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VALIDATION OF REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION FOR INDIRECT ASSESSMENT OF FMD VIRUS TITRE

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SUMMARY

Procedure for validation of the method for indirect assessment of cultural FMD virus titre (T_{FMDV}) in raw materials used for vaccine production with real-time reverse transcription polymerase chain reaction (RT-PCRrt) based on determined amplification threshold cycle value (C_t) using linear regression model, $T_{FMDV} = -0.2956C_t + 11.4650$, is described in the paper. Testing results for 390 samples of cultural FMD virus were analyzed and basic validation characteristics of the proposed method: specificity, accuracy, precision, limit of detection and limit of quantification, application scope and linearity, were defined. Validation results for the method for indirect assessment of FMD virus titre with RT-PCRrt met the acceptance criteria.

Key words: foot-and-mouth disease (FMD), virus infectivity titre, validation, RT-PCRrt, amplification threshold cycle.

INTRODUCTION

Production of vaccines for veterinary use shall fully guarantee the conformity of products to its purpose and requirements. To achieve these goals the establishments operate a system of quality assurance of intermediate raw materials and finished products, which includes detailed control of vaccine manufacturing process at all stages.

Analytical control of raw materials plays an important role in the production of FMD vaccines, in particular, the determination of the infectivity titre of FMD virus reproduced in the suspended continuous cell culture from Syrian baby hamster kidney (BHK-21/2-17). One of the methods of indirect assessment of FMD virus titre in vaccine raw materials is real-time reverse transcription polymerase chain reaction (real-time RT-PCR) [9, 10] which allows determining the amount of genome copies and whole infectious virus particles inducing signs of cytopathic effect (CPE) in sensitive cell cultures [1, 6, 8, 9]. The research results of Zh. A. Shazhko (1980), A. P. Ponomaryova, M. V. Kotova et al. (1983) confirm that whole FMD virus particles are mainly responsible for infectivity. Virus suspensions are known to contain both infectious and non-infectious 146S particles but the number of the latter is insignificant and is approximately 1:10,000 [6]. Data of scientific publications by T. R. Doel (1982), N. S. Mamkova, V. L. Uzyumova et al. (1983), R. R. Rückert (1989) indicate a correlation between the amount of FMDV 146S particles and the degree of its cytopathic effect in infected cell culture [1, 6]. The information presented indicates the possibility of real-time RT-PCR application for indirect determination of the FMD virus titre in vaccine raw materials [8].

Assessment of the suitability of quantitative real-time RT-PCR using the previously proposed linear regression model: $T_{FMDV} = -0.2956Ct + 11.4650$, where T_{FMDV} is the FMD virus titre; Ct is the threshold amplification cycle [8], is carried out on the basis of validation results, involving the determination of specificity, trueness, precision, range of application and linearity of the analytical method, as well as the limit of analyte detection and quantification [2–4, 9].

The considered real-time RT-PCR method specificity is understood as the ability to unequivocally quantify

infectious FMD virus particles that induce CPE in the porcine kidney cell line (IB-RS-2) [4] if the tested sample contains foreign antigenic components.

Obtaining results with a high confidence level is only possible when using an accurate method by which the measurement results are close to the reference value of the parameter [2]. The accuracy of quantitative methods discloses the concepts of trueness and precision. In this case trueness is considered as the proximity of the mean value of the FMD virus titre obtained on the basis of a large series of experiments to the reference value. The precision of the proposed method is understood as the degree of variation in measurements in terms of threshold amplification cycles between the series of measurements carried out for a number of samples taken from the pooled sample under the conditions regulated by the method [2, 7]. To assess the level of the required quality of test results, the repeatability test is carried out under the conditions of repeatability and reproducibility, thus determining the absolute and relative variation values [2, 3, 7, 11, 12].

The detection limit of the analytical method is considered the least number of infectious FMD virus particles in the sample that induce CPE, which can be detected, but not necessarily quantified [5, 7]. The limit of quantification in this case is the lowest value of FMD virus titre, which can be quantified with the appropriate trueness and precision of the method [2].

Linearity is an indication that there is a directly proportional relationship between the threshold amplification cycle values and the FMD virus titre within the analytical range of the method [2, 7, 11]. The range of application of the developed method is the interval between the upper and lower values of the virus titre within which the proposed algorithm for analyte determination has a suitable level of precision, trueness and linearity [2, 12].

The aim of the study is to assess suitability of the method for indirect assessment of the infectivity titre of the cultural FMD virus in vaccine raw material by real-time RT-PCR.

MATERIALS AND METHODS

Tested material. The cultural FMD virus of the following vaccine strains was used: A № 2187/Kuti/2013, A № 2029/Turkey/2006, A № 2171/Kabardino-Balkarsky/2013, O № 2212/Primorsky/2014, O № 2047/08/Saudi Arabia 120/29, O_1 № 1618/Chechen-Ingushsky/66, Asia-1 № 2145/Tajikistan/2011, Asia-1 № 1946/Shamir Israel 3/89, SAT-2/Kenya 183/74, SAT-2/Saudi Arabia/2000, that were reproduced in continuous suspension cell culture BHK-21/2-17.

Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR). The test was performed as described by A. P. Ponomaryov et al. [6]. The results of real-time RT-PCR were presented as calculated titre values. The estimated virus titre was the value obtained by analyzing the sample threshold amplification cycle of the collected samples with regard to the FMDV calibration samples with a known titre. Calibration curves were constructed using Quantitative Analysis function of amplifier Rotor-Gene 6000 Series Software v 1.8.17.5.

FMD virus titration in a sensitive cell line. To determine the infectivity titre (lg TCD_{50}/cm^3) of cultural FMD virus vaccine strains, continuous monolayer cell culture IB-RS-2 was used [4].

Method specificity assessment. The specificity of the method was evaluated based on the relative error (e) of the results obtained simultaneously with application of the validated real-time RT-PCR method [6] and virus titration test in IB-RS-2 cell line [4]. Ten model mixtures of cultural FMD virus which contained a different number of infectious particles that induced CPE in IB-RS-2 cell line mixed with 75S, 12S and 3.8S components that did not have a similar effect on the cells, were tested [1, 4, 9]. The fractionation of the cultural FMDV into components was performed using sucrose density gradient ultracentrifugation [9]. The test was performed in 6 replicates. The relative error was determined by the formula: $e = (T_{FMDV}^{I} - T_{FMDV})/T_{FMDV}$, where T_{FMDV}^{I} is the virus titre based on real-time RT-PCR; T_{FMDV} – the virus titre based on titration results in cell culture IB-RS-2.

The method specificity at $e \leq 5\%$ was considered high [7, 11].

Method trueness assessment. To determine the trueness of the method, 390 samples of the cultural FMD virus with known titre values were tested by quantitative real-time RT-PCR. The data were presented as a linear relationship between the experimental values obtained in real-time RT-PCR (y) and the reference values of virus titre (x): $y = k \times x + b$. For the function obtained, we tested the hypotheses on equality of the tangent of the angle of inclination (k) to unity, as well as equality of the free term (b) to zero. When proving the truth of these hypotheses with a level of reliability equal to 0.05, the implementation of a validated method gives error-free results [2, 5, 7, 12].

Method precision analysis. To evaluate the precision of the proposed real-time RT-PCR methodology under repeatability and reproducibility conditions, absolute and relative variation values were calculated.

Determination of absolute variation values. The range of variation (R) was determined as the difference between the upper and lower values of the amplification threshold cycle: $R = Ct_{max} - Ct_{min}$. Individual linear deviation (d_i) was obtained by the formula: $d_i = | Ct_i - Ct_{mean} |$. The mean linear deviation (d_{mean}) was calculated as the arithmetic mean of individual linear deviations: $d_{mean} = \Sigma | d_i | / N$, where d_i is the individual linear deviation of the threshold amplification cycles; N is the total volume. Estimation of the dispersion (δ^2) of values was performed using the formula: $\delta^2 = (\Sigma d_i^2) / N$. To characterize the size of the variation Ct, the standard quadratic deviation (δ) was calculated using the formula: $\delta = \sqrt{(\delta^2)}$ [2].

Determination of relative variation values. The oscillation coefficient (V_R) was calculated using the formula: $V_R = R / Ct_{mean} \times 100$. The linear coefficient of variation (C_d) was calculated using the formula: $C_d = d_{mean} / Ct_{mean} \times 100$. To evaluate the variability of individual values of the threshold amplification cycles, the coefficient of variation was determined (C_δ) according to the formula: $C_\delta = \delta / Ct_{mean} \times 100$ [2]. The method is considered reliable at $C_\delta < 2\%$ under conditions of repeatability and $C_\delta < 3\%$ under conditions of reproducibility [2, 7].

Estimation of the FMDV titre limit of detection (LOD_{TFMDV}). The least amount of FMDV infectious particles of virus using a validated method was obtained by the formula: $LOD_{TFMDV} = 3{,}3 \times S_b / k$, where S_b is the standard deviation of the analytical signal, that corresponds to the standard deviation of the free term (b); k is the tangent of the angle of inclination [5, 7].

The free term b was obtained in the study of 10 model samples with FMD virus titres as follows: 1.00; 2.00; 3.00; 4.00; 5.00; 6.00; 7.00; 7.50; 8.00; 9.00 lg TCD_{50}/cm^3.

Table 1
Evaluation of method specificity for indirect assessment of FMDV titre using real-time RT-PCR
($n = 6, M \pm m$)

Mixture No.	Correlation of mixture components		FMDV titre, lg TCD_{50}/cm^3		Relative error (e) in titre estimates by real-time RT-PCR, %
	146S particles	75S + 12S + 3.8S components	in IB-RS-2 cell line	Real-time RT-PCR results as virus titre estimates	
1	1	0	9.00 ± 0.09	8.98 ± 0.02	0.11–0.44
2	1	10	8.03 ± 0.08	8.02 ± 0.04	0.12–0.63
3	1	10^2	7.02 ± 0.13	7.00 ± 0.02	0.14–0.57
4	1	10^3	6.04 ± 0.18	6.02 ± 0.03	0.17–0.83
5	1	10^4	5.04 ± 0.15	5.02 ± 0.03	0.20–0.79
6	1	10^5	4.00 ± 0.14	3.99 ± 0.03	0.50–1.00
7	1	10^6	3.01 ± 0.18	3.05 ± 0.02	0.66–1.99
8	1	10^7	2.05 ± 0.14	2.01 ± 0.03	0.49–3.41
9	1	10^8	1.00*	0.98 ± 0.03	1.00–5.00
10	1	$10^{8.7}$	0.50*	0.53 ± 0.05	4.00–16.00

* titre values were obtained by theoretical calculations based on dilution factor.

Estimation of FMDV titre limit of quantification (LOQ_{TFMDV}). The minimum value of the FMDV titre estimated with the appropriate level of trueness and precision of the validated method was calculated using the formula: $LOQ_{TFMDV} = 10 \times S_b / k$ [7, 11]. The obtained value of LOQ_{TFMDV} was validated in direct experiment by testing 13 model suspensions of cultural virus with titres close to the obtained value of the limit of quantification. The test was performed in five replicates. The test results were considered reliable at $p < 0.005$.

Determination of the application range of the method. To evaluate the analytical range of the validated method, 11 model samples with the following FMDV titres: 0.50; 1.00; 2.00; 3.00; 4.00; 5.00; 6.00; 7.00; 8.00; 9.00; 9.50 lg TCD_{50}/cm^3 were tested in five replicates.

Evaluation of the method linearity. The presence of linear relationship between the threshold amplification cycle and FMDV titre within the application range of the method was experimentally tested, wherein Ct was determined in five replicates for 40 samples containing different amounts of analyte. The obtained data were processed using the least squares regression method: $Ct = k \times T_{FMDV} + b$, where k is the angular coefficient; b is the free term. The reliability of the analysis results was confirmed by calculating the correlation coefficient (r), which should be ≥ 0.99 based on the model [5, 7].

