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METHOD FOR FMD VIRUS 146S COMPONENT CONCENTRATION

DETERMINATION WITH REAL-TIME REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION IN VACCINE RAW MATERIALS

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SUMMARY

Method for determination of foot-and-mouth disease (FMD) virus 146S component concentration with real-time reverse transcription – polymerase chain reaction (rtRT-PCR) in vaccine raw materials is developed. Negative regression model of $C_{146S} = (30.269 – C_t)/4.155$ type allowing determination of FMDV 146S particle concentrations based on the amplification threshold cycle values (C_t) is proposed. It has been experimentally proven that the quantity of the 146S component determined by the real-time RT-PCR method using developed regression model and contained in the inoculation dose of FMD vaccine confers protection to the vaccinated animals against generalized FMD of A, O, Asia-1 types. rtRT-PCR method is highly sensitive and allows rapid and highly reliable estimation of the 146S antigen concentration in FMD vaccine. The method for 146S particle quantity determination by real-time RT-PCR using the regression model is reliable and demonstrates high correlation (95.5–99.0%) with the complement fixation test results.

Key words: foot-and-mouth disease (FMD) virus, concentration of 146S component, rtRT-PCR, amplification threshold cycle.

INTRODUCTION

Foot-and-mouth disease is a highly contagious vesicular virus disease of cloven-hooved animals that is classified to transboundary infections [3, 11]. The agent is a non-enveloped RNA-virus that belongs to *Picornavirales* order, *Aphthovirus* genus of *Picornaviridae* family [11]. FMD virus is highly antigenically variable due to mutations in capsid protein genes and has 7 types and various subtypes [11, 13].

FMD causes huge economic losses associated with costs for the disease eradication and stringent measures imposed on domestic and international trade in animal products. Complex measures for FMD prevention and control include mass immunization of animals as well as control of postvaccinal immunity level [6, 13].

FMD virus forms 4 variants of its specific components when it is reproduced in suspension continuous baby hamster kidney cell line (BHK-21/2-17): 146S component (whole particles) containing one viral RNA molecule and 60 polypeptide copies, each is a complex of VP_1-VP_2-VP_3-VP_4 proteins; 75S particles lacking RNA and comprising 60 copies of VP_1-VP_3-VP_0 polypeptide; 12S component consisting of VP_1, VP_2, VP_3 proteins; 3.85S subunits represented by non-structural VP_g protein [8, 10].

Concentration of highly immunogenic and resistant 146S particles in raw materials is of great importance for FMD vaccine production [3, 12]. Therefore, each batch of FMD vaccine raw materials is tested by quantitative complement fixation test (CFT) to determine 146S component concentration. However, this method has some disadvantages: it is labour and time consuming (test duration is at least 2-3 days) especially when many samples are tested simultaneously [1].

Therefore, it is reasonable to propose a real-time reverse transcription – polymerase chain reaction (rtRT-PCR)-based method for determination of FMDV whole particle concentrations in vaccine raw materials that is highly sensitive and specific and more rapid that will allow simultaneous testing of several dozens of virus-containing raw

Table 1
Correlation between FMDV 146S component concentrations determined by CFT and amplification threshold cycle values determined by rtRT-PCR
n=3

Tested sample	146S component concentrations determined by CFT, μg/cm³				Amplification threshold cycle values determined by rt RT-PCR			
	C_1	C_2	C_3	C_{mean}	C_{t1}	C_{t2}	C_{t3}	C_{tmean}
Контрольные образцы								
Positive control 146S component concentration: 0.1 μg/cm³	0.10	0.10	0.15	0.12 ± 0.03 ($p < 0.005$)	29.78	29.84	29.73	29.78 ± 0.06 ($p < 0.005$)
0.5 μg/cm³	0.60	0.58	0.60	0.59 ± 0.01 ($p < 0.001$)	27.84	27.81	27.72	27.79 ± 0.06 ($p < 0.005$)
1.0 μg/cm³	1.05	1.10	1.10	1.08 ± 0.03 ($p < 0.001$)	25.78	25.84	25.86	25.83 ± 0.04 ($p < 0.001$)
2.0 μg/cm³	1.90	1.85	2.10	1.95 ± 0.13 ($p < 0.005$)	22.09	22.14	22.19	22.14 ± 0.05 ($p < 0.005$)
3.0 μg/cm³	3.00	2.91	3.13	3.01 ± 0.11 ($p < 0.005$)	17.77	17.69	17.81	17.76 ± 0.06 ($p < 0.005$)
4.0 μg/cm³	4.10	3.95	3.95	4.00 ± 0.09 ($p < 0.005$)	13.66	13.6	13.74	13.67 ± 0.07 ($p < 0.005$)
5.0 μg/cm³	4.85	5.00	4.95	4.93 ± 0.08 ($p < 0.005$)	9.78	9.88	9.85	9.84 ± 0.06 ($p < 0.005$)
Negative control	n/f	n/f	n/f	n/f	n/d	n/d	n/d	n/d
Non-inactivated antigen for anti-FMD vaccine								
raw material for emulsion vaccine against type A FMD based on A No. 2029/Turkey/2006 strain	1.39	1.55	1.51	1.48 ± 0.08 ($p < 0.01$)	23.91	23.74	23.79	23.81 ± 0.09 ($p < 0.005$)
raw material for emulsion vaccine against type O FMD based on O No. 2212/Primorsky/2014 strain	0.92	1.10	1.06	1.03 ± 0.09 ($p < 0.01$)	25.91	26.16	25.99	26.02 ± 0.13 ($p < 0.005$)
raw material for emulsion vaccine against type Asia-1 FMD based on Asia-1 No. 2145/Tajikistan/2011 strain	3.31	3.09	3.27	3.22 ± 0.12 ($p < 0.01$)	17.01	16.68	16.64	16.78 ± 0.20 ($p < 0.005$)

n/f – 146S component of FMD virus was not found;
n/d – FMD virus genome was not detected.

material samples and reduce testing time to 4 hours [7, 9]. Considering rtRT-PCR advantages as compared to CFT a negative linear correlation between FMDV 146S component quantity in vaccine raw materials and amplification threshold cycle values (C_t) is to be established in the form of regression model. As for rtRT-PCR, C_t value depends on FMDV RNA concentration and number of viral RNA copies is proportional to number of 146S particles. Experimental demonstration of correlation between rtRT-PCR and CFT results will enable development of rapid and cost-effective method for determination of whole virus particle concentrations in vaccine raw material.

The study was aimed at assessment of potential rtRT-PCR use for determination of FMDV 146S component concentration in vaccine raw materials.

MATERIALS AND METHODS

Virus. Culture FMDV A No. 2029/Turkey/2006, O No. 2212/Primorsky/2014, Asia-1 No. 2145/Tajikistan/2011 vaccine strains deposited to the FGBI "ARRIAH" Collection of Microorganism Strains were used. The virus was reproduced in suspension continuous BHK-21/2-17 cell culture.

Complement fixation test (CFT). Quantitative CFT variant with FMDV antigen and strain-specific guinea pig sera against the above-said FMDV strains was used for determination of 146S component concentration (μg/cm³) [1].

RNA extraction from the vaccine raw materials was performed with commercial RIBO-sorb kit (FBSE "Central Research Institute of Epidemiology" of the Rospotrebnadzor) in accordance with manufacturer's instructions.

rtRT-PCR. Real-time RT-PCR was used for detection of FMD virus genome and determination of 146S particle concentration in the virus-containing suspension. Primers and TaqMan DNA probe designed for highly conservative segment of FMDV 3D-gene were used for analysis. The reaction results were analyzed using Rotor-Gene 1.8.17.5 software that determines amplification threshold value that is reciprocal to number of viral RNA copies and 146S component concentration [4].

Vaccines. Three emulsion monovalent ARRIAH-VAC vaccines against type A, O, Asia-1. FMD virus were used. The vaccine against type A FMDV contained 6.15 ± 0.11 μg/dose, vaccine against type O FMDV contained 6.03 ± 0.12 μg/dose and vaccine against Asia-1 FMDV contained 6.43 ± 0.12 μg/dose of 146S component as determined by CFT; and rtRT-PCR-determined concentration of 146S component was 6.20 μg/dose in vaccine against type A FMDV, 6.12 μg/dose in vaccine against type O FMDV and 6.50 μg/dose in vaccine against Asia-1 FMDV.

Animals. Twenty-one Landrace and Duroc gilts weighing 30–40 kg were used.

Immunization and challenging of animals. All animals were divided into 3 test groups, 5 gilts per group. Animals of test group 1 were immunized with emulsion monovalent vaccine against type A FMD, animals of test group 2 were immunized with the vaccine against type O FMD and animals of test group 3 were immunized with the vaccine against type Asia-1 FMD. Three control groups, 2 animals per group, were formed for type A, O, Asia-1 FMDV, respectively. The non-diluted vaccine was injected intramuscularly at a dose of 2 cm^3 in the upper third of neck. Animals of test group 1, test group 2 and test group 3 were challenged with gilt-adapted homologous FMDV A No. 2029/Turkey/2006, O No. 2212/Primorsky/2014 and Asia-1 No. 2145/Tajikistan/2011 strains, respectively. The said virus strains were injected at a dose of 10^4 ID_{50}/0.2 cm^3 in tongue mucosa 28 days after immunization. The animals were subjected to postmortem examination 7 days after challenge.

Determination of antibody titres in animal sera after immunization was performed with neutralization test in accordance with approved guidelines [2].

Statistical data processing. Obtained results were processed and arithmetical mean, confidence level for statistical difference between mean values determined with differential Student's-Fischer test as well as determination coefficient were identified [5]. Diagrams were constructed using Microsoft Excel 2010 software application package.

RESULTS AND DISCUSSION

At the first stage a calibration panel of control positive samples containing amounts of FMDV RNA equivalent to the following 146S component concentrations: 0.1; 0.5; 1.0; 2.0; 3.0; 4.0; 5.0 μg/cm^3 as well as negative control that was BHK-21/2-17 cell suspension not infected with FMDV were prepared to identify correlation between 146S component concentrations determined by CFT and amplification threshold cycle values determined with rtRT-PCR. Two specimens were taken from each sample: one – for amplification threshold cycle value (C_t) determination with rtRT-PCR and the other one – for estimation of FMDV 146S component concentration (C_{146S}) with CFT. The experiment was repeated thrice. Parallelly determined 146S particle concentrations and amplification threshold cycle values were selected based on the analysis results (Table 1).

Table 1 shows that amplification threshold cycle values for all dilutions of FMDV 146S component at concentration of 0.1–5.0 μg/cm^3 were in the range of 29.78 ± 0.06 – 9.84 ± 0.06, respectively. No FMD virus genome was detected in negative control sample. Based on the obtained data negative linear correlation between C_{146S} and C_t was identified as a $C_{146S} = (30.269 - C_t)/4.155$ regression model plotted as linear function in Figure. Actual determination coefficient (R^2) for the said correlation tends to 1 and is 0.9891 that indicates high reliability of the obtained results.

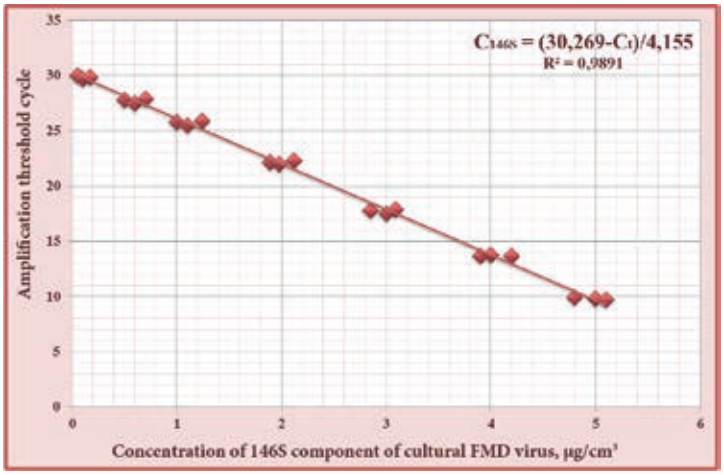

Fig. Graph of correlation between amplification threshold values and cultured FMDV 146S component concentrations (n=3)

At the next stage proposed regression model was tested by determining 146S particle concentrations in non-inactivated raw materials for vaccines against FMDV of three types (A, O, Asia-1) with rtRT-PCR method.

Non-inactivated virus-containing material intended for production of type A FMD vaccine based on A No. 2029/Turkey/2006 was tested. The specimen was parallelly tested in triplicate with rtRT-PCR and CFT. Obtained results given in Table 1 show that amplification threshold cycle value was 23.81 ± 0.09. In accordance with regression model $C_{146S} = (30.269 - C_t)/4.155$ 146S component concentration in the given specimen was 1.55 ± 0.02 μg/cm^3 that correlated to the CFT results (1.48 ± 0.08 μg/cm^3). In other words, developed model based on rtRT-PCR method allows estimation of 146S component quantity in non-inactivated raw material for vaccine against type A FMD.

Prepared material was inactivated, concentrated twice and 146S antigen quantity was estimated per dose (2 cm^3). Test results are given in Table 2 and show that 146S component quantity in inactivated material theoretically estimated based on rtRT-PCR results considering initial whole virus particle quantity and concentration factor was 6.20 μg per dose that correlated with experimental CFT results (6.15 ± 0.11 μg per dose).

Prepared monovalent emulsion vaccine against type A FMD was used for inoculation of 5 gilts and tested for its immunogenicity according to standard procedure. Neutralizing antibody titre in immunized animals of test group 1 was 6.10 ± 0.14 log_2 (Table 2). Animal challenge test results evidences that quantity of immunogenic components contained in a FMD vaccine dose and determined by regression model-rtRT-PCR protects 5 out of 5 immunized pigs from generalized type A FMD. All control animals demonstrated generalized FMD form.

At the next stage non-inactivated virus-containing material intended for production of type O FMD vaccine based on O No. 2212/Primorsky/2014 was tested with CFT and rtRT-PCR using proposed regression model; C_t value for the sample was 26.02 ± 0.13 (Table 1). Based on the developed $C_{146S} = (30.269 - C_t)/4.155$ model 146S component concentration was 1.02 ± 0.03 μg/cm^3 that was consistent to CFT results (1.03 ± 0.09 μg/cm^3). Therefore, proposed

Table 2
Tests of three inactivated emulsion monovalent type A, O, Asia-1 FMD vaccines for their immunogenicity
n=5

Group of animals	Name of vaccine	Concentration of FMDV 146S component in a vaccine, μg/dose		Animal number	Anti-FMDV antibody titre, $\log_2$	Presence of generalized disease
		CFT	Theoretically estimated based on rtRT-PCR results			
Test group No. 1	Type A FMD vaccine based on A No. 2029/Turkey/2006	6.15 ± 0.11 ($p < 0.010$)	6.20	1	6.00	–
				2	6.25	–
				3	6.00	–
				4	6.25	–
				5	6.00	–
				M ± *m*	6.10 ± 0.14 ($p < 0.010$)	
Control group No. 1	not immunized	–	–	6	<0.5	+
				7	<0.5	+
Test group No. 2	Type O FMD vaccine based on O No. 2212/Primorsky/2014	6.03 ± 0.12 ($p < 0.010$)	6.12	8	6.25	–
				9	6.00	–
				10	5.75	–
				11	6.00	–
				12	5.75	–
				M ± *m*	5.95 ± 0.21 ($p < 0.010$)	
Control group No. 2	not immunized	–	–	13	<0.5	+
				14	<0.5	+
Test group No. 3	Type Asia-1 FMD vaccine based on Asia-1 No. 2145/Tajikistan/2011	6.43 ± 0.12 ($p < 0.010$)	6.50	15	6.25	–
				16	6.25	–
				17	6.00	–
				18	6.50	–
				19	6.25	–
				M ± *m*	6.25 ± 0.18 ($p < 0.010$)	
Control group No. 3	not immunized	–	–	20	<0.5	+
				21	<0.5	+

Antibody titres in sera of animals immunized with not-diluted FMD vaccine are indicated.

model based on rtRT-PCR allows estimation of 146S component quantity in non-inactivated antigen intended for type O FMD vaccine production.

Prepared material was inactivated, concentrated thrice and 146S antigen quantity was estimated in a dose of 2 cm^3. Test results are given in Table 2 and show that quantity of 146S component in inactivated material theoretically estimated based on rtRT-PCR results considering initial whole virus particle quantity and concentration factor was 6.12 μg per dose that correlated with CFT results (6.03 ± 0.12 μg per dose).

Prepared emulsion monovalent vaccine against type O FMD was used for inoculation of 5 pigs. Neutralizing antibody titre in immunized animals of test group 2 was 5.95 ± 0.21 $\log_2$ (Table 2). Results of animal challenge test evidenced that quantity of immunogenic components contained in a FMD vaccine dose and determined by rtRT-PCR protected 5 out of 5 immunized pigs from

generalized type O FMD. All control animals demonstrated generalized FMD.

Then, non-inactivated virus-containing material intended for production of type Asia-1 FMD vaccine based on Asia-1No. 2145/Tajikistan/2011 was tested with CFT and rtRT-PCR using proposed regression model. Amplification threshold value (C_t) for the sample was 16.78 ± 0.20 (Table 1). According to developed $C_{146S} = (30.269 – C_t)/4.155$ model 146S component concentration was 3.25 ± 0.05 μg/cm^3 that was consistent to CFT results (3.22 ± 0.12 μg/cm^3). Thus, proposed model based on rtRT-PCR method allows estimation of 146S component quantity in non-inactivated raw materials intended for type Asia-1 FMD vaccine production.

Prepared material was inactivated and 146S antigen quantity was estimated in a dose of 2 cm^3. Test results are given in Table 2 and show that quantity of 146S component in inactivated material theoretically estimated based on rtRT-PCR results considering initial whole virus particles content and concentration factor was 6.50 μg per dose that correlated with CFT results (6.43 ± 0.12 μg per dose).

Prepared emulsion monovalent vaccine was used for inoculation of 5 gilts. Neutralizing antibody titre in immunized animals of test group 3 was 6.25 ± 0.18 $\log_2$ (Table 2). Results of animal challenge test evidenced that regression-model-rtRT-PCR-determined quantity of immunogenic components contained in a FMD vaccine dose protected 5 out of 5 immunized pigs from generalized type Asia-1 FMD. All control animals demonstrated generalized FMD.

Thus, rtRT-PCR using $C_{146S} = (30.269 – C_t)/4.155$ regression model is a reliable and rapid method for 146S component concentration determination. Test system for detection of vaccine FMDV strains was highly sensitive and demonstrated high correlation (95.5–99.0%) with CFT results.

CONCLUSION

New approach to the determination of 146S component concentration with rtRT-PCR method and regression model developed based on the said method in raw materials for vaccines against FMD was proposed. Based on CFT and rt RT-PCR results, negative correlation between 146S component quantity and amplification threshold value was identified in the form of regression and plotted as a linear function graph, $C_{146S} = (30.269 – C_t)/4.155$. As for rtRT-PCR, threshold value depends on FMDV RNA concentration and number of viral RNA copies is proportional to 146S component content. Experimental evidence of correlation between CFT and rtRT-PCR results allowed development of rapid and cost-effective method for determination of whole virus particle concentration in vaccine raw materials.

The testing indicated that 146S component quantity determined with rtRT-PCR using proposed regression model and contained in an inoculation dose of FMD vaccine protected vaccinated animals from generalized type A, O, Asia-1 FMD.

rtRT-PCR method is highly sensitive and allows rapid and highly reliable determination of 146S antigen concentration in FMD vaccine. Proposed method for 146S particle quantity determination is reliable and demonstrates high correlation (95.5–99.0%) with CFT results.

It is shown that the said method can be used for determination of cultural FMDV 146S component quantity in vaccine raw material.

REFERNCES

1. Bondarenko A. F. Qualitative and quantitative immunochemical assay of virus proteins [Kachestvennyj I kolichestvennyj immunohimicheskij analiz virusnyh belkov]. Suzdal, 1994 (in Russian).

2. Methodical instructions on neutralization test procedure for determination of immune status of animals with regard to FMD [Metodicheskie ukazanija po postanovke reakcii nejtralizacii dlja opredelenija immunnogo statusa zhivotnyh pri jashhure]: approved by USSR Ministry of Agriculture on 26.12.1983 (in Russian).

3. Ponomaryov A. P., Uzyumov V. L., Gruzdev K. N. Foot-and-mouth disease virus: structure, biological and physical and chemical properties [Virus jashhura: struktura, biologicheskie i fizikohimicheskie svojstva]. Vladimir: Foliant, 2006 (in Russian).

4. Lozovoy D. A. , Shishkova A. A. , Mikhalishin D. V. et al. Real-time RT-PCR for determination of concentration of 146S component of FMD virus types O/Saudi Arabia and Asia-1/Shamir in semi-finished vaccine [Primenenie OT-PCR-RV dlya opredeleniya koncentracii 146S komponenta virusa yashchura tipov O/Saudovskaya Araviya i Asia-1/Shamir v polufabrikate vakciny]. *Veterinariya*. 2017; 6: 57–61 (in Russian).

5. Rumyantzev P. O., Sayenko V. A., Rumyantzeva U. V., Chekin S. Yu. Statistical analysis methods in clinical practice [Statisticheskie metody analiza v klinicheskoj praktike]. Obninsk: GU MRRC RAMS. URL: http://www.kantiana.ru/medicinal/help/StatMethodsInClinics.pdf (in Russian).

6. Annual OIE/FAO FMD Reference Laboratory Network Report. Pirbright, 2007. URL: http://www.wrlfmd.org/ref_labs/fmd_ref_lab_reports.htm.

7. Shaw A. E., Reid S. M., King D. P. et al. Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. *Rev. Sci. Tech. OIE*. 2004; 23 (3): 1003–1009.

8. Gartwright B., Brown F. Chapman W. G. Serological and immunological relationships between the 146S and 12S particles of foot-and-mouth disease virus. *J. Gen. Virol.* 1980; 50: 369–375.

9. Shaw A., Reid S. M., Ebert K. et al. Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. *J. Virol. Methods*. 2007; 143(1): 81–85. https://doi.org/10.1016/j.jviromet.2007.02.009.

10. Lubroth J., Rodriguez L., Dekker A. Vesicular diseases. *Diseases of Swine*. ed. B. E. Straw, J. J. Zimmerman, S. D'Allaire, D. J. Taylor. 9th ed. Ames, Iowa; 2006: 517–536.

11. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.Vol. 1. 7th ed. Paris, France: OIE; 2012; Chapter 2.1.5: 166–169.

12. Alexandersen S., Zhang Z., Donaldson A. I., Garland A. J. M. The pathogenesis and diagnosis of foot-and-mouth disease. *J. Comp. Path*. 2003; 129: 1–36. DOI: 10.1016/S0021-9975(03)00041-0.

13. Wernike K., Beer M., Hoffmann B. Rapid detection of foot-and-mouth disease virus, influenza A virus and classical swine fever virus by high-speed real-time RT-PCR. *J. Virol. Methods*. 2013: 193(1): 50–54. DOI: 10.1016/j.jviromet.2013.05.005.

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