Quantitative PCR to determine the titer of infectious activity of the canine hepatitis virus

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Abstract. This article presents data on the development and validation of a method for the indirect determination of the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for culture vaccines by real-time polymerase chain reaction using the Cq quantification cycle, including the following steps: eluting DNA of canine infectious hepatitis virus genotype CAV-1; performing amplification of a specific fragment orf 16 of canine infectious hepatitis virus genotype CAV-1 DNA using the original specific forward and reverse primers, as well as a molecular probe labeled with fluorescent dye FAM and luminescence quencher RTQ-1: CAV-1-T-F-primer with 5'-CGTAATGGGGAAACCTAGGGG-3' design, CAV-1-T-R-primer with 5'-TCTGTGTTGTTTCTGTCTTGG-3' design, and CAV-1-T-Pb-probe with 5'-FAM- CCAATCATCATCTCAACTCAACTAAATGCCGTG-RTQ1-3' design; calculation of Cq quantification cycle from real-time PCR data; determination of the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 using a logarithmic function expressed as the equation lg $T_{CAV-1} = -0.2979 \times C_t + 9.2595$ with an approximation reliability of 0.9941 and amplification efficiency of 99.38%. The analysis time is reduced to 3 h, and the analytical sensitivity is at least 1.0 lg TCD_{50}/cm^3.

1 Introduction

Infectious respiratory diseases of dogs occur predominantly in animals kept in groups, including in shelters, kennels and veterinary hospitals. Several studies evaluating natural outbreaks have demonstrated a complex etiology and infection by various viruses and bacteria [1].

Canine adenovirus infections manifest as two distinct diseases: infectious hepatitis caused by canine adenovirus serotype 1 (CAV-1) and adenovirosis caused by canine adenovirus serotype 2 (CAV-2) [2].

The first to describe the etiology and symptoms of CAV-1 in detail was the Swedish scientist Rubart (1947), whose name is sometimes given to the disease [3].

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Infectious hepatitis (CAV-1) (contagious inflammation of the liver of dogs, Rubart's disease, viral hepatitis) is an acute contagious disease with fever, inflammatory processes of the mucous membranes of the eyes, nasal cavity, gastrointestinal tract, liver and gallbladder, sometimes accompanied by disruption of the central nervous system. Canine infectious hepatitis virus belongs to the family Adenoviridae, genus Mastadenovirus, species Canine Mastadenovirus (CAV-1) [4].

CAV-1 virions, like all adenoviruses, are isometric particles of cubic type symmetry with a virion diameter of 70-90 nm. There are outgrowths (fibers) at the tips of the icosahedron. The virion capsid includes 252 capsomeres without a suparcapsid shell. The capsid contains 12 structural proteins. There is also a core protein bound to virion DNA. The virion nucleic acid is represented by a double-helical linear DNA molecule of about 30,500 bp, which encodes orf 1-orf 30 proteins [5, 6]. In the genome of canine infectious hepatitis virus of genotype CAV-1, various conserved regions are identified, in particular, the gene responsible for the synthesis of orf 16 protein (positions in the genome 16757...19474 bp).

No antigenic affinity of CAV-1 virus with human adenovirus was found. CAV-1 virus strains isolated in different regions of the country are antigenically related. The virus contains precipitating, hemagglutinating, and complement-binding antigens and induces the formation of appropriate antibodies. CAV-1 strains successfully reproduce in cell lines of the kidney of dog, fox, and fox puppies [7, 8]. The culture of Madin Darbey canine kidney MDCK (Madin Darbey canine kidney) cells is highly sensitive to this virus among transplantable cultures. Using this cell line, the cytopathic effect reaches 95-100% after 48 h and is characterized by cell rounding, formation of conglomerates resembling bunches of grapes. Intranuclear inclusions are found in the cells. Most epizootic strains of CAV-1 virus have hemagglutinating activity against guinea pig and human erythrocytes [9, 10, 11].

The system of measures to control and prevent canine infectious hepatitis of the CAV-1 genotype provides for immunization of animals and control of the level of postvaccinal immunity [12, 13, 14].

Various vaccines against infectious hepatitis of the CAV-1 genotype in dogs are used for immunization of puppies [15]. In the manufacture of these vaccines, the virus-containing suspension is assayed for infectivity titer to evaluate its activity in cells. In 1.0 cm3 of virus suspension, the number of cell culture infectious doses causing 50% cellular damage is determined, which actually reflects the concentration of complete viral particles containing DNA in the active state.

Traditionally, to determine the titer of infectivity of infectious hepatitis virus of genotype CAV-1, the titration method has been used in a monolayer Madin-Darby canine kidney (MDCK) transplantable cell line, which is used to calculate the minimum virus dose capable of causing lysis of 50% of cells (prototype) [10]. This method has some limitations in its application, namely: 1) long assay procedure associated with the development of cytopathic action; 2) a certain degree of subjectivity in the evaluation of assay results; 3) high cost of MDCK cell line as a test system and costs of its maintenance; 4) high risk of MDCK cell culture contamination.

In this regard, it is reasonable to search for an alternative method of determining the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccines using real-time polymerase chain reaction (PCR-RV) by Cq quantification cycle.

The aim of the work is to develop and validate the method of determining the infectivity titer of canine infectious hepatitis virus of CAV-1 genotype in raw materials for vaccines using real-time polymerase chain reaction (PCR-RV) by Cq quantification cycle.

2 Materials and methods

Standards and control samples. To determine the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1, a control panel of standards of this virus is prepared, as which lyophilically dried suspensions of the causative agent of canine infectious hepatitis of genotype CAV-1 were used with titers: 0.0; 1.0; 2.0; 3.0; 4.0; 5.0; 6.0; 7.0; 8.0 lg TCD_{50}/cm^3, which were diluted with 1/15 M phosphate-buffered saline to the required volume and titer of infectivity. A suspension of MDCK cells not infected with virus was used as a negative control.

DNA extraction. DNA was extracted using 5 M guanidinisothiocyanate solution (ratio of sample to GTC = 1/5) during detering on glass fiber filters for 10 min followed by washing three times with 80% isopropyl alcohol solution (500 µl per sample).

Real-time PCR. The composition of the reaction mixture for real-time PCR is presented in Table 1. For elongation we used primers and probe at a concentration of 5 pM, deoxyribonucleoside triphosphates with their concentration in the reaction mixture of 0.2 mM each. A buffer solution (5x) having a content of 20% of the total volume of the reaction mixture is used as a base. The buffer solution includes potassium ions (K+) (5×10-2 M) and dimethyl sulfoxide (DMSO) (1.3%) in its composition. Magnesium chloride is also added to the mixture to a concentration of 3 mM. Tag DNA-dependent DNA polymerase (1 unit) is used as the catalyst for real-time PCR. The DNA eluates of each sample are added to the reaction mixture in 5 µL increments. The total volume of the mixture for one reaction is 25 µl.

Table 1. Composition of the reaction mixture for the indirect determination of the infectivity titer of canine infectious hepatitis virus genotype CAV-1 in raw material for culture vaccines by real-time polymerase chain reaction using the Cq quantification cycle.

Name of the component of the mixture	Component concentration
CAV-1-T-F	5 pM
CAV-1-T-R	5 pM
CAV-1-T-Pb	5 pM
Deoxyriboadenosine triphosphate (dATP)	0.20 mM each
Deoxyriboguanosine triphosphate (dGTP)	
Deoxyribocytidine triphosphate (dCTP)	
Deoxyribotimidine triphosphate (dTTP)	
Magnesium chloride	3 mM
Taq-buffer solution Colorless Flexi (x 5)	20% of the reaction mixture volume
Taq DNA-dependent DNA polymerase	1 unit

Note: the volume of DNA eluate to be added is 5 µl, volume of the reaction mixture - 25 µl.

Table 2. Time and temperature modes of real-time PCR for determining the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccine.

Reaction stage	Reaction substage	Temperature, ºC	Reaction time	Number of cycles
Preheating	-	96	5 min.	1
Real-time PCR	Denaturation	95	10 s.	40
	Annealing of primers and probe	55	17 s.	
	Elongation *	60	20 s.	

Note: * - the degree of fluorescent signal is recorded at this sub-step.

The reaction was carried out in a detecting thermocycler of any brand at temperature and time parameters, the information about which is presented in Table 2.

3 Results and discussion

3.1 Detection of the titer dependence of the infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw material for culture vaccines and Cq quantification cycle

DNA was extracted from all standard positive samples and negative controls as well as test samples. In the next step, real-time PCR was performed to examine control samples and test samples. The primers and probe were calculated based on the nucleotide sequences of the orf gene of 16 strains of canine infectious hepatitis virus of genotype CAV-1, published in GenBank databases and obtained as part of research at the "Federal Center for Animal Health Protection" (FGBU "VNIIZZh").

The following oligonucleotides developed by us were used as homologous to the orf 16 gene region:

- CAV-1-T-F-primer (5'-CGTAATGGAAACCTAGGGG-3'),
- CAV-1-T-R-primer (5'-TCTGTGTTTCTGTCTTGG-3') и
- CAV-1-T-Pb-probe (5'-FAM-CCAATCATCTCAACTAAATGCCGTG-RTQ1-3') (Figure 1) at a concentration of 5 pM per reaction.

Fig. 1. Nucleotide sequence of forward primer, reverse primer, and molecular DNA probe when aligned with DNA from canine infectious hepatitis virus genotype CAV-1. Note: Using alignments of other isolates and production strains obtained the same oligonucleotide design.

Oligonucleotides for cDNA matrix were selected according to a number of general rules, which are reflected in the works of B. Deiman and R. Sooknanan [14]. The lengths of CAV-1-T-F-primer, CAV-1-T-R-primer and CAV-1-T-Pb-probe are 19, 18 and 25 n.o. (Table 3). The molecular weights of the forward, reverse primer and probe oligonucleotides are 59.01.9; 5493.6; 7570.0, respectively. The primers and probe were purified in a polyacrylamide gel and by high-performance liquid chromatography, respectively. The nucleotide sequence of the probe is not complementary to the oligonucleotide primers. There are 4 or more consecutive identical nucleotides missing in the primer and probe chain. The fluorophore FAM is attached to the 5'-end and the fluorescence quencher RTQ1 is attached to the 3'-end. These conditions meet the requirements for oligonucleotide primers and molecular probe that are involved in real-time PCR. FAM with a wavelength of maximum fluorescence of 520 nm was chosen as the fluorescent dye. RTQ1 fluorescence quencher with a wavelength of maximum absorption at 520 nm and a possible quenching range of 470-570 nm was used to quench the luminescence. Thus, a suitable fluorophore-quencher pair was selected.

Table 3. Physical, thermodynamic parameters and melting point of oligonucleotide primers and molecular probe for determining the infectivity titer of canine infectious hepatitis virus genotype CAV-1 in vaccine raw material.

Parameters of oligonucleotides	**Characteristics of oligonucleotides**		
	CAV-1-T-F - primer	**CAV-1-T-R – primer**	**CAV-1-T-Pb - probe**
Length (L), n.o.	19	18	25
Molecular weight (Mw)	5901.9	5493.6	7570.0
Entropy differential (dH), kcal/mol	158.1	142.1	198.9
Gibbs energy differential (dG), kcal/mol	23.8	21.0	31.7
Enthalpy differential (dS), cal/ºK×mol	417	374.3	523.1
Melting point (Tm), ºC: - with salt concentration correction	57	51	64
- by nearest neighbor algorithm	49	49	57

Note: thermodynamic parameters of the developed oligonucleotide primers were calculated under the following conditions: NaCl concentration 1 M, temperature 25 ºC, hydrogen index (pH) 7.0.

When analyzing the nucleotide sequences of oligonucleotides, we found that primers and probe are not characterized by the formation of "hairpins", as well as no 3'-complementarity and sites annealing on themselves. The probability of formation of "hairpins" and oligonucleotide dimers was calculated under the condition that the minimum number of base pairs required for dimerization is 5 and for formation of "hairpins" is 4.

The melting temperatures (Tm) for oligonucleotide primers and probe were determined. Accurate determination of melting temperature plays an important role in molecular biological studies, including the selection of DNA primers and probe for real-time PCR of canine infectious hepatitis virus DNA of CAV-1 genotype. To estimate the melting temperature of oligonucleotides, the method of accounting for the concentration of salts in the buffer solution and the nearest-neighbor method were used.

The physical, thermodynamic constants and calculation of melting temperatures of the developed oligonucleotide DNA primers and molecular probe are presented in Table 3. It shows that entropy, Gibbs energy and enthalpy for the forward primer were 158.1 kcal/mol, 23.8 kcal/mol, 471 cal/(ºK×mol), respectively. Entropy, Gibbs energy and enthalpy for the reverse primer were 142.1 kcal/mol, 21.0 kcal/mol, 374.3 cal/(ºK×mol), respectively. Entropy, Gibbs energy and enthalpy for the molecular DNA probe were 198.9 kcal/mol, 31.7 kcal/mol, 523.1 cal/(ºK×mol), respectively. These values are necessary to calculate the melting temperatures of the presented oligonucleotides. Tm using the nearest neighbor algorithm for forward, reverse primer and probe were 49, 49 and 57ºC, respectively.

Using a simpler method that takes into account the concentrations of K+ ions and dimethyl sulfoxide (DMSO) Tm for forward, reverse primers and probe were 57, 51 and 64ºC.

Experimentally, it was found that the optimal annealing temperature of the considered oligonucleotides was 55ºC. For real-time PCR, it was decided to hybridize primers and probe with the orf 16-gene region of DNA of canine infectious hepatitis virus of CAV-1 genotype at 55ºC.

The sequences of the designed primers and molecular probe were checked for unwanted matches with other nucleic acid sequences using the CAV-1 Nucleic Acid Sequence Data Bank. The primer and probe sequences were also analyzed for the presence of internal

secondary structures using the Mfold nucleic acid folding program. It was found that for the designed oligonucleotides, no undesirable matches with other nucleic acid sequences as well as the presence of internal secondary structures were detected.

Real-time PCR data were analyzed by evaluating and comparing plots of fluorescent signal accumulation by the values of Cq quantification cycles determined by crossing the threshold line and logarithmic mapping of the function Fl = f (Cq). Counting of the results in the reaction occurs at each cycle. The fluorimeter determines the fluorescence level and constructs a kinetic curve in the coordinates: fluorescence level - amplification reaction cycle. In case of presence of specific DNA-matrix in the sample under study, the kinetic curve has an exponential dependence. Samples are considered positive if they correspond to sigmoid obtained by analyzing the fluorescence of the dye included in the molecular probe. Samples are considered negative if there is no exponential curve in their analysis. The data obtained are shown in Figure 2 and expressed as a logarithmic function:

$$\lg T_{CAV\text{-}1} = -0{,}2979 \times C_t + 9{,}2595$$

with high approximation reliability (R2 = 0.9941) and amplification efficiency of 99.38%. The proposed model allows to indirectly determine the infectivity titer of canine infectious hepatitis virus of CAV-1 genotype in the raw material for culture vaccines.

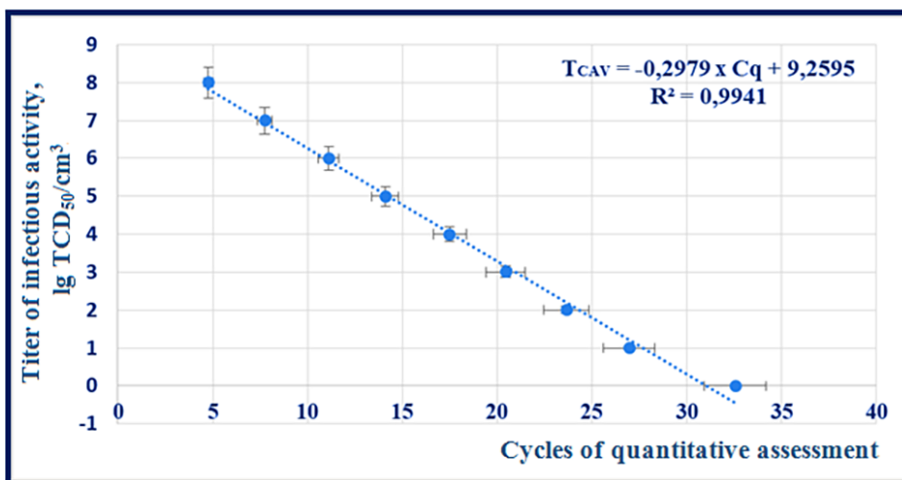

Fig. 2. Dependence of titer of infectivity of canine infectious hepatitis virus of CAV-1 genotype (TCAV-1) and the value of quantification cycle (Cq) by real-time PCR by Cq quantification cycle (n=12, points representing average Cq values are marked).

3.2 Application of the method of mediated determination of the infectivity titer of canine infectious hepatitis virus of CAV-1 genotype in raw materials for vaccines by real-time polymerase chain reaction using the Cq quantification cycle

The study used 6 suspensions of canine infectious hepatitis virus culture of CAV-1 genotype with infectivity titers of 6.50; 6.75; 7.00; 7.25; 7.50; 7.75 lg TCD50/cm3, respectively (samples No. 1-6). As a positive control we used a suspension of culture virus of infectious canine hepatitis virus of genotype CAV-1 with a titer of infectivity 7.00 lg TCD50/cm3. A suspension of MDCK cells not infected with microorganisms was used as negative controls. Test samples and control samples were examined in 12 repetitions. DNA isolation steps, and real-time PCR staging were performed as described above.

The mean values of quantification cycles for samples #1-6 were 9.31±0.01, 8.48±0.02, 7.64±0.02, 6.81±0.01, 5.98±0.01, 5.14±0.02, respectively. Using the developed logarithmic function, we calculated the mean values of the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 for samples #1-6, which were 6.49; 6.75; 7.00; 7.25; 7.50; 7.74 lg TCD50/cm3, respectively. For positive controls, the amplification threshold cycle value was 7.64±0.01, which corresponded to an infectivity titer of canine infectious hepatitis virus of CAV-1 genotype equal to 7.00 lg TCD50/cm3. No exponential plots were generated for the negative controls, which meant the absence of canine infectious hepatitis virus of CAV-1 genotype in these samples.

The studied samples were tested in parallel using the classical titration method in the MDCK monolayer cell line. It was found that the data obtained using the developed method correlated with the titration method in cell culture by 99-100% (n=12). The obtained results indicated a high degree of accuracy of the developed method for mediated determination of the titer of infectivity of canine infectious hepatitis virus of CAV-1 genotype in the raw material for culture vaccines using real-time polymerase chain reaction by Cq quantification cycle. Thus, the developed method allows estimating the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccines by real-time PCR.

3.3 Determination of the degree of reliability of determining the titer of infectious activity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccines using the developed method

For the analysis 210 suspensions of culture of canine infectious hepatitis virus of CAV-1 genotype with the titer of infectivity from 1.00 to 8.00 lg TCD50/cm3 were used. As a positive control, a suspension of culture virus of infectious canine hepatitis virus of CAV-1 genotype with a titer of virus infectivity of 7.00 lg TCD50/cm3 was used. A suspension of MDCK cells not infected with microorganisms was used as negative controls. Test samples and control samples were examined in three repetitions. The nucleic acid extraction steps and real-time PCR were performed as described above. The results of the analysis are presented in Table 4.

Table 4. Reliability of mediated determination of canine infectious hepatitis virus titer of CAV-1 genotype in vaccine raw material by real-time PCR by Cq quantification cycle (n=12).

Values of infectivity titer of canine infectious hepatitis virus of genotype CAV-1 (standards), lg TCD_{50}/cm^3	**Reliability (%) of T detection of canine infectious hepatitis virus of CAV-1 genotype in the raw material for vaccine**	
	in cell culture MDCK (prototype)	**Real-time PCR (developed method)**
8.0-6.0	94-100	99.57-100.00
5.9-4.0	94-97	98.13-99.56
3.9-2.0	93-96	96.23-98.14
2.0-1.5	91-93	95.41-98.12
1.4-1.0	84-89	94.21-97.56

Note: MDCK of monolayer transfected Madin-Darby canine kidney cell line; T, titer of infectivity of canine hepatitis virus of CAV-1 genotype.

The results were interpreted using the developed logarithmic function to obtain T_{CAV-1} values for each of 210 samples. For positive controls, the amplification threshold cycle value was 7.64±0.01, which corresponded to an infectivity titer of canine infectious hepatitis virus of CAV-1 genotype equal to 7.00 lg TCD50/cm3. No exponential plots were generated for

the negative controls, which meant that there was no infectious canine hepatitis virus of CAV-1 genotype in the samples analyzed.

The analyzed samples and controls were also examined by classical titration method in MDCK monolayer cell line and by real-time PCR. It was found that the data obtained by the developed method correlated with the standard values by 99.57-100, 00% for 8.0-6.0 lg TCD_{50}/cm^3 (n=42), by 98.13-99.56% for 5.9-4.0 lg TCD_{50}/cm^3 (n=42), by 95.41-98.14% for 3.9-1.5 lg TCD_{50}/cm^3 (n=84), by 94.21-97.56% for 1.4-1.0 lg TCD_{50}/cm^3 (n=42). The obtained results indicated a high degree of accuracy of the developed method of mediated determination of the infectivity titer of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccines. Thus, the developed method allows to estimate with a high degree of reliability the titer of infectious activity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccines.

3.4 Determination of analytical sensitivity of the method of indirect determination of the titer of infectious activity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for culture vaccines using real-time polymerase chain reaction by the cycle of quantitative assessment Cq

In determining the analytical sensitivity of the method of indirect determination of the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for culture vaccines by real-time polymerase chain reaction by Cq quantification cycle, a series of virus standards with T_{CAV-1} equal to 1.00-8.00 lg TCD_{50}/cm^3 with a step of 0.1 lg $TCD_{50}/cm^3$was prepared. Control samples were tested in 5 repetitions. Nucleic acid elution steps and real-time PCR staging as described above. It was confirmed that the reliability of the assay performed using the developed method is 99.57-100.00% for 8.0-6.0 lg TCD_{50}/cm^3, by 98.13-99.56% for 5.9-4.0 lg TCD_{50}/cm^3, by 95.41-98.14% for 3.9-1.5 lg TCD_{50}/cm^3, 94.21-97.56% for 1.4-1.0 lg TCD_{50}/cm^3. In the manufacture of vaccines, virus-containing raw materials are used only with virus infectivity titers ≥6.00 lg TCD_{50}/cm^3. For these samples, the reliability of determining the infectivity titer of canine infectious hepatitis virus of genotype CAV-1 was 99.57-100%. Analytical sensitivity of the test system developed for the method of indirect determination of the titer of infectious activity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for vaccines is not less than 1.0 lg TCD_{50}/cm^3 with reliability of the results of the study not less than 94.21%.

3.5 Study of the specificity of the developed method of mediated determination of the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for culture vaccines using real-time polymerase chain reaction by the cycle of quantitative assessment of Cq

When assessing the specificity of the developed method of mediated determination of the titer of infectivity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for culture vaccines by real-time polymerase chain reaction according to the cycle of quantitative estimation Cq, suspensions of canine infectious hepatitis virus of genotypes CAV-1 and CAV-2, calicivirus FCV, rabies virus RabV, canine alpha-coronavirus CCoV were examined. The amount of infectious doses of viruses in suspensions was at least 6.0 lg TCD_{50}/cm^3. The studies were performed in 5 repetitions.

The steps of nucleic acid elution and real-time PCR were performed as described above. For samples containing other viruses, no formation of exponent plots was observed and they did not exceed the threshold level of the fluorescent signal (0.01 cfu). Thus, the developed method is specific to canine infectious hepatitis virus of genotype CAV-1 and can be used for its quantification.

4 Conclusion

The main advantages of the developed method are the possibility to exclude the probability of contamination during the reaction, to reduce the time of analysis of virus-containing raw materials for vaccine up to 3 h, to increase the specificity and sensitivity of the analysis to determine the titer of infectious activity of canine infectious hepatitis virus of CAV-1 genotype. In the proposed test between the titer of infectious activity of canine infectious hepatitis virus of CAV-1 genotype (T_{CAV-1}) and the cycle of quantification (Cq) the dependence is established, reflected as a logarithmic function $\lg T_{CAV-1} = -0.2979 \times C_t + 9.2595$ with high approximation reliability ($R^2 = 0.9941$) and amplification efficiency of 99.38%. The proposed model allows to indirectly determine the infectivity titer of canine infectious hepatitis virus of CAV-1 genotype in raw materials for culture vaccines. The developed method allows quickly and with a high degree of reliability to determine the titer of infectious activity of canine infectious hepatitis virus of genotype CAV-1 in raw materials for culture vaccines.

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