RESULTS AND DISCUSSION

At the initial stage of the study the specificity of the validated method for the indirect assessment of the FMDV titre in vaccine raw material using real-time RT-PCR was evaluated. Ten model samples were prepared by adding 146S component to the suspensions, determining the virus infectivity [1, 9], and a mixture of 75S, 12S and 3.8S components to identify their possible effect on the specificity of this method. The qualitative and quantitative composition of model samples, as well as the relative error (e) values based on real-time RT-PCR are shown in Table 1.

As Table 1 shows, the relative error of the indirect assessment of FMDV titre using a validated method with the number of infectious particles at 9.00–8.03; 8.03–7.02; 7.02–6.04; 6.04–5.04; 5.04–4.00; 4.00–3.01; 3.01–2.05; 2.05–1.00; 1.00–0.50 lg TCD_{50}/cm^3 was 0.11–0.63; 0.12–0.57; 0.14–0.83; 0.17–0.79; 0.20–1.00; 0.50–1.99; 0.66–3.41; 0.49–5.00; 1.00–16.00% respectively. Thus, a high level of method specificity for FMDV suspensions with the virus titres at 1.00–9.00 lg TCD_{50}/cm^3 was noted, while the relative error shown in the study did not exceed 5%.

At the next working stage the validated method was assessed for trueness by testing of 390 samples of cultural FMD virus with titres of infectious activity from 1.00 to 9.00 lg TCD_{50}/cm^3 (at the intervals of 0.25 lg TCD_{50}/cm^3) with real-time RT-PCR. The values of titres exceeding the indicated range were not used in the analysis as in industrial preparation of FMD viral suspensions the infectivity titre tends to be within the range of 6.0–8.5 lg TCD_{50}/cm^3. The research results are presented in Figure 1 as a linear function $y = 0.9985x + 0.0113$, which with confidence level of (R^2) 0.9995 confirms that the tangent of the angle of inclination (k) tends to one ($k = 0.9985$) and a free term (b) – to zero ($b = 0.0113$). The analysis of the obtained data suggests that the use of the validated method allows getting reliable results.

Precision of the method for indirect determination of FMD virus infectivity titre in raw materials for vaccines with real-time RT-PCR and with calculation of absolute and relative variation parameters of the analysis data was evaluated.

Precision under repeatability conditions was assessed on the basis of variability results of real-time RT-PCR in one

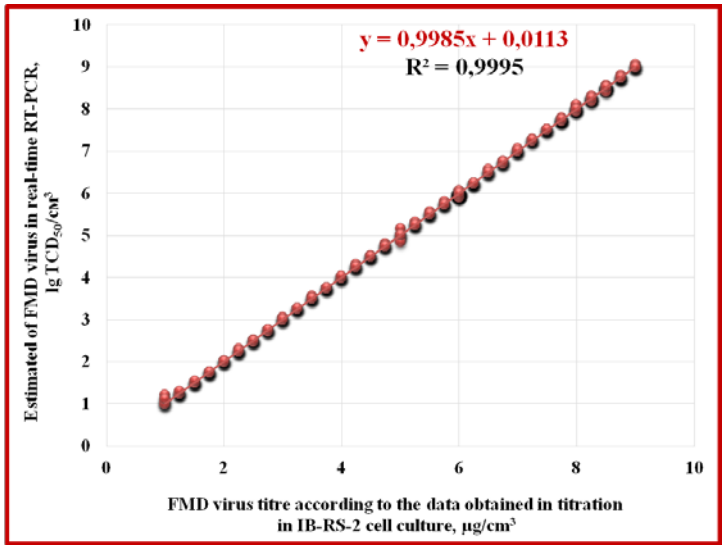

Fig. 1. Analysis of trueness of method of indirect assessment of FMD virus titre with real-time RT-PCR

test of five suspensions of cultural FMD virus with the following titres: 1.0; 5.0; 7.0; 8.0; 9.0 lg TCD_{50}/cm³, which were simultaneously tested by an operator with one device in 20 replicates. The results of the statistical analysis of the total of threshold amplification cycles (Ct_j) are shown in Table 2 and in Figure 2. The data of Table 2 demonstrates that the variation range was 0.05–0.22; mean linear deviation – 0.007–0.042, i. e., variation level Ct_j in the obtained samplings with the indicated virus titres was low in comparison with the mean value of the parameter. Coefficient of variation was within the range of 9.4×10^{-5}–3.9×10^{-3}, mean quadratic deviation stood at 0.010–0.056, which confirms an insignificant deviation level of individual values Ct_j from the arithmetic mean (Ct_{av}). Thus, according to the results of the analysis of absolute variation values the variability of individual values in samples is low and the values of threshold amplification cycles constitute a reliable total.

In order to analyze the variability of the obtained values of Ct_j within the general total, relative variation parameters were determined. Oscillation coefficient was 0.427–0.662%, consequently, relative variation of extreme values of Ct_j in comparison with the mean value in all samples is low. Linear variation coefficient was within the range of 0.089–0.118%, consequently, the share of an averaged value of deviation from Ct_{av} in each total is negligible. Variation coefficient of individual values of threshold cycles stood at 0.116–0.158%, which corresponds with general requirements for validated methods (C_δ < 2%) [2, 5].

As follows from Table 2 and Figure 2, variation of values of each parameter in samples decreased when the virus titre rose from 1.00 to 9.00 lg TCD_{50}/cm³ and the confidence of indirect quantity determination of the analyte with real-time RT-PCR increased. Thus, during testing suspensions of FMD virus with titres from 1.00 to 9.00 lg TCD_{50}/cm³ in 20 replicates with the use of the proposed method an operator received homogeneous and reliable total values of threshold cycles which can be used for indirect expression in the form of a virus titre.

Table 2
Precision test of the method for indirect assessment of FMD virus titre with real-time RT-PCR

Validation parameters of the method in precision assessment	Values of validation parameters of tested suspensions with different FMD virus titres, lg TCD_{50}/cm³									
	repeatability conditions					reproducibility conditions				
	1.0	5.0	7.0	8.0	9.0	1.0	5.0	7.0	8.0	9.0
Volume of amplification threshold cycle sampling (*N*)	20	20	20	20	20	40	40	40	40	40
Mean value of amplification threshold cycles (Ct_{av})	35.390	21.847	15.110	11.710	8.353	35.397	21.848	15.107	11.71	8.354
Total value of individual linear deviation in modulus ($\Sigma \mid d_i \mid$)	0.833	0.471	0.304	0.232	0.148	1.612	1.002	0.652	0.490	0.316
Total value of squared modulus of individual linear deviation (Σd_i^2)	0.063	0.021	0.009	0.004	0.002	0.158	0.051	0.018	0.010	0.005
Maximum value of a threshold cycle (Ct_{max})	35.50	21.94	15.16	11.74	8.37	35.58	21.94	15.17	11.74	8.39
Minimum value of a threshold cycle (Ct_{min})	35.28	21.80	15.06	11.69	8.32	35.21	21.78	15.06	11.68	8.33
Variation range (*R*)	0.22	0.14	0.10	0.05	0.05	0.37	0.16	0.11	0.06	0.06
Mean linear deviation (d_{av})	0.042	0.024	0.015	0.012	0.007	0.040	0.025	0.016	0.012	0.008
Variation (δ^2)	3.0×10^{-3}	1.0×10^{-3}	4.0×10^{-4}	2.0×10^{-4}	9.4×10^{-5}	3.9×10^{-3}	1.2×10^{-3}	4.5×10^{-4}	2.5×10^{-4}	1.2×10^{-5}
Mean quadratic deviation (δ)	0.056	0.032	0.021	0.014	0.010	0.063	0.035	0.021	0.016	0.011
Oscillation coefficient (V_R), %	0.622	0.640	0.662	0.427	0.599	1.045	0.732	0.728	0.512	0.718
Linear variation ratio (C_d), %	0.118	0.108	0.100	0.097	0.089	0.114	0.112	0.108	0.105	0.095
Variation ratio (C_δ), %	0.158	0.148	0.137	0.123	0.116	0.177	0.164	0.141	0.134	0.132

