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TEST-SYSTEM FOR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS GENOME BY REVERSE TRANSCRIPTION REAL-TIME PCR

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Abstract

Peste des petits ruminants (PPR) is a highly contagious transboundary infection disease of small ruminants, characterized by fever, anorexia, ocular and nasal discharge, erosions and ulcers in digestive mucosa, diarrhoea and marked leucopoenia with immunosuppression. Because of the complexity of the PPR epizootic situation in neighboring countries (Tajikistan, China, Mongolia, Kazakhstan, Afghanistan) the risk of occurrence of this disease on the territories of Siberian, Ural, Far Eastern, North Caucasian and Southern Federal Districts of Russian Federation is very high. So, the development of molecular-genetic methods for the diagnosis of PPR and methods for stabilizing of biological samples represents scientific relevance. This article presents data on the development of a test system for detecting the PPR virus genome by reverse transcription Real-Time PCR. This technique is based on the amplification of a fragment (148 bp) of hemagglutinin gene of PPR virus using the original oligonucleotide primers and fluorescent-labeled hybridization probe. Analytical specificity of the developed test system was evaluated by testing the strains of PPR virus, rinderpest virus, bluetongue virus, as well as biological samples from clinically healthy animals and intact cell cultures. Positive results were obtained only with samples containing PPR virus (strains Epizooticheskiy, Nigeria 75/1 and 45G37/35-K). The analytical sensitivity of the developed test-system, determined using ten-fold serial dilutions of cultural virus-containing material, is 0.83±0.22 lg $TCID_{50}$/ml. To create a positive amplification control, a fragment of hemagglutinin gene synthesized usign Real-Time PCR was cloned in plasmid pTZ57 R/T in *Escherichia coli*. It was established that the plasmid DNA concentration for amplification linearity ranges from 2.4×10^7 to 24 molecules/μl. To assess the practical suitability of the developed test system for the diagnosis of PPR, the blood samples from sheep experimentally infected with PPR virus (strain Epizooticheskiy) were tested. As a result, the PPR virus genome was detected in blood samples from day 5 to day 12 after infection. Since transportation of biological samples over long distances may require during survey, we have developed "dry" blood method to collect and store blood samples. It has been shown that "dry" blood drops are stable at room temperature for a month and can be used in Real-Time PCR testing.

Keywords: peste des petits ruminants, virus, reverse transcription, PCR, cell culture, recombinant plasmid, experimental infection

Peste des petits ruminants (PPR) is a highly contagious transboundary infection disease of small ruminants, which is endemic for most of African, Asian and Middle Eastern countries. It jeopardizes their livestock industry. PPR elimination is considered to be an essential component of global food security and poverty reduction [1, 2]. PPR is characterized by fever, anorexia, ocular and nasal discharge, erosions and ulcers in digestive mucosa, diarrhea and marked

leucopoenia with immunosuppression. Abortions can be observed in pregnant sheep and goats [3, 4]. Direct viral affection with PPR virus (PPRV), decreased immunity and reproduction of other pathogens (e.g., *Pasteurella* spp., *Escherichia coli*, *Mycoplasma* spp.) can lead to an animal's death. Depending on an infected animal's species, breed, age and extension of secondary infectious agents, mortality varies substantially (10-90%) [5].

A causative agent is a RNA-containing virus (family *Paramyxoviridae*, genus *Morbillivirus*) [6, 7] whose antigens are related to contagious bovine typhus (CBT) (i.e., another *Morbillivirus*) [8]. PPRV genome represents a negative single-strand RNA molecule (15,948 nucleotides). PPRV genome RNA contains open reading frames for 6 structural proteins (i.e., nucleocapsid, phosphoprotein, matrix, fusion protein and haemagglutinin) and 2 non-structural ones (V and C) [9]. Although PPR strains and isolates are not divided into serotypes, they are clustered in 4 genetic lines [10] according to nucleotide genetic sequences of nucleocapsid protein and phosphoprotein. It was found that lines I and II are common in West Africa, line III is typical for East Africa, Middle East and South India; line IV is widespread in Asia [11, 12].

For the first time, PPR was reported in West Africa. Then, the infection got across to Central and East Africa. In 1980 it was reported on the Arabian Peninsula. Nowadays, the disease is wide-spread in Africa and Asia. Moreover, it was mentioned in our neighboring countries (Tajikistan, China, Mongolia, Kazakhstan, Afghanistan, Georgia and Turkey). According to analysis of PPR epizootic situation in Russia, the risk of occurrence of this disease on the territories of Siberian, Ural, Far Eastern, North Caucasian and Southern Federal Districts of the Russian Federation is quite high [13, 14].

As per the International Epizootic Bureau (IEB, Office International des Epizooties, OIE, France) guidelines, PPR can be diagnosed in virtue of the viral genome revealed by a polymerase chain reaction (reverse transcription PCR), virus extraction with permissive cell line, and viral antigen detection by enzyme-linked immunosorbent assay (ELISA), fluorescent antibody test (FAT), agarose gel diffusion test and immunoelectrophoresis. Serological methods of specific antibody detection (neutralization test and competitive ELISA) are applicable to monitoring studies and determination of immune status of a vaccinated animal. IEB points out that reverse transcription PCR and ELISA methods to detect viral antigens are the most suitable for confirmation of PPR clinical cases [15]. Reverse transcription Real-time PCR combined with electrophoresis or hybrid fluorescent detection of amplification products is the most common diagnostic method. Sustainable reverse transcription PCR schedules with electrophoretic detection showed good results. Proposed by Shaila et al. [16] and Couacy-Hymann et al. [17] in 1996 and 2002, respectively, they are based on amplification of gene fragments related to nucleocapsid protein and phosphoprotein. Further, Bao et al. [18], Kwiatek et al. [19] and Batten et al. [20] offered several schedules of highly efficient and more sensitive reverse transcription Real-Time PCR on the basis of amplification of highly conserved regions of nucleocapsid protein gene. Analytical sensitivity of these test systems is 30-10 RNA copies/reaction. They can detect genomes of PPRV strains falling into all the four lines [18-20]. In 2010, Balamurugan et al. [21] published a reverse transcription Real-Time PCR protocol where matrix protein gene fragment is amplified at 0.1-1.0 $TCID_{50}/cm^3$.

To diagnose PPR viral genome, conjunctival, nasal and oral discharges, as well as blood samples are collected in animals. To avoid a non-specific degradation of the viral nucleic acid, biomaterial must be shipped in cooled state [22]. If temperature conditions cannot be met during sample shipping to a diagnostic

laboratory, a paper medium should be used to stabilize blood samples (i.e., "dry" blood method). Dry spot method is very common in veterinary and medical practice to collect samples of various biological fluids intended for virological, serological and biochemical tests in domestic environment and to ship them to a laboratory [23, 24].

Even though molecular-genetic methods are widely used in veterinary practice for PPR diagnosis, alternative target gene-based confirmatory tests must be performed. Validated as per IEB standards, these tests must detect PPR viral genome in samples of biomaterial collected in infected animals.

The paper presents results related to development of a test system to detect PPR viral genome based on amplification of hemagglