required to induce antibodies in goats for following reasons. First, the ELISA kit used most commonly worldwide to detect NSP antibodies is the PrioCHECK FMDV NS kit (Prionics AG, Zurich, Switzerland), and it was designed to detect the 3B epitope [11]. MAb 4G24, which was generated in this study using the 3B peptide as the immunogen, also showed reactivity specific to the 3B epitope in indirect ELISA and western blot experiments (Fig. 1 and Table 1). Second, the OIE manual specifies that 3AB and 3ABC are the most reliable indicators of FMDV infection in serological analyses [12,13]. Third, previous reports showed that

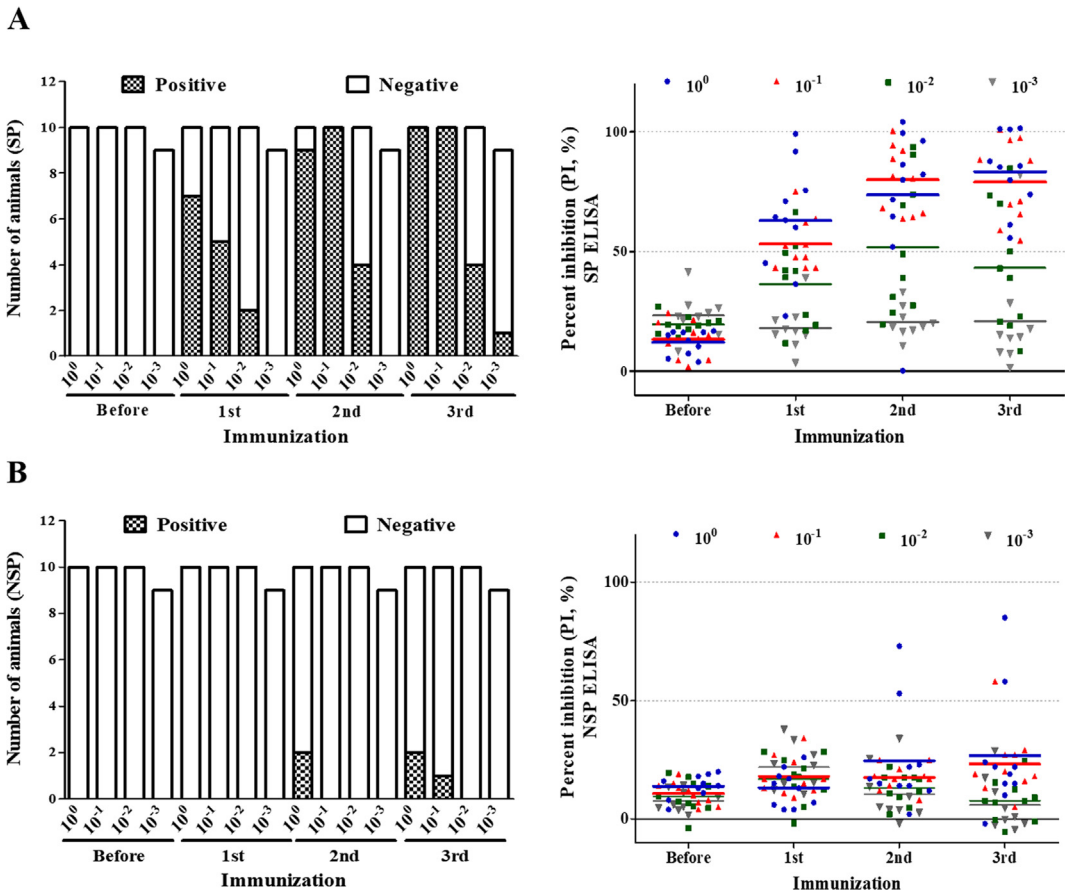


Fig. 3. Determination of the minimal amount of NSP in virus culture supernatant required to induce NSP antibodies in goats. The results are expressed as the number of animals and the percent-inhibition values (PI < 50%, negative; PI ≥ 50%, positive), as determined using (A) the PrioCHECK FMDV Type O ELISA Kit and (B) the PrioCHECK FMDV NS ELISA Kit.

Table 2
Repeatability tests of LFD with POCT reader.