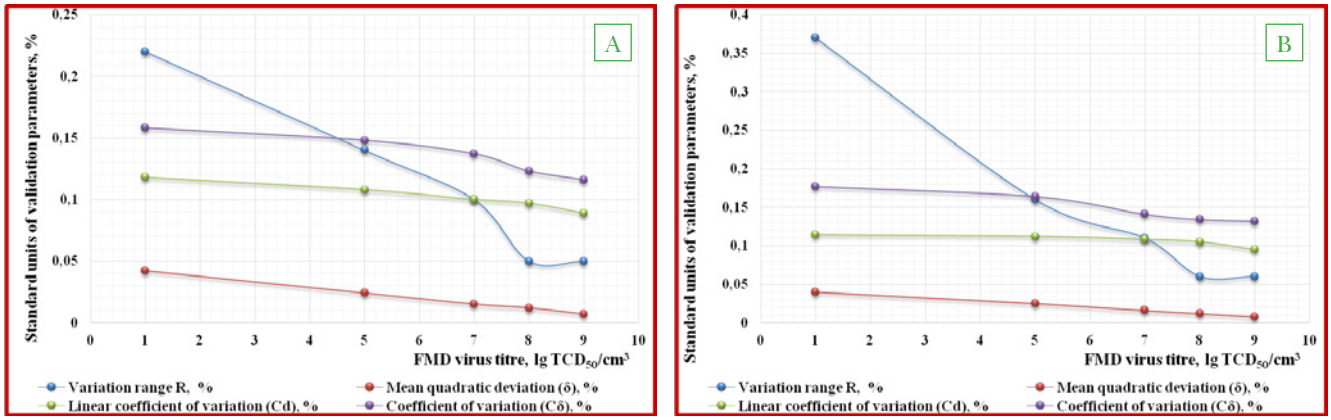

Fig. 2. Variation of validation parameters values during validation of the method for indirect assessment of FMD virus titre with real-time RT-PCR under repeatability (A) and reproducibility conditions (B)

The next working stage was devoted to the precision test of the real-time RT-PCR method under reproducibility conditions. To this end, the analysis of FMD cultural virus suspension was performed by two operators at different time in 20 replicates (40 measurements for each sample). The obtained results are presented in Table 3, from which it follows that for a general total number of amplification threshold cycles the variation range (R) stood at 0.06–0.37, mean linear deviation (d_{meat}) – 0.008–0.040, in other words, variation level Ct_i in obtained samples with respect to the mean level of the parameter is low. Upon the results of the statistical analysis, variation (δ^2) in total quantities was 1.2×10^{-5}–3.9×10^{-3}, mean quadratic deviation (δ) – 0.011–0.063, which confirms the insignificant deviation level of individual values of amplification threshold cycles with respect to the arithmetic mean and a high confidence level of results in each sampling.

Assessment of relative variation parameters Ct_i determined that oscillation coefficient (V_R) stood at 0.512–1.045%, linear variation ratio (C_d) – 0.095–0.114%, variation ratio (C_δ) – 0.132–0.177%, which corresponds to widely accepted standards ($C_\delta < 3\%$) [2, 7]. Proceeding from the

Table 3
Determination of a threshold of quantitative indirect determination of FMD virus titre with the validated method of real-time RT-PCR
($n = 5, M \pm m$)

FMD virus suspension No.	FMD virus titre		Testing of the suspension with real-time RT-PCR		Fact of experimental proof: $LOD_{TFMDV} = 0.00$ lg TCD_{50}/cm^3; $LOQ_{TFMDV} = 0.48$ lg TCD_{50}/cm^3
	TCD_{50}/cm^3	lg TCD_{50}/cm^3	threshold cycle	results presented as calculated values of virus titre, lg TCD_{50}/cm^3	
1	0.63	–0.2	39.31 ± 0.45 ($p > 0.01$)	n/d	confirmed
2	0.79	–0.1	39.15 ± 0.41 ($p > 0.01$)	n/d	confirmed
3	1.00	0.0	38.79 ± 0.69 ($p > 0.01$)	n/d	confirmed
4	1.26	0.1	38.49 ± 0.52 ($p > 0.01$)	n/d	confirmed
5	1.58	0.2	38.15 ± 0.66 ($p > 0.01$)	n/d	confirmed
6	2.00	0.3	37.79 ± 0.42 ($p > 0.01$)	n/d	confirmed
7	2.51	0.4	37.47 ± 0.48 ($p > 0.01$)	n/d	confirmed
8	3.16	0.5	37.09 ± 0.09 ($p < 0.005$)	0.50 ± 0.02	confirmed
9	4.00	0.6	36.76 ± 0.06 ($p < 0.005$)	0.60 ± 0.02	confirmed
10	5.00	0.7	36.42 ± 0.06 ($p < 0.005$)	0.70 ± 0.02	confirmed
11	6.31	0.8	36.08 ± 0.05 ($p < 0.005$)	0.79 ± 0.01	confirmed
12	7.94	0.9	35.74 ± 0.05 ($p < 0.005$)	0.90 ± 0.01	confirmed
13	10.00	1.0	35.40 ± 0.05 ($p < 0.005$)	1.01 ± 0.02	confirmed

n/d – virus titre was not determined with certainty.

Table 4
Assessment of validation criteria of the method of indirect FMD virus titre determination in raw materials for the vaccine with real-time RT-PCR

Criteria of validation method	Validation parameter	Validation parameter values of the method when testing FMD virus with different titres, lg TCD_{50}/cm³					
		0.5	1.0	5.0	7.0	8.0	9.0
Specificity	relative error (*e*), %	4–16	1–5	0.2–0.79	0.14–0.57	0.12–0.63	0.11–0.44
Repeatability	mean quadratic deviation (*δ*), %	n/t	0.056	0.032	0.021	0.014	0.010
	variation ratio ($C_δ$), %	n/t	0.158	0.148	0.137	0.123	0.116
Reproducibility	mean quadratic deviation (*δ*), %	n/t	0.063	0.035	0.021	0.016	0.011
	variation ratio ($C_δ$), %	n/t	0.177	0.164	0.141	0.134	0.132
Linearity	correlation ratio (*r*)	n/t	0.9997				
Accuracy	confidence level (R^2)	n/t	0.9995				
	tangent of the angle of inclination (*k*)	n/t	0.9985				
	free term (*b*)	n/t	0.0113				
Analytical range	range of indirect quantitative determination of FMD virus, lg TCD_{50}/cm³	0.50–9.00					

n/t – not tested.

obtained results, relative variation of extreme values of amplification threshold cycles and share of a mean value of absolute deviations in comparison to a mean in each total are insignificant. With that, the variation of validation parameter values when testing FMD virus suspensions decreased with the increase of the titre from 1.00 to 9.00 lg TCD_{50}/cm³ and the confidence level of the results increased.

Comparative analysis of data acquired during precision testing of the method created an opportunity to prove that under repeatability conditions the confidence level of results of indirect virus titre determination was higher compared to the assessment under reproducibility conditions which corresponds to widely-accepted statistical expectations [7]. With that, for all absolute and relative variation parameters the validated real-time RT-PCR method satisfied the acceptance criteria [2, 7, 11].

