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Indirect determination of FMDV 146S component concentration in non-inactivated suspension by comparison of graphs of the second derivative for real-time RT-PCR curves

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SUMMARY
During reproduction in biological systems, FMD virus forms four variants of components, three of which do not include RNA of the virus. In the process of industrial production of FMD vaccines, special attention is paid to the number of whole virions, which have the most important biological properties of FMD virus and are the main components that determine the immunogenicity of vaccine preparations. Raw materials for vaccines at various stages of the technological process are tested for concentration of FMDV 146S component. The traditional method of determination is quantitative complement fixation test. In recent years, real-time RT-PCR has been used for indirect determination of FMDV 146S component concentration in a virus-containing suspension. The article presents a new approach to indirect determination of FMDV 146S component concentration in a non-inactivated suspension by comparing the maximum extreme points of the graphs of the second derivative of the fluorescence signal accumulation curves and the number of amplification reaction cycles. The dependence between FMDV 146S component concentration and the maximum extreme points of the graphs of the second derivative of the fluorescence signal accumulation curve is presented in the form of a square function: $C_{146S\ FMDV} = 0.0111(C_p)^2 - 1.0157C_p + 20.446$ with a high accuracy of approximation ($R^2 = 0.993$). The proposed model allows to quantitatively estimate the content of 146S component in virus-containing vaccine raw materials. The presented method allows studying a large number of samples of non-inactivated raw materials for FMD vaccine in 4–5 hours. The main advantage of the proposed method is the capacity to determine the concentration of FMDV 146S component in a suspension with a high level of ballast proteins (more than 7.00 mg/cm^3) and complete viral particles (from 0.01 to 5.00 μg/cm^3).

Key words: FMDV virions, real-time RT-PCR, amplification threshold cycle, graph of second derivative of amplification curve.

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Опосредованное определение концентрации 146S компонента вируса ящура в неинактивированной суспензии при сравнении графиков второй производной для кривых ОТ-ПЦР-РВ

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РЕЗЮМЕ

При репродукции в биологических системах вирус ящура формирует 4 варианта компонентов, три из которых не включают в себя РНК вируса. В процессе промышленного производства противоящурных вакцин особое внимание уделяют количеству цельных вирионов, которые обладают важнейшими биологическими свойствами вируса ящура и являются основными компонентами, определяющими иммуногенность вакцинных препаратов. Сырье для вакцин на различных этапах технологического процесса исследуют с целью определения концентрации 146S компонента вируса ящура. Традиционным методом определения является количественный вариант реакции связывания комплемента. Для опосредованного определения концентрации 146S компонента вируса ящура в вируссодержащей суспензии в последние годы стали использовать полимеразную цепную реакцию с обратной транскрипцией в режиме реального времени. В статье представлен новый подход к опосредованному определению концентрации 146S компонента вируса ящура в неинактивированной суспензии при сравнении максимальных экстремумов графиков второй производной кривых накопления сигнала флуоресценции относительно количества циклов реакции амплификации. Существование зависимости между концентрацией 146S компонента вируса ящура и максимальными экстремумами графиков второй производной кривой накопления флуоресцентного сигнала представлено в виде квадратичной функции $C_{146S\,ВЯ} = 0{,}0111(C_p)^2 - 1{,}0157C_p + 20{,}446$ с высокой достоверностью аппроксимации ($R^2 = 0{,}993$). Предложенная модель позволяет количественно оценивать содержание 146S компонента в вируссодержащем сырье для вакцины. Представленный способ позволяет исследовать большое количество образцов неинактивированного сырья для противоящурной вакцины за 4–5 ч. Основным преимуществом предлагаемого способа является возможность определения концентрации 146S компонента вируса ящура в суспензии, содержащей высокое количество балластного белка (более 7,00 мг/см^3) и количество полных вирусных частиц от 0,01 до 5,00 мкг/см^3.

Ключевые слова: вирионы вируса ящура, ОТ-ПЦР-РВ, пороговый цикл амплификации, график второй производной кривой амплификации.

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INTRODUCTION

In many countries foot-and-mouth disease (FMD) is at the top of the list of animal viral diseases control and prevention measures. FMD is a highly contagious viral acute disease of wild and domestic cloven-hoofed animals and tylopods and is a global problem, which a special attention of international organizations (FAO, OIE) and veterinary services of many countries is paid to [1, 2].

FMD virus (FMDV) genome is represented by a single-stranded positive RNA consisting of approximately 8,500 n. r. (nucleotide residues), surrounded by an icosahedral capsid consisting of 60 copies, each of which is represented by four structural proteins: VP_1 (1D-gene), VP_2 (1B-gene), VP_3 (1C-gene), VP_4 (1A-gene) [3–5].

