Determination of non-structural protein level for Turkey foot-and-mouth disease vaccine antigens during in-process

Beyhan Sareyyüpoğlu[1],
Can Çokçalışkan[1], Aydın Çoskuner[1],
Veli Gülyaz[2]

[1]Ministry of Agriculture and Forestry, Institute of Foot and Mouth disease (SAP), Ankara; [2]Ministry of Agriculture and Forestry, Directorate-General for Agriculture and Rural Development, Ankara, Turkey

Corresponding author:
Beyhan Sareyyüpoğlu, DVM, PhD
Ministry of Agriculture and Forestry, Institute of Foot and Mouth disease (SAP), Ankara, Turkey
Tel: +90-312-2873600-2511, Fax: +90-312-2873606
E-mail: beyhan.sar@gmail.com

K O R E A N V A C C I N E S O C I E T Y

Purpose: The success of foot-and-mouth disease (FMD) serological serosurveillance greatly depends on the FMD vaccine which does not include any non-structural proteins (NSPs) of the FMD virus. Since pure FMD vaccines from NSPs are used with the FMD eradication programs using DIVA (Differentiating Infected from Vaccinated Animals) tests. Apart from the *in-vivo* test defined in the World Organisation for Animal Health, two different test kits were developed in-process NSP detection purposes. The first test kit was developed in 2010 and the second one has been very recently developed in 2019.

Materials and Methods: In this study, the level of NSP has been examined by first-chemiluminescent filtration assisted (FAL)-enzyme-linked immunosorbent assay (ELISA) based *in-vitro*, in-process test kit for Turkey FMD vaccine antigen samples. A total of 94 samples were used. The critical maximum acceptable levels of NSP were determined after purification stage of samples.

Results: As a maximum NSP level, 70 ng NSP for the polyethylene glycol concentrated samples and 30 ng NSP for the vaccine antigen mixture samples were accepted. A mini repeatability study was also performed. The correlation between the NSP, total protein, and 146S particul quantity of samples were analyzed.

Conclusion: As a conclusion, the chemiluminescent FAL-ELISA based test kit can be used for the NSP purity level determination of in-process samples.

Keywords: Foot-and-mouth disease, Vaccine, Non-structural protein, Determination, In-process

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease which affects the cloven-hooved animals causing serious economic losses [1]. FMD virus belongs to the family *Picornaviridae*. It has an approximately 8 kB RNA genome encoding a single polyprotein that is cleaved into 12 different proteins by the help of viral proteases. Among these proteins, there are structural virus capsid proteins (SPs); VP1-4, and the non-structural proteins (NSPs); L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. Since the NSPs are clarified during the vaccine production, vaccinated animals elicit antibodies only against SPs, but FMD infected animals elicit antibodies against both SPs and NSPs [1-3]. Developed from this principle, Differentiating Infected from Vaccinated Animals (DIVA) tests are differentiated the vaccinated animal from the infected one. These tests are used in the serosurveilance activities of endemic countries, and have been

one of the main disease eradication tools in recent years [4-8].

Purification of FMD vaccines began in 1989 with the elimination of cell culture allergens from the vaccine [9]. After that, the World Organisation for Animal Health (OIE) declared that it is necessary to investigate the NSP content of FMD vaccines. This is currently performed on the *in vivo* animal experiment test declared by OIE [8]. But in recent years, *in-vitro* tests have been developed according to the principle of 3R (refine-reduce-replace animals) [10]. As an alternative to this *in-vivo* test, two test kit developed to use in- process. At first, the filtration-assisted chemiluminometric enzyme-linked immunosorbent assay (FAL-ELISA) method and as a second, a newly developed test kit based on lateral flow assay device using monoclonal antibody against the 3B NSP [11,12].

Chemiluminescent based immunoassays have a wide dynamic range, quantitative, highly sensitive-specific, and suitable for automation. They have an advantage over absorbance in that the spectrophotometry is an absolute measurement whereas the chemiluminescent is a relative measurement. However, the low background level of emission in the condition of the absence of analyte in the samples and high cost are a few limitations of this method [13]. FAL-ELISA is a chemiluminescent ELISA. The NSPs content of a vaccine sample are concentrated through the monoclonal 3 ABC NSP antibody, and the contaminants of it are discarded by filtration. The specificity of the test is increased using anti-NSP conjugate, and the sensitivity is increased by luminol peroxidase [11]. In the present study, the vaccine samples from different steps of the FMD vaccine production were analyzed with the first developed test kit (chemiluminescent FAL-ELISA test kit) and determined acceptable maximum NSP limits for in process-samples of Turkey FMD vaccine.