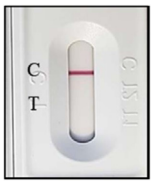
Sample ID. (actual conc.)[1]	Test 1[2]	Test 2	Test 3	Mean	SD[3]	CV%[4]
Intra-run repeatability test						
#1 (50 ng/ml)	52.40	56.56	58.67	55.88	3.19	5.71
#2 (25 ng/ml)	31.92	30.44	30.44	30.93	0.85	2.76
#3 (12.5 ng/ml)	13.07	13.50	15.16	13.91	1.10	7.93
#4 (6.25 ng/ml)	9.35	7.86	8.87	8.69	0.76	8.75
#5 (1 ng/ml)	N.D.[5]	N.D.	N.D.	N.A.[6]	N.A.	N.A.
Inter-run repeatability test						
#1 (50 ng/ml)	52.40	50.03	52.30	51.58	1.34	2.60
#2 (25 ng/ml)	31.92	28.59	30.05	30.19	1.67	5.53
#3 (12.5 ng/ml)	13.07	11.89	13.61	12.86	0.88	6.84
#4 (6.25 ng/ml)	9.35	7.23	8.74	8.44	1.09	12.93
#5 (1 ng/ml)	N.D.[5]	N.D.	N.D.	N.A.[6]	N.A.	N.A.

[1] Actual concentration of rec.3AB quantified by the bicinchoninic acid (BCA) protein assay.
[2] Detected value of 3AB by LFD with POCT reader.
[3] Standard deviation.
[4] Coefficient of variation.
[5] N.A., not applicable.
[6] N.D., not detected.

3AB is the major precursor protein containing 3B in FMDV-infected cells [14,15]. Direct detection of 3B has been reported to be difficult due to its small size and hydrophilic nature, which results in its loss during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [16,17]. Furthermore, 3B, also known as viral genome-linked protein (VPg), tends to be rapidly degraded when it exists freely in FMDV-infected cells [17,18]. Therefore, 3AB, as a relatively stable and much more easily detectable precursor protein of 3B, was considered to be a representative NSP. As expected, we observed only one protein (3AB) consisting of $3AB_1$, $3AB_{12}$, and $3AB_{123}$ (Fig. 1) by western blot analysis, as reported previously [14,19].

The dilution series for rec.3AB (170, 42.5, 10.6, and 2.6 ng/dose) that we chose in this study was based on a previous report describ-

Table 3
Quantification of 3AB in the virus culture supernatant using LFD with POCT reader.

Rec.3AB	Conc. (ng/ml)	85	42.5	21.3	10.6	5.3	2.6
	Applied LFD						
	NSP Ab response in goats	N.A.[1)]	post 2nd I.M.[2)]	N.A.	post 2nd I.M.	N.A.	None
	Peak value	1674.16	932.912	435.509	199.24	82.016	0
	Standard curve	$y = 6E\text{-}09x^3 - 7E\text{-}06x^2 + 0.0447x + 2.2021$, $R^2 = 0.9999$					
FMDV culture supernatants	Dilution of stock	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Cell supernatant
	Applied LFD						
	Peak value (n = 3)	996.0 ± 40.27	297.1 ± 5.52	0	0	0	0
	3AB (ng/ml)	45.73 ± 1.95	15.03 ± 0.24	N.D.[3)]	N.D.	N.D.	N.D.
	NSP Ab response in goats	post 2nd I.M.	post 3rd I.M.	None	None	N.A.	N.A.

[1)] N.A., not applicable.
[2)] I.M., intramuscular injection.
[3)] N.D., not detected.

ing repeated vaccinations in cattle [13]. Two out of eight goats vaccinated twice with 10.6 ng rec.3AB showed positivity for NSP antibodies, indicating that it did not meet the requirements for FMD vaccine purity test of the OIE guidelines. This outcome contrasted with that of a previous report, which described the lowest amount of 3ABC that could elicit specific antibodies in cattle vaccinated three times ranged between 10.2 and 42.6 ng/dose [7,20]. This slight discrepancy might be attributable to the fact that various factors including the purity of antigen, adjuvants, and inherent variations of individual animal responses can affect the level of antibody formation [21]. According to preliminary data generated in our institute, goats are prone to produce NSP antibodies more rapidly and sensitively than cattle in repeated-vaccination experiments. Briefly, goats began to produce NSP antibodies after the fourth vaccination and cattle did after the fifth vaccination, using an FMD trivalent vaccine composed of serotype O, A, and Asia 1 strains (data not shown). Therefore, the quantity of NSP established as a cutoff value for goats in this study would rarely induce NSP antibodies in cattle after repeated vaccination.