During reproduction in biological systems FMD virus forms four variants of components: 146S component (whole virion, full particle), consisting of one whole viral RNA molecule and 60 copies of polypeptide, each of which is represented by a complex of proteins VP_1 (1D-gene), VP_2 (1B-gene), VP_3 (1C-gene), VP_4 (1A-gene); 75S particle ("blank" capsid), consisting of 60 copies of polypeptides VP_0 (1AB-gene), VP_1 (1D-gene), VP_3 (1C-gene); 12S particle (capsomer), consisting of structural proteins VP_1 (1D-gene), VP_2 (1B-gene), VP_3 (1C-gene); 3.8S subunit, represented by non-structural protein VP_g. 75S, 12S and 3.8S components do not include FMDV RNA [1, 3].

In the process of industrial production of vaccines, special attention is paid to the number of whole virions, which have the most important biological properties of FMD virus and are the main components that determine the potency of vaccine preparations [2, 3]. Therefore, raw materials for vaccines at different stages of technological process are studied to determine the concentration of 146S component of FMD virus. Traditionally for this purpose quantitative variant of complement fixation test (CFT) is used and the results are evaluated according to methodical recommendations [6]. In recent years, real-time reverse transcription polymerase chain reaction (real-time RT-PCR) has been used for indirect determination of concentration of FMDV 146S component concentration in a non-inactivated virus-containing suspension [1, 7–12]. The presented method is highly sensitive, specific, economical and rapid, and also allows to investigate several dozens of samples of the virus-containing material simultaneously. However, this modification has some disadvantages: if the content of 146S component in the tested sample is less than 0.1 mg/cm^3, the sensitivity of the reaction decreases; if there are a lot of proteins and lipoproteins in the sample, the sorbent particles are sensitized by excess of ballast components that reduces the possibility of virus RNA sorption and decreases the sensitivity of the analysis; where trace amounts of sorbent may end up in the reaction mixture, the background value of the fluorescence may increase and there may be a distortion of the analysis results when determining the a proportionality coefficient between the fluorescence signal and the amount of the whole virus in the sample.

It is therefore an important and challenging task to improve the method for indirect determination of concentration of FMDV 146S component in a non-inactivated suspension on the basis of the method for comparing the maximum extrema of the graphs of the second derivative for real-time amplification reaction curves.

The aim of the research is to develop a method for indirect determination of concentration of FMDV 146S component in the viral suspension by comparing the maximum extrema of the second derivative graphs for the real-time amplification reaction curves.

MATERIALS AND METHODS

Virus. FMD culture virus of Asia-1/Shamir Israel 3/89 strain was used. The virus was propagated in the suspension continuous cell line of baby hamster kidney BHK-21. Non-inactivated FMDV suspensions with ballast proteins contents of more than 7.00 mg/cm^3 were used.

Complement fixation test (CFT). Quantitative CFT was used to determine FMDV 146S component concentration [6].

Determination of 146S component concentration. FMDV 146S component concentration was determined with real-time RT-PCR using values of threshold cycle of amplification (*C*t) according to the above-mentioned requirements [7].

Coating of plates with polyclonal strain-specific antibodies against FMDV. A six-well plate was coated with highly purified strain-specific polyclonal antibodies against FMDV in the volume of 1.5 cm^3 of the suspension with concentration of immunoglobulins G of 5.0 μg/cm^3 at 4 ± 2 °C for 18–20 hours. Open binding sites were blocked with 1% gelatine suspension at 37 ± 1 °C for 30 minutes and the wells were washed with 1/15 M phosphate-buffered saline (PBS) five times.

Strain-specific binding of FMD virus. Samples of suspensions in the volume of 2.4 cm^3 were added to the wells coated with strain-specific FMDV antibodies and incubated at 37 ± 1 °C for 30 minutes. The wells were washed to remove ballast components three times using 1/15 M PBS. The obtained immune complexes were resuspended in 1.0 cm^3 of Eagle's medium (MEM).

Isolation of FMDV virion RNA bound by immune complex. The process of isolation of RNA of 146S FMDV component was based on method by P. Chomczynski [13, 14]. The process resulted in obtaining per 0.2 cm^3 of 12-fold extracts of viral RNA.

Evaluation of purity of eluates of FMDV RNA. The spectral absorption capacity of RNA extracts was measured at wave lengths within the range of 205–325 nm and temperature of 22–25 °C. In the isolated extracts the content of residues of phospholipids, polysaccharides and guanidine isothiocyanate (GITC), carbolic acid, polypeptides and large suspended particles was estimated, determining the optical density (OD) values at 205, 235, 270, 280 and 320 nm, respectively [15]. The RNA eluate was considered free of protein and carbolic acid impurities if the extinction coefficient R_1 (OD_{262}/OD_{280}) was within the range of 1.8–2.2 and was optimally about 2.0. Lower R_1 values indicated the presence of DNA, protein components and carbolic acid residues in the eluate. Higher values of R_1 coefficient indicated the degradation of RNA and the presence of free ribonucleotides. FMDV nucleic acid extract was considered uncontaminated with polysaccharides if the extinction coefficient R_2 (OD_{262}/OD_{235}) was close to 2.000. When 1% RNA is substituted for polysaccharide components, R_2 decreases by 0.002 [16]. Values of R_2 coefficient greater than 2.000 may indicate degradation of RNA molecules. The absence of coarse particle suspension in the eluate is confirmed if OD_{320} is close to zero [15, 16]. If the purity requirements are not met, the stages of serological binding and isolation of FMDV RNA from the source material are repeated.

Real-time RT-PCR for quantitative determination of FMDV 146S particles. Forward-3D-FMDV-primer (5′-ACT-GGT-TTT-ACA-AAC-CTG-TGA-GGT-3′), Reverse-3D-FMDV-primer (5′-GCG-AGT-CCT-GCC-ACG-GAG-TTG-GTT-3′) and 3D-FMDV-ROX/BHQ2-probe (5′-ROX-TCC-TTT-GCA-CGC-CGT-GGG-ACG-3′) were used in the test as oligonucleotides homologous to 3D FMDV gene in 15 pM concentrations per reaction. The concentration of each of the deoxyribonucleoside triphosphates was 0.2 mM. DreamTaq buffer (10×), magnesium chloride and dimethyl sulfoxide in the amounts of 4 mM and 3% of the volume of real-time RT-PCR-mixture, respectively, were used as the basis. MMLV-reverse transcriptase (10 units) and Thermus aquaticus DNA polymerase (1 unit) were used as catalysts for reverse transcription and amplification reaction. The temperature and time parameters of real-time RT-PCR were set according to the above-mentioned requirements [7].

Control during testing of the method. Control during testing of the method. As a positive control, a non-inactivated suspension of culture FMD virus with the content of virions of 1.00 μg/cm^3. A non-inactivated suspension of BHK-21 cells with a concentration of 2.5–3.0 mln/cm^3 was used as negative control.

RESULTS AND DISCUSSION

At the first stage of the study the control panel of ready dilutions of the standard was obtained. A non-inactivated suspension of FMD culture virus of Asia-1/Shamir Israel 3/89 strain with concentrations of 146S component of the virus: 0.01; 0.05; 0.10; 0.20; 0.50; 1.00; 2.00; 2.50; 3.00; 3.50; 4.00; 4.50; 5.00 μg/cm^3 was used as a standard. Then, a viral suspension containing FMD virus was introduced into plates coated with antibodies against FMD virus of a given strain and then viral RNA was isolated from the obtained complexes of "FMDV virion – strain-specific antibodies". As a result, 12-fold viral RNA extracts of each standard dilution were obtained and their purity was assessed by spectral analysis in ultraviolet light. A record of the absorption spectrum of standard dilutions of RNA at wavelengths from 205 to 325 nm is shown in Figure 1.

Based on the results of analysis of control samples in the above-mentioned dilutions demonstrated that $OD_{205\text{-}259}$ and $OD_{263\text{-}325}$ values did not exceed $OD_{260\text{-}262}$, which attests to a high level of purity of the obtained RNA eluates ($n = 3$). The data of spectral study of the standards, shown in Figure 1, showed the absence of marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, which indicated almost complete absence of contamination of RNA extracts with phospholipids, polysaccharides and residues of GITC, carbolic acid, polypeptides and large conglomerates, respectively. The values of R_1 extinction coefficient for the standards are close to the norm of 2.000 (R_1 was 1.995–1.999), which confirmed the absence of DNA and the presence of only trace amounts of protein impurities and carbolic acid residues. Degradation of nucleic acid and presence of free nucleotides in eluates were not observed, as R_1 did not exceed 2.000. The viral RNA extracts of the standard dilutions were not contaminated with

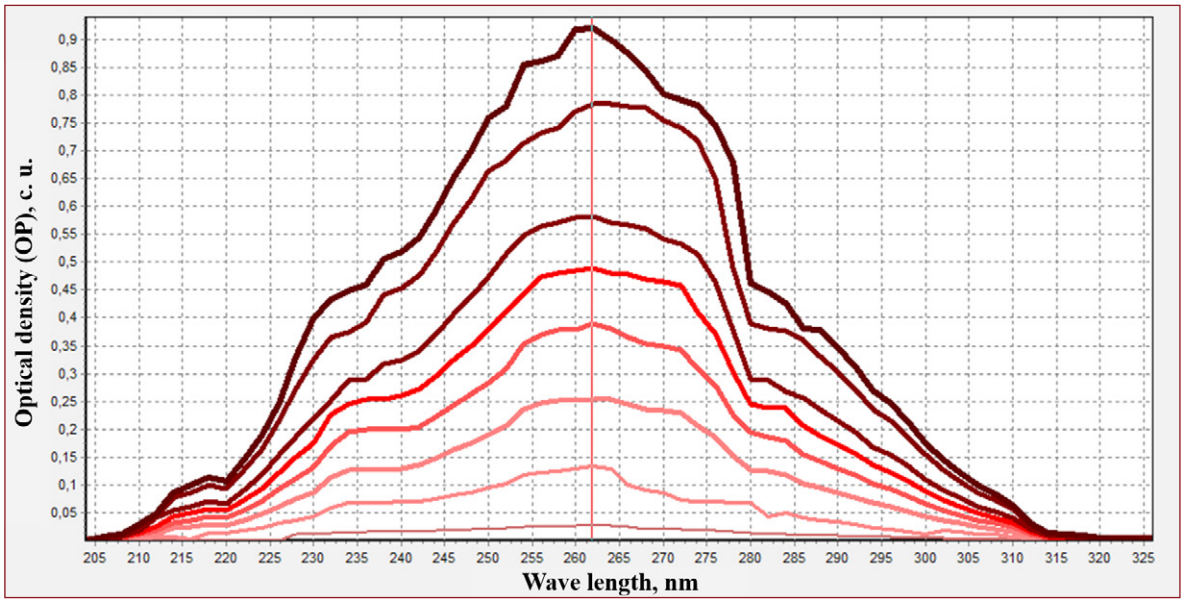

Fig. 1. Spectograms of diluted eluates of FMDV RNA (Asia-1/Shamir Israel 3/89 strain). From bottom to top see graphs for diluted extracts corresponding to the following concentrations of virions: 0.01; 0.10; 0.50; 1.0; 2.0; 3.0; 4.0; 5.0 μg/cm³

Рис. 1. Спектрограммы разведений элюатов РНК вируса ящура штамма Азия-1/Шамир Израиль 3/89. Снизу вверх отражены графики для разведений экстрактов, соответствующих следующим концентрациям вирионов: 0,01; 0,10; 0,50; 1,0; 2,0; 3,0; 4,0; 5,0 мкг/см³

polysaccharides and GITC, as the values of R_2 extinction coefficient were close to the norm of 2.000 and corresponded to 2.000–2.001. Taking into account that when 1% of RNA is substituted for carbohydrates, R_2 value decreases by 0.002 [16], polysaccharide impurities were not detected in the extracts obtained. The degree of RNA destruction in the extracts was not more than 0.5% ((2.001–2.000)/0.002), which is admissible. Thus, FMDV RNA extracts extracted from standard dilutions and used for further studies were characterized by a high purity level.

At the next stage of the work, real-time RT-PCR was carried out according to the above-mentioned recommendations. The analysis was based on the use of 5'-exonuclease activity of Thermus aquaticus of DNA polymerase. In the absence of a target, fluorophore ROX and fluorescence extinguisher BHQ2 in the 3D-FMDV-probe were brought closer due to the maximum use of hydrogen bonds between atoms of H, O and N oligonucleotides. Due to the mechanism of fluorescence-resonance energy transfer the glow is suppressed. Due to the 5'-exonuclease activity of Thermus aquticus of DNA polymerase after annealing the Forward-3D-FMDV-, Reverse-3D-FMDV-primers and 3D-FMDV-ROX/BHQ2-probe the hybridized probe and amplicon were destroyed, their spatial separation was observed, which led to the growth of the detected signal. The increase in the fluorescence level (*Fl*) was proportional to the number of reaction products produced. Monitoring of the signal during 40 cycles (*C*) of real-time PCR allowed to construct kinetic fluorescence curves, which are set by functions of type $Fl = f(C)$.

The obtained data were analyzed with the help of Rotor-Gene FRT-Manager software, which allows to construct graphs of the accumulation of fluorescent signal in real time during the specified number of amplification cycles.

Using the technology of "Maxima" software (or analogue), the graphs of the first and second derivatives for obtained eluates of FMD RNA of each dilution of the standard with known concentrations of 146S component were plotted and average values of maximum extrema (C_p) of the graphs of the second derivative $Fl = f(C_p)$ with projection on the abscissa axis "O-cycles" were calculated.