Materials and Methods

Preparation of vaccine antigen samples
Antigen eluate samples were called as FSE. FSE is the samples just before the vaccine formulation stage and it is an aqueous phase sample before the vaccine emulsification process. FMD vaccine concentration was performed with polyethylene glycol (PEG) 6000 (Sigma-Aldrich, St. Louis, MO, USA). Briefly, a sterile 50% PEG 6000 solution was added to vaccine antigen bulk and stirred for 2 hours. Then the supernatant was separated from the antigen. After PEG concentration, filtration was performed by serial filtration steps (end of its 0.2 µM). Finally, antigen eluate (called as FSE) was pre-

pared with the elution buffer and stored at -80°C [8]. Vaccine antigen mixture samples were called as AK. the AK is the sample collecting during the vaccine formulation. After PEG concentration and filtration steps, vaccine formulation was done with Montanid ISA-206 adjuvant, double oil emulsion principal in water in oil in water (W/O/W).

Vaccine eluate samples were called as eluate. Eluate is the final product a vaccine sample, just before the vaccine bottling stage. A total of 15 mL vaccine was transferred to the 50 mL tube, and then 7.5 mL chloroform added, stirred with a magnetic stirrer in 5–10 minutes at room temperature. After that, it was centrifuged for 30 minutes at 3,500 rpm and the supernatant (an aqueous portion of antigen fraction) collected to a sterile tube. Finally, a vaccine eluate is gently mixed, aliquoted to a small part, and stored at -80°C until analyzed [8].

The filtration-assisted chemiluminometric enzyme-linked immunosorbent assay analysis of samples
A total of 94 vaccine antigen samples collected from a different stage of production were analyzed with the chemiluminescent FAL-ELISA test kit. Thirty-nine of these were PEG concentrated samples, 40 of these were vaccine antigen mix-AK samples, and 15 of these were vaccine eluate samples tested with Prionics PrioCHECK FMD IPC-3ABC kit, and FMD IPC basic kit (Prionics AG, Zurich, Switzerland) [11]. Microplate luminometer LB 960 (Berthold Technologies, Bad Wildbad, Germany) was used. The test was performed as recommended by the kit [11]. Kit confirms that if the NSP level of the sample is ≥10 ng; it is positive, and if the sample's NSP level ≤10 ng; it is negative for multivaccinated areas. We evaluated the NSP result of samples according to these criteria. Furthermore, some FSE and AK samples of Turkey FMD vaccine were selected to determine for the maximum accepted NSP level. For this purpose, a total of 39 vaccine samples that had been collected after the PEG concentration (called FSE) were analyzed with the kit. After that, a FSE sample consisting of 70 ng NSP was selected to analyze with the kit to determine whether that how much 70 ng NSP level reduced to the critic level (≤10 ng or ≥10 ng) towards the downstream processing during vaccine production. The forward sample after the FSE is the antigen mixture sample (called AK). The same approach was used for the AK samples. For this aim, a total of 40 AK samples were analyzed with the kit. Then, an AK sample consisting of 30 ng NSP levels was selected to analyze with the kit to determine whether that how much 30 ng NSP level reduced to the critic level (≤10 ng or ≥10 ng) towards the downstream

processing during vaccine production. The forward sample after AK is the vaccine eluate sample (called eluate).