At the next stage of the test, limit of detection (LOD_{TFMDV}) and limit of quantification (LOQ_{TFMDV}) of the FMD virus titre in raw materials for the vaccine were determined indirectly with real-time RT-PCR method. The following values of a free term were received upon testing in 10 replicates (*b*): 11.461; 11.465; 11.469; 11.470; 11.465; 11.466; 11.465; 11.465; 11.452; 11.461. Taking into account that a standard deviation of a free term (S_b) was 0.005 and the tangent of the angle of inclination (*k*) equaled –3.38, calculations were carried out and it was established that LOD_{TFMDV} by the given method stood at 1 TCD_{50}/cm³ (or 0 lg TCD_{50}/cm³) and LOQ_{TFMDV} – 3 TCD_{50}/cm³ (or 0.48 lg TCD_{50}/cm³).

The established value of LOQ_{TFMDV} was confirmed experimentally during testing of 13 model samples of FMD cultural virus with titres from –0.2 to 1.0 lg TCD_{50}/cm³ in five replicates. The analysis results are presented in Table 3.

As follows from the data of Table 3, it was experimentally confirmed that minimum LOQ_{TFMDV} with the method in validation is 0.50 lg TCD_{50}/cm³.

During testing of 11 model samples with FMD virus titres from 0.50 to 9.50 lg TCD_{50}/cm³ it was determined that analytical domain of indirect infectivity titre assessment with real-time RT-PCR ranged from 0.50–9.00 lg TCD_{50}/cm³ with threshold cycle values from 37.08 ± 0.09 to 8.34 ± 0.03 ($n = 5$, $p < 0.005$). It seems that with titres more than 9.00 lg TCD_{50}/cm³ the suggested regression model did not allow for an accurate indirect quantitative analysis due to a high content of full virus particles ($n = 5$, $p > 0.01$).

The next working stage was devoted to checking of linear dependence of an amplification threshold cycle and virus titre within the analytical range of the method. To do that, 40 virus-containing suspensions with titres from 1.00 to 9.00 μg/cm³ in five replicates were tested. Suspensions with virus titres less than 1.0 lg TCD_{50}/cm³ were not used for the analysis as they are not used in the production work. The analysis produced a regression in the following form: $T_{FMDV} = –0.2956C_t + 11.465$ with correlation ratio (*r*) of 0.9997 which allows assessing the accuracy of statistical analysis results.

Main validation parameters for indirect FMD virus titre determination in raw materials for the vaccine with real-time RT-PCR were assessed based upon the results of the carried out tests. Final results of the test are presented in Table 4 which shows that real-time RT-PCR method range was 0.50–9.00 lg TCD_{50}/cm³. When virus-containing material with titres from 1.00 to 9.00 lg TCD_{50}/cm³ is tested, the method in validation is characterized with a high specificity (*e* stands at 0.11–5.00%) and a high precision under repeatability conditions (*δ* was within the range of 0.010–0.056%, $δ < 2\%$, $C_δ$ – 0.116–0.156%, $C_δ < 2\%$) and reproducibility conditions (*δ* constituted 0.011–0.063%, $δ < 3\%$, $C_δ$ – 0.132–0.177%, $C_δ < 3\%$). Assessment of linearity and validity proved that the method in validation gave error-free results with a high correlation ratio ($r = 0.995$, $r \rightarrow 1$) and confidence level ($R^2 = 0.991$, $R^2 \rightarrow 1$).

CONCLUSION

Main validation characteristics of the method of indirect FMD virus titre determination in raw materials for the vaccine with real-time RT-PCR with the use of the regression model $T_{FMDV} = -0.2956C_t + 11.4650$ were assessed. It was established that the analytical range of the method lies within the limits of 0.50–9.00 lg TCD_{50}/cm^3. A high specificity of real-time RT-PCR was proved during testing of FMD virus suspensions with titres from 1.00 to 9.00 lg TCD_{50}/cm^3. The developed method is characterized with a high precision both under repeatability and reproducibility conditions and complies with generally recognized linearity and accuracy requirements [2, 7, 11, 12].

Real-time RT-PCR validation results meet all acceptance criteria. Thus, the proposed method is reliable and can be used for indirect quantitative assessment of FMD virus titre in raw materials for the vaccine.

Conflict of interest. The authors declare that there is no conflict of interest.

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