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USE OF REAL-TIME qRT-PCR
FOR FMD VIRUS DETECTION IN CATTLE MILK

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SUMMARY

FMDV can be shed with milk of infected animals and infect susceptible animals. The possibility of using real-time qRT-PCR for FMD virus detection in cattle milk was assessed. To prepare samples of FMDV infected milk the following strains were used: A No. 2177/Amursky/2013, O No. 2123/South Ossetia/2011, Asia-1 No. 1987/Amursky/2005, C No. 564, SAT-1 No. 2033/Botswana, SAT-2/Saudi Arabia, SAT-3/Bech 1/65. For specificity test swine vesicular disease O-72 strain, Vesicular Exanthema of Swine A-48 strain and vesicular stomatitis virus Indiana strain from FGBI "ARRIAH" collection were used. The obtained data showed that real time qRT-PCR can be successfully used for the virus detection in milk of infected cows as well as in epithelium and cell cultures. Analytical sensitivity of real-time qRT-PCR in milk samples is 1–10 TCD_{50}/ml, the method specificity is 100%. When identifying basic validation characteristics of the method it was proven that real-time qRT-PCR demonstrates precision and sensitivity and can detect FMDV in its genetic diversity. So, the detected data showed that the real-time qRT-PCR is a precious method of milk and dairy product control.

Key words: FMD virus, milk, real-time qRT-PCR, validation.

INTRODUCTION

FMD is a highly contagious disease of domestic and wild cloven hoofed animals capable of causing great economic damage nationwide. FMD free countries are under constant risk of the disease introduction from endemic regions, such as Asia, Africa and South America.

The disease agent is non-enveloped RNA-containing FMDV, *Aphthovirus* Genus, *Picornaviridae* family. There are seven types of the virus: O, A, C, Asia-1, SAT-1, SAT-2, SAT-3. Within each type there are many genetic and antigenic variants of the virus.

FMD is not endemic in Russia, however there is a constant threat of the virus introduction from the neighboring Asian countries, primarily from China and Mongolia. FMD outbreaks caused by the infection introduction from other countries were reported in Russia in 1995, 2000, 2004–2006 and 2010–2018 [3].

Rapid and precise diagnosis is a key element of the control of the disease having high contagiousness and hard social and economic consequences.

In milk cows the disease is characterized by vesicular lesions in the mouth, on the udder, on the coronary band, in the interdigital cleft, by fever and decreased milk yield. FMDV is shed with milk of infected animals facilitating the infection transmission [9].

Milk is a perfect medium for laboratory diagnosis as it is available in any quantity and its samples are easily collected. Several researchers applied different diagnostic approaches when using milk samples for cattle infection control and identification of the disease status of individual animals [5, 8].

According to the researches of S. M. Reid and et al., FMDV can be observed in cow milk on day 2–3 after infection until the experimentally infected animals demonstrate clinical signs. It was also established that the virus can replicate in the mammary gland and shed with milk for up to 23 days. The authors showed benefits of real-time qRT-PCR tests of cow milk as an instrument of preclinical FMD diagnosis [9].

P. M. Tomasula and R. P. Konstance's investigations demonstrated that FMDV is detected in milk after sample incubation at 37 °C, after storage at room temperature and at 4 °C for two weeks. Besides, FMDV can be detected in pasteurized products. Such method as virus isolation can not detect the viable virus in heat treated samples [7].

According to the previous researches real-time qRT-PCR is a valuable method for laboratory FMDV detection in epithelium vesicules, nasal swabs and esophageal samples [3, 6, 8].

The purpose of the research was to assess the possibility of real-time qRT-PCR use for FMDV detection in milk and to identify its basic validation characteristics.

MATERIALS AND METHODS

Viruses. The following viruses were used in the research: FMD viruses: A No. 2177/Amursky/2013, O No. 2123/South Ossetia/2011, Asia-1 No. 1987/Amursky/2005, C No. 564, SAT-1 No. 2033/Botsvana, SAT-2/Saudi Arabia, SAT-3/Bech 1/65, as well as swine vesicular disease virus strain O-72, VESV A-48 strain and vesicular stomatitis virus Indiana strain from FGBI "ARRIAH" collection.

FMDV infected milk samples. 10-fold FMDV serial dilutions of three circulating serotypes (A, O, Asia-1) with the concentration of 10^{-1}–10^{-10} lg TCD_{50}/ml were prepared using milk of normal milk cows.

RNA isolation from cattle was performed using 6 M guanidine isothiocyanate and fibrous glass filter GF/F as described by A. Sherbakov et al. [4].

Real-time qRT-PCR. 25 µl of reaction mixture contained 5 pm direct and reverse primers, 5 pm TaqMan probe, 5 µl of RNA, 20 µl of the reaction mixture for real time PCR (Sintol, Moscow), containing 2.5 µl of PCR buffer (10-fold concentration); 2.5 µl of 25 mM $MgCl_2$; 2.5 µl of 2.5 mM dNTP; 1 unit. Taq-DNA-polimerase; 10 units MMLV-reverse transcriptase and 12 µl of water. The components were mixed in 96-well optical plates (Axygen). PCR was

performed using amplifier C1000 Touch Thermal Cycler (Bio-Rad) at the following temperature conditions: 15 minutes – reverse transcription, 15 minutes – preliminary denaturation at 95° C and 40 cycles of reaction (denaturation at 95 C°– 15 s, annealing at 55 °C – 15 s, elongation at 60 °C – 20 s). CFX96 Real-Time PCR Detection System was used for recording fluorescence signals. The obtained data were analyzed using Bio-Rad CFX Manager software and Microsoft Excel. To establish C_t threshold cycle two basic parameters were used: basic line determined for each individual curve; threshold line, calculated for this phluorophore. The threshold cycle value is inversely proportional to log of a matrix in the reaction mixture – the less C_t value the higher the matrix concentration [3].

Statistical data processing. Precision in repeatability and reproducibility was determined as the level of consistency of results of multiple analyses of one sample [1, 2]. Coefficient of variation (C) was calculated basing on the following formula [1]:

$$C = \frac{\sigma}{\overline{X}} \times 100\%,$$

where $\overline{X}$ – mean value of the threshold cycle determined by real-time PCR;

σ – mean square deviation, calculated using the following formula:

$$\sigma = \frac{X_{max} - X_{min}}{K}.$$

RESULTS

The research aimed at assessment of real-time qRT-PCR use for FMDV detection in cattle milk was performed according to the Research Plan. To implement this task FMDV infected cattle milk samples were prepared. The samples were prepared using 10-fold serial dilutions. Each sample contained 10% virus suspension so that the virus final concentration in the sample was 10^{-1}–10^{-10} lg TCD_{50}/ml. Basing on the epidemic situation three relevant FMD strains were selected: A No. 2177/Amursky/2013, O No. 2123/South Ossetia/2011, Asia-1 No. 1987/Amursky/2005 – and 10 virus dilutions in milk were prepared.

To determine the method specificity milk samples containing 10% virus suspension of seven virus serotypes and heterologous vesicular viruses were used. All analyses were performed in triplicates.

During the investigation such parameters as analytical sensitivity of the reaction, precision and analytical specificity were determined.

Determination of the reaction analytical sensitivity. Analytical sensitivity of real-time qRT-PCR was determined using 10-fold serial dilutions of three FMDV cultural FMDV virus preparations in milk samples (A No. 2177/Amursky/2013, O No. 2123/South Ossetia/2011, Asia-1 No. 1987/Amursky/2005 strains) with known original concentration. The virus concentration in the milk samples was determined basing on the threshold cycle values (C_t). Real-time qRT-PCR detected FMDV strains A No. 2177/Amursky/2013 and Asia-1 No. 1987/Amursky/2005 at the concentration of up to 0.1 lg TCD_{50}/ml (i. e. 1 TCD_{50}/ml), and O No. 2123/South Ossetia/2011 – at the concentration of up to 1 lg TCD_{50}/ml (i. e. 10 TCD_{50}/ml) (Table 1).

So, the performed experiments showed that when detecting FMDV in milk analytical sensitivity of real-time qRT-PCR is 1–10 TCD_{50}/ml. Difference in analytical sensitivity of the reaction for different strains is probably explained by the chosen 10-fold sample dilution and possible inaccuracy of the virus titer determination in the cell culture.

Table 1
Determination of FMDV concentration in milk samples using real-time qRT-PCR

FMDV concentration in samples, lg TCD_{50}/ml	Threshold cycle mean value (C_t), in triplicates		
	A No. 2177/ Amursky/2013	O No. 2123/South Ossetia/2011	Asia-1 No. 1987/ Amursky/2005
6.0	16.09	16.76	18.36
5.0	17.74	20.30	21.00
4.0	21.58	23.81	24.09
3.0	23.73	27.67	27.66
2.0	26.69	30.80	29.84
1.0	29.40	**36.83**	32.48
0.1	**32.38**	n/d	**34.71**
0.01	n/d	n/d	n/d
0.001	n/d	n/d	n/d
0.0001	n/d	n/d	n/d

n/d – C_t not determined, which means that real-time qRT-PCR result is negative.

The obtained results confirm that real-time qRT-PCR can detect FMDV in milk and the sensitivity is the same as in epithelium tissues and cell cultures [3].

Assessment of the reaction precision. Precision of the reaction was determined under repeatability and reproducibility conditions. Precision under repeatability conditions was assessed by measuring the variability of the results within one run. For that purpose the same milk sample containing 6 lg TCD_{50}/ml of FMDV A No. 2177/Amursky/2013, was tested simultaneously in 20 replicates by one operator using the same machine.

The coefficient of variation was 1.23% when real-time qRT-PCR as performed by one operator in 20 replicates, 5.6% – when performing reaction by one operator using two machines at a different time, and 6.72% when performing the reaction by two operators using two machines (Table 2).

The obtained results are indicative of high real-time qRT-PCR precision.

Determination of the analytical specificity of the reaction. Analytical specificity of real-time qRT-PCR was determined using RNA from cattle milk samples containing seven serotypes of FMDV. Other viruses (SVD, VES, VS), diluted in milk and unskimmed milk from normal cows were also used. A positive result was obtained when testing milk samples containing seven FMDV serotypes (figure). Samples containing RNA of other viruses as well as milk from normal animals demonstrated negative results which is indicative of the method specificity.

DISCUSSION

The previously performed tests showed that real-time qRT-PCR is a contemporary method for FMDV genome detection in epithelium of infected animals and cell cultures [3]. Results obtained during this research showed that real-time qRT-PCR can be successfully applied for FMDV detection in milk of infected cows.

Table 2
Threshold cycle values and real-time qRT-PCR coefficients of variation for the milk sample containing FMDV A No. 2177/Amursky/2013

Within one run		Different machine		Different operator	
No Run	C_t	No repeat	C_t	No repeat	C_t
1	16.12	1	16.24	1	16.32
2	16.30	2	17.02	2	16.74
3	16.14	3	16.66	3	**16.08***
4	16.17	4	16.06	4	16.16
5	16.21	5	16.14	5	16.81
6	16.27	6	17.01	6	16.27
7	16.31	7	16.83	7	16.98
8	16.30	8	16.41	8	16.22
9	16.28	9	16.29	9	17.11
10	16.27	10	17.04	10	16.17
11	16.23	11	16.21	11	17.01
12	16.23	12	16.47	12	16.37
13	16.18	13	16.32	13	16.49
14	16.17	14	16.94	14	16.47
15	16.19	15	16.83	15	16.29
16	16.21	16	16.81	16	16.64
17	16.27	17	16.19	17	16.36
18	16.12	18	16.71	18	16.18
19	16.32	19	16.29	19	16.41
20	16.26	20	16.28	20	**17.19****
C = 1.23					
C = 5.6					
		C = 6.72			

* minimum C_t; ** maximum C_t.

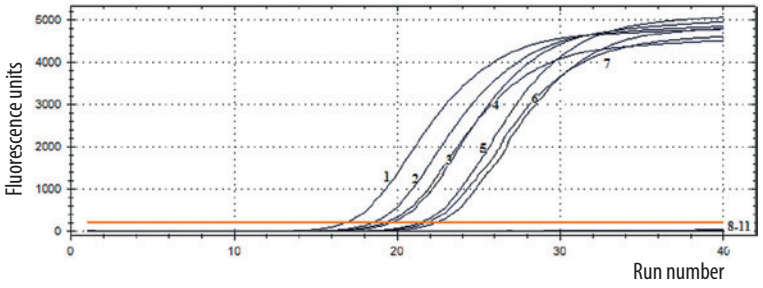

Figure. Analytical specificity of real-time qRT-PCR for detection of FMDV RNA in milk

1–7 – milk samples containing seven FMDV serotypes;
8–11 –milk from normal cows and milk samples containing SVDV, VESV, VSV.

As a result of the performed research several validation characteristics of real-time qRT-PCR were determined: precision under repeatability and reproducibility conditions, analytical specificity and sensitivity. Also the results of the research demonstrate the possibility to apply the method for FMDV detection in milk of a dairy herd.

The performed tests showed that real-time qRT-PCR has high precision, specificity and sensitivity. Coefficient of variation was 6.72%.

Sensitivity of the method was 1–10 TCD_{50}/ml depending on the FMDV strain. Real-time qRT-PCR allows detection of 1:1,000,000 FMDV dilution in milk. According to S. M. Reid et al. [9] such sensitivity allows detecting an infected animal in the herd, so, the method can be used for testing composite milk samples. It is also possible to detect the infection even after the milk from different has been pooled into one tanker.

FMDV is characterized by a high level of genetic diversity. The performed tests show, that all seven FMDV serotypes are successfully detected in milk as well as in epithelium of sick animals [3], which confirms the capability of the method to detect a wide spectrum of the FMD virus.

CONCLUSIONS

The possibility of real-time qRT-PCR use as a contemporary method for FMDV detection in milk was demonstrated.

Major validation characteristics of the method for detection of RNA of all FMDV serotypes were determined.

Results of the experimental tests prove that the method has high precision, specificity and sensitivity and can detect FMDV in its genetic diversity even if it is diluted.

Conflict of interests. The authors claim no conflict of interests.

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