The value of C_p is an important characteristic of the reaction, which is directly proportional to the number of copies of the original RNA matrix and, consequently, to the concentration of the 146S component of FMD virus, since each such particle contains one molecule of viral RNA [3, 17, 18]. Taking into account that the second derivative of the function $f(C_p)$ ($f''(C_p)$) is continuous in some neighborhoods of the point $C_p = C_{p1}$ and is set at the amplification cycle interval from 0 to 40, there is a certain interval near the point C_p for which in all coordinates on the O-C_p axis the second derivative of the function $f(C_p)$ will be negative. Since $f''(C_p)$ is the first derivative of $f'(C_p)$, it follows from the condition $(f'(C_p)) < 0$ that $f'(C_p)$ on some small interval containing point $C_p = C_{p1}$ will decrease. Taking into account that $f'(C_p) = 0$, on the segment at $C_p < C_{p1}$ the first derivative of the function $f(C_p) > 0$, and at $C_p > C_{p1} - f'(C_p) < 0$. In other words, the first derivative of the function $f(C_p)$ changes the sign from plus to minus when passing through the point $C_p = C_{p1}$, therefore, at the point C_{p1} the function reflecting the process of fluorescence signal accumulation has the maximum extremum [17]. Thus, if the graph of the real-time amplification reaction is represented by the function $Fl = f(C_p)$, $f'(C_p) = 0$ and $f''(C_p) < 0$, then provided that $C_p = C_{p1}$ the obtained function has a maximum at the point with the argument C_{p1}, the value of which is taken into account to establish the dependence between the concentration of FMDV 146S component and the value of C_p.

The advantage of using the second derivative in this case is that when the function of the amplification curve $Fl = f(C_p)$ is multiplied by any multipliers, including the proportionality coefficient α [8, 14], the position of the maxima of the derivatives does not change. The maximum extremum of the second derivative is within the exponential

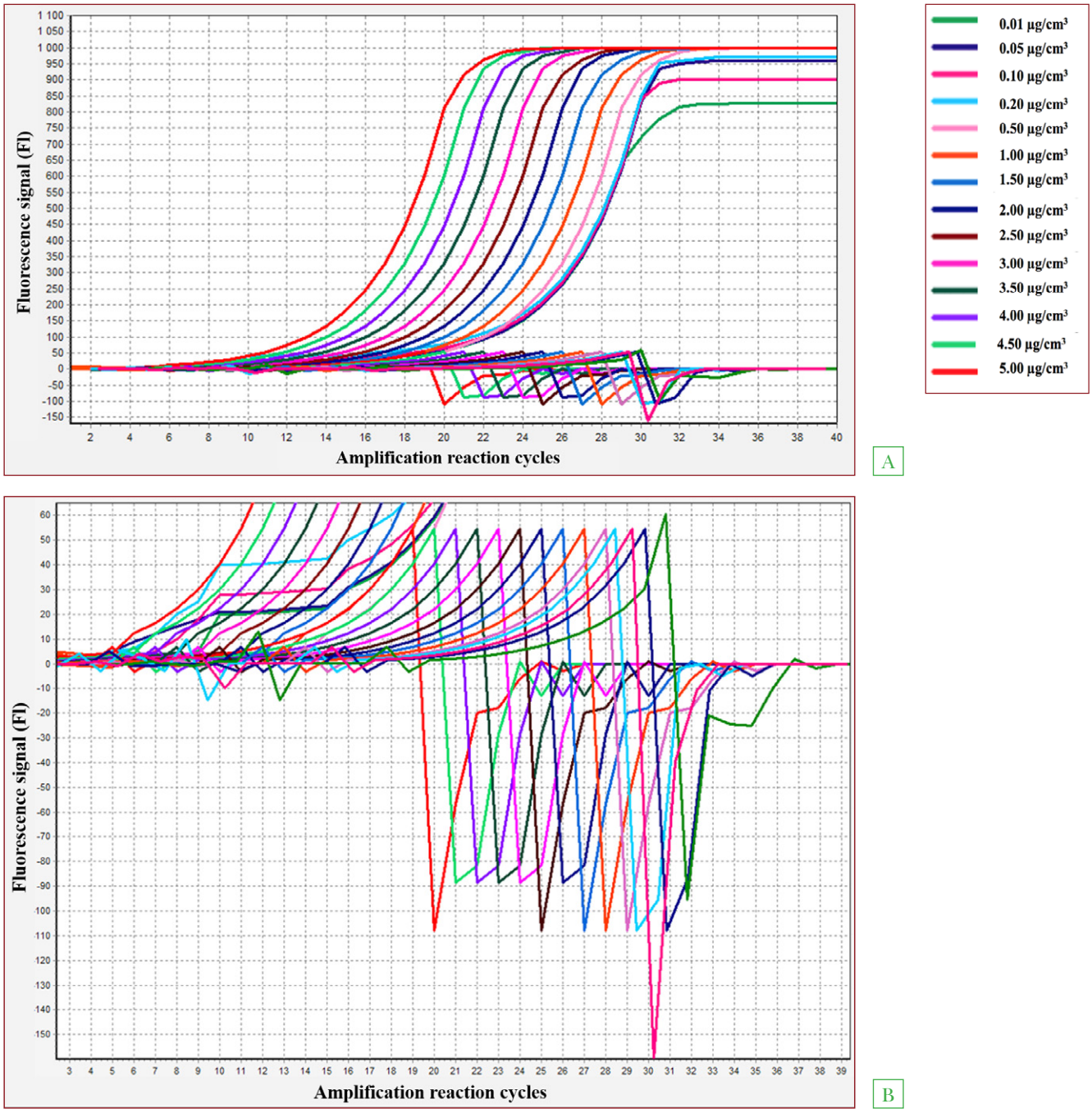

Fig. 2. Mean values of critical points C_p, calculated with the second derivative for real-time amplification reaction graphs during analysis of RNA of standard FMD virus, Asia-1/Shamir Israel 3/89 strain, with the following concentrations of 146S component: 0.01, 0.05, 0.10, 0.20, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00 µg/cm³ (n = 3) (A – graphs of accumulation of fluorescent signal, first and second derivatives; B – graphs of second derivatives for amplification reaction curves)

Рис. 2. Средние значения критических точек C_p, рассчитанные с помощью второй производной для графиков реакции амплификации в реальном времени при анализе РНК стандарта вируса ящура штамма Азия-1/Шамир Израиль 3/89 с концентрациями 146S компонента 0,01; 0,05; 0,10; 0,20; 0,50; 1,00; 1,50; 2,00; 2,50; 3,00; 3,50; 4,00; 4,50; 5,00 мкг/см³ (n = 3) (А – графики накопления флуоресцентного сигнала, первой и второй производных; В – графики второй производной для кривых реакции амплификации)

region of the fluorescence accumulation graph, i.e., in the exponential region, during the analysis of which the efficiency of the amplification reaction does not change [17].

Graphs of the first and second derivatives of the obtained eluates of FMDV RNA of each dilution of the standard with known concentrations of virions of FMD virus are shown in Figure 2.

The results of the experiment on presentation of the system of parallel evaluation of maximum extrema of the graphs of the second derivative for real-time amplification reaction curves (C_p) and the concentration of 146S component of FMDV ($C_{146S\ FMDV}$) in control samples are presented in the table from which it follows that C_p values for all dilutions of the standard of culture FMDV

Table
Relationship between concentration of FMDV 146S component and values of maximum extreme points of real-time amplification reaction curves determined with the second derivative ($n = 3$)

Таблица
Зависимость концентрации 146S компонента вируса ящура и значений максимальных экстремумов кривых реакции амплификации в реальном времени, определенных с помощью второй производной ($n = 3$)

Type of sample	FMDV 146S component concentration, μg/cm³	Determination of FMDV 146S component concentration with real-time RT-PCR							
		according to critical point C_p (crossing point) (proposed method)					according to amplification threshold cycle Ct (threshold cycle) (prototype)		
		C_{p1}	C_{p2}	C_{p3}	C_{pcp}	$C_{virions}$, μg/cm³	Ct_{cp}	$C_{virions}$, μg/cm³	
								per 12× eluate	per 1× eluate
Standard with known concentrations of FMDV 146S component (according to CFT)	0.01	29.85	29.92	29.81	29.86 ± 0.06	0.010 ± 0.003	29.19 ± 0.08	0.058 ± 0.069	0.005 ± 0.005
	0.05	29.82	29.78	29.71	29.77 ± 0.06	0.050 ± 0.010	29.12 ± 0.07	0.304 ± 0.098	0.025 ± 0.009
	0.10	29.59	29.63	29.61	29.61 ± 0.04	0.103 ± 0.020	29.01 ± 0.06	0.660 ± 0.083	0.055 ± 0.038
	0.20	23.34	29.31	29.35	29.33 ± 0.02	0.204 ± 0.030	28.78 ± 0.06	1.440 ± 0.038	0.120 ± 0.050
	0.50	28.53	28.51	28.56	28.53 ± 0.03	0.503 ± 0.041	27.74 ± 0.05	4.985 ± 0.024	0.415 ± 0.050
	1.00	27.21	27.19	27.28	27.23 ± 0.05	1.019 ± 0.032	25.93 ± 0.05	11.160 ± 0.013	0.931 ± 0.052
	1.50	26.02	26.05	26.08	26.05 ± 0.03	1.520 ± 0.031	24.28 ± 0.04	16.741 ± 0.015	1.395 ± 0.048
	2.00	25.03	25.01	25.00	25.01 ± 0.02	1.990 ± 0.030	22.57 ± 0.04	22.560 ± 0.011	1.882 ± 0.051
	2.50	23.94	23.88	23.92	23.91 ± 0.03	2.506 ± 0.028	20.83 ± 0.03	28.503 ± 0.014	2.375 ± 0.039
	3.00	22.99	22.95	23.00	22.98 ± 0.03	2.970 ± 0.027	19.26 ± 0.03	33.840 ± 0.015	2.820 ± 0.045
	3.50	22.01	22.04	21.99	22.01 ± 0.03	3.470 ± 0.034	17.72 ± 0.05	39.060 ± 0.012	3.255 ± 0.044
	4.00	20.99	20.98	21.04	21.00 ± 0.03	4.010 ± 0.030	16.22 ± 0.05	44.160 ± 0.014	3.682 ± 0.038
	4.50	20.10	20.07	20.05	20.07 ± 0.03	4.530 ± 0.031	14.60 ± 0.05	49.684 ± 0.019	4.144 ± 0.061
	5.00	19.29	19.21	19.26	19.25 ± 0.04	5.007 ± 0.033	13.15 ± 0.05	54.601 ± 0.017	4.556 ± 0.055
Negative control	0.00	0.00	0.00	0.00	0.00	0.000	0.00	0.000	0.000

p-criteria: for C_p samples with concentrations of 0.01–0.10 μg/cm³ is less than 0.010, with concentrations of 0.10 –5.00 μg/cm³ – less than 0.005 (for the developed method); for Ct samples with concentrations of 0.01–0.10 μg/cm³ – less than 0.020, with concentrations of 0.10–0.20 μg/cm³ – less than 0.10 μg/cm³, and with concentrations of 0.50–5.00 μg/cm³ – less than 0.005 (for the prototype). To calculate the concentration of 146S particles in 12-fold sample using Ct method (initial method) the following formula was used: $C_{146S} = -3.401(Ct) + 99.333$, to evaluate the content of virions in a one-fold sample the obtained value was divided by 12.

p-критерий: для C_p образцов с концентрациями 0,01–0,10 мкг/см³ составляет менее 0,010, с концентрациями 0,10–5,00 мкг/см³ – менее 0,005 (для разработанного метода); для Ct образцов с концентрациями 0,01–0,10 мкг/см³ – менее 0,020, с концентрациями 0,10–0,20 мкг/см³ – менее 0,10 мкг/см³ и с концентрациями 0,50–5,00 мкг/см³ – менее 0,005 (для прототипа). Для расчета концентрации 146S частиц в 12-кратном образце методом Ct (первоначальный способ) применяли формулу: $C_{146S} = -3,401(Ct) + 99,333$, для расчета содержания вирионов в однократном образце полученное значение делили на 12.

with concentrations of 146S component from 0.01 to 5.00 μg/cm³ are within the range of 29.86 ± 0.06 to 19.25 ± 0.04 respectively. The study of negative control did not reveal fluorescent signal accumulation, which confirmed the absence of FMD virus in the sample. In the presented studies *p*-level of significance is less than 0.010 for control samples of the standard with concentrations of FMDV 146S component from 0.01 to 0.10 μg/cm³ and $p < 0.005$ – from 0.20 to 5.00 μg/cm³.

The dependence of FMDV 146S component concentration and the values of maximum extrema of the graphs of the second derivative for the fluorescence signal accumulation curves is shown in Figure 3 and is presented as a square function $C_{146S\ FMDV} = 0.0111(C_p)^2 - 1.0157C_p + 20.446$ with high accuracy of approximation (R^2 = 99.3%). The model was developed to analyze 12-fold eluates of FMDV RNA obtained by this method. Thus, the existence of dependence between FMDV 146S component concentration in non-inactivated vaccine raw materials and the maximum extreme of the graph of the second derivative for amplification reaction curve in real time was found.

The control samples were studied simultaneously with CFT and classical real-time RT-PCR method with determination of threshold amplification cycle value (Ct) and calculation of 146S component concentration (n = 3) [7]. It should be noted that the preparation of eluates and real-time RT-PCR for the developed method and the prototype differ, in this connection the values of