Total protein and 146S analysis of samples

In this study, we also checked the correlation between the NSP result and total protein (TP) and between the NSP result and 146S of samples. BCA protein assay kit (Pierce 23225; Thermo Scientific, Rockford, IL, USA) was used to determine the TP amount in the samples. The 146S content of antigens was determined according to the method of Barteling and Meloen [14]. The samples (0.5 mL) were placed on the sucrose gradients (15%–45%) in 12 mL polyallomer tubes and centrifuged in Optima XP 90 ultracentrifuge for 2 hours at 40,000 rpm with Beckman SW-41 Ti swinging-bucket rotor (Beckman Coulter Life Sciences, Indianapolis, IN, USA). The tubes were placed on a tube piercer (Teledyne-isco Teledyne Technologies, Lincoln NE, USA; Fisher Scientific, Waltham, MA, USA) linked to a ultraviolet (UV) detector (1260 Infinity; Agilent Technologies, Santa Clara, CA, USA). The gradient samples were transferred to flow cell by 60% sucrose solution with a syringe pump at a speed of 0.75 mL/min. The UV absorbance was read at 259 nm with sensitivity of 0.5. The Openlab Chromatography Data System software (Agilent Technologies, Santa Clara, CA, USA) was used to plot the peak and calculate the peak area. To evaluate correlation, receiver operating characteristic (ROC) curve analyses (IBM SPSS ver. 22.0; IBM Corp., Armonk, NY, USA) were used for 45 samples.

Repeatability test

Repeatability performance of the kit was also studied. For this aim, a total of 13 samples, which had been previously worked with the kit, were used. The repeatability experiments were performed on two different days and by two different persons.

Results

The filtration-assisted chemiluminometric enzyme-linked immunosorbent assay

In conclusion, the NSP result of 94 vaccine antigen samples is shown in Table 1. A total of 10 ng NSP was determined in the AK of the 70 ng NSP consisting FSE sample. Therefore, as a maximum accepted NSP level for FSE samples, 70 ng were defined. A total of 3 ng NSP was determined in the vaccine eluate of the 30 ng NSP consisting AK sample. Therefore, as a maximum accepted NSP level for AK samples, 30 ng were defined.

Table 1. NSP test results of vaccine antigen samples

Sample code	Evaluation criteria		
	NSP positive (>10 ng)	NSP negative (<10 ng)	Total
FSE	13	26	39
AK	1	39	40
Vaccine eluate	-	15	15
Total			94

Data shows the NSP test result of FMD Institute-Turkey vaccine antigen samples. Samples were called FSE, AK, and vaccine eluate. FSE means the samples before the vaccine formulation steps and these samples include the polyethylene glycol concentrated and eluated samples after the filtration step. AK means the samples after the vaccine formulation steps. These samples include the vaccine antigen mixture samples. Vaccine eluate means the vaccine antigen in the water in oil emulsion.
NSP, non-structural protein.

Table 2. Repeatability study result (non-structural protein, ng)

Sample	Day 1		Day 2	
	Person 1	Person 2	Person 1	Person 2
1	-12	-12	-27	-27
1	-12	-12	-27	-27
2	-12	-12	-27	-27
2	-12	-12	-27	-27
3	-12	-12	-27	-27
3	-12	-12	-28	-27
4	-8	-4	-26	-19
4	-7	-7	-25	-19
5	-11	-12	-27	-26
5	-12	-11	-27	-27
6	-10	-11	-26	-25
6	-9	-10	-26	-25
7	-4	-6	-25	-20
7	-6	-3	-25	-20
8	-12	-12	-27	-27
8	-12	-12	-27	-27
9	-12	-12	-27	-27
9	-12	-12	-27	-27
10	25	26	30	30
10	21	24	24	25
11	11	10	9	11
11	10	10	12	9
12	1	2	2	1
12	1	1	1	2
13	1	1	2	1
13	1	1	1	2