As shown in Fig. 2, the lowest amount of rec.3AB (2.6 ng/ml) did not induce antibody in goats after immunization three times, and the PI values did not increase over the pre-immunization values. Even after the fifth immunization, no positivity for NSP antibody responses were found, and the PI value remained at the baseline level (data not shown). Based on this, it could be assumed that if the NSP content in FMD vaccines is maintained below < 2.6 ng/ml during the vaccine-manufacturing process, then samples positive for NSP antibodies detected during serological surveillance in South Korea would not be attributable to repeated vaccination, but rather to FMDV infection, since serological surveillance in South Korea is mainly conducted for animals that were vaccinated less than five times.

Based on the current OIE guidelines for vaccine-purity testing, the crude FMDV culture supernatants failed to the test because they induced NSP antibodies in two goats after the second immunization. Goats immunized with virus culture supernatant diluted 10^1, however, did not show NSP antibody responses after the second immunization. After the third immunization, two goats were positive for NSP antibodies indicating that, even after obtaining an acceptable vaccine-purity result, it might be difficult to differentiate between repeatedly vaccinated animals and infected animals in the field. This prediction was verified in Fig. 3B, which shows that the mean PI values increased as goats were vaccinated repeatedly with virus culture supernatant, diluted 10^0 or 10^1. In contrast, the virus culture supernatant diluted 10^{-2} did not show increased PI values, suggesting that if the amount of NSP in FMD vaccine is <2.6 ng, one cannot detect positivity on the test line of the LFD by either the naked eye or the POCT reader and, further, can expect that FMD-susceptible animals would not produce enough NSP antibodies to induce seroconversion after at least three vaccinations, although an additional herd test in the field condition is required for validation.

The results of the animal experiment obtained using rec.3AB correlated with those obtained using virus culture supernatant. The quantity of rec.3AB that did not induce antibodies in goats after three immunizations ranged between 10.6 ng and 2.6 ng. Therefore, the virus culture supernatant diluted 10^{-1} was expected to induce NSP antibodies after the second immunization based on the quantity of 3AB protein (15.03 ± 0.24 ng/ml), based on the formula for the standard curve for rec.3AB. However, in practice, it induced NSP antibodies after three immunizations in goats. This negligible gap between the virus culture supernatant and rec.3AB, however, can exist as the FMDV culture supernatant may include 3B-containing cleavage intermediates other than 3AB, for which we could not determine the exact *in vivo* immunogenicity [22].

For a simple FMD vaccine-purity test, we set the cutoff value of the LFD at 2.6 ng/ml, which was shown to be much more rigorous in practical terms, compared to the current OIE guidelines, considering the fact that cattle in FMD-vaccination areas (including South Korea) normally are vaccinated at least five times before slaughtering age. Data from a recent study also suggested that the current purity-testing method of the OIE guidelines and the even more

stringent Brazilian method [23] seemed inadequate for detecting low chances of NSP seropositivity induced by vaccination [24]. The authors recommended using larger numbers of animals to obtain reliable results, but using more animals is problematic, not only due to the cost, but also in terms of conforming to the principle of the 3Rs (replacement, reduction, and refinement) for animal experiments [25].

Taken together, our data suggest that the present LFD could be effectively employed for testing the purity of FMD vaccines with a cut-off value of 2.6 ng/ml, as an ancillary pre-test method to compensate for the limited assurance of the current *in vivo* purity test. Although it would be necessary to validate the usefulness of the LFD with a final vaccine product containing various amount of NSP and various types of adjuvants with animal experiments using goats and cattle because the present results were from particular experimental conditions, this novel LFD should help FMD vaccine manufacturers save time and costs for vaccine purity testing by easily tracking down the NSP content on the in-line process, in cases of poor purity.

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Declaration of Competing Interest

None

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