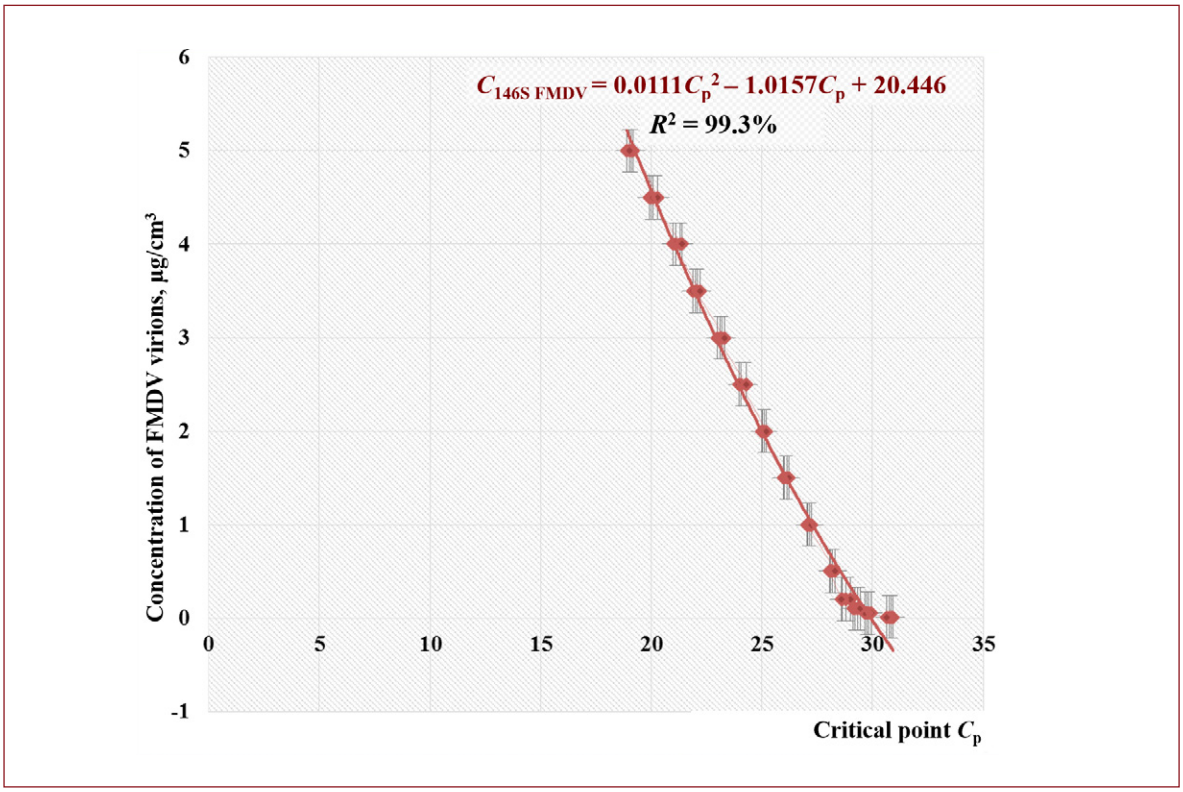

Fig. 3. Relationship between critical point C_p detected with the second derivative for real-time amplification curve and concentration of FMDV 146S component (standard error specified) (n = 3)

Рис. 3. Зависимость критической точки C_p, детектируемой с помощью второй производной для кривой амплификации в режиме реального времени, от концентрации 146S компонента вируса ящура (с указанием стандартной погрешности) (n = 3)

threshold amplification cycles for the obtained graphs of fluorescent signal accumulation of the same sample will be different.

The obtained data are presented in the table, from which it follows that the degree of difference between the results of control analysis by the developed method and the true values of positive standards is 0.00–2.91%. Differences in determination of FMDV 146S component concentration of positive controls with ballast protein content more than 7.00 mg/cm^3 by the initial method in comparison with expected values for samples with 146S component concentrations from 0.01 to 0.10 μg/cm^3 were 45–52%, from 0.01 to 0.10 μg/cm^3 – 17–45%, with full viral particles content from 0.50 to 5.00 μg/cm^3 – 5–17%. In the negative control, FMD virus was not detected with any of the methods presented. Thus, the developed method of indirect determination of FMDV 146S component concentration in the non-inactivated suspension by comparing the maximum extremes of the graphs of the second derivative for the amplification reaction curves in real time allows studying the virus-containing material with concentrations of the 146S component from 0.01 to 5.00 μg/cm^3 with the presence of the ballast protein in the samples more than 7.00 mg/cm^3 within 4–5 hours and with high accuracy.

CONCLUSION

A new approach to indirect determination of FMDV 146S component concentration in a non-inactivated suspension when comparing the maximum extrema of the graphs of the second derivative of the fluorescence signal accumulation curves with the number of amplification reaction cycles is proposed.

The proposed method allows to: 1) increase specificity of sample analysis due to strain-specific binding of FMD virus virions; 2) exclude the possibility of increasing the background fluorescence values due to the use of P. Chomczynski method of sample fraction separation; 3) increase the reliability of the conducted analysis by determining the dependence between the values of FMDV 146S component concentration ($C_{146S\ FMDV}$) and the maximum extrema of the second derivative graphs for amplification reaction curves (C_p).

The existence of dependence between the quantities of 146S particles of FMDV and maximum extrema of the graphs of the second derivative of the fluorescence signal accumulation curve in the form of a square function $C_{146S\ FMDV} = 0.0111(C_p)^2 - 1.0157C_p + 20.446$ with high accuracy of approximation ($R^2 = 0.993$) was determined. The proposed model allows us to quantitatively estimate the content of FMDV virions in non-inactivated raw materials for the vaccine in 4–5 hours.

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