Table 2 shows the repeatability study result of the test kit. Two different persons tested the same samples with the test kit. Day 1 and day 2 show the relative light unit results of two different days.
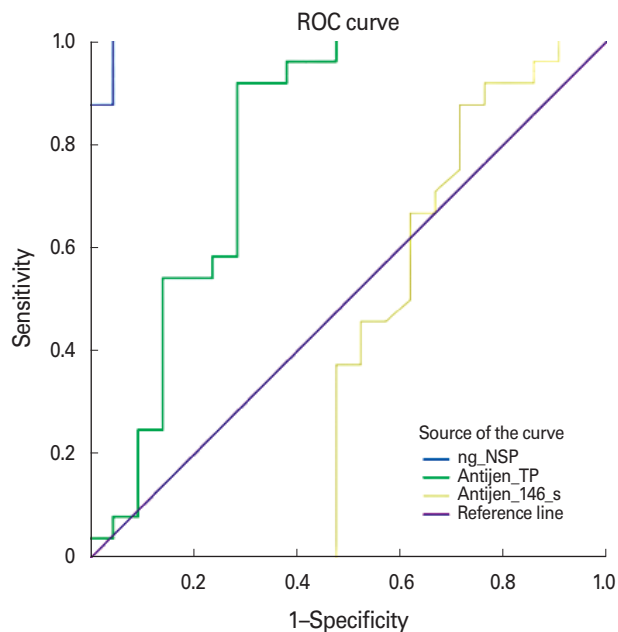
Fig. 1. ROC curve analyses of FSE sample (146S, TP, and NSP correlations). ROC, receiver operating characteristic; NSP, non-structural protein; TP, total protein.

Repeatability

Approximately, a similar result was obtained with the repeatability study. The covariances found within the normal ranges of limits on the same day and the different day test results as suggested by Capozzo et al. [11] in 2010. Repeatability result is shown in Table 2.

Correlation between the 146S, total protein, and non-structural protein of antijen samples

It was determined a good correlation between the TP and NSP of a sample and we found that if the TP of the FSE sample is higher than 4,500 (μg/mL), NSP of this FSE sample can be risky for vaccine production (Fig. 1).

Discussion

FMD vaccine free from NSPs is a crucial parameter for endemic countries using serological DIVA tests for its serosurveillance activities. Furthermore, this purity is also important for the Good Manufacturing Process certification of a FMD vaccine. For this reason, the new in-process tests have been developed in recent years [11,12]. These tests were capable of the determination of NSPs during the process (in-process tests). Thus, producers can easily be informed early information about the NSP level of vaccine during the process, and so

the financial inputs of the vaccine production can be reduced. In this study, we evaluated an in-process test kit performance for Turkey FMD vaccine antigens. For this aim, a total of 94 FMD vaccine samples were analyzed with the kit. Besides, we determined roughly accepted NSP levels for some Turkey FMD vaccine samples after vaccine purification.

Here, we used the chemiluminescent FAL-ELISA kit. The test period of this kit is shorter than the OIE recommended in-vivo test and also it is advantageous because of its NSP detection capability for in-process samples. Nevertheless, it is not as easy to perform. Many blocking steps are time-consuming. This test requires expertise to analyze and evaluate the result. Besides, it might be required at least 2 or 3 times test repetitions to get a more exact result for some complex (like AK samples) vaccine antigen samples.

When we look at the ROC curve correlation, we were not to find a good correlation between the NSP and 146S results of the sample. It was presumed that when the 146S of a sample is low, in other words when the capsid of FMD antigen deteriorated, their NSP also might be deteriorated. In fact, this non-correlation between the 146S and NSP is probably linked to the test principle of the sucrose density gradient method. Since the field of absorption peak in the gradient doesn't include any NSPs. Not surprisingly, it was found a good correlation with TP and NSP value of the sample. Since, TP part of the sample consists of both NSP and SP (capsid-146S part) of the FMD vaccine samples. Unfortunately, there has been no previous research about the *in vitro* (in-process) NSP level determination of any FMD vaccine in the world. Therefore, here, it is difficult to compare and evaluate the NSP results of our study. Future studies are warranted to clarify this.

In conclusion, the chemiluminescent FAL-ELISA test kit is capable of determining the NSP purity of the Turkey-FMD vaccine samples although a few negative sides of it like additional blocking steps, additional repetition of test, etc. It should be better to analyze our vaccine also with the newly developed NSP purity test based on lateral flow assay device, and then, to compare all results of this two *in-vitro* in-process test kit to the OIE defined *in vivo* NSP purity determination test.

ORCID

Beyhan Sareyyüpoğlu *https://orcid.org/0000-0002-0279-1673*

Can Çokçalışkan *https://orcid.org/0000-0002-3839-0986*
Aydın Çoşkuner *https://orcid.org/0000-0001-6511-2685*
Veli Gülyaz *https://orcid.org/0000-0002-9089-919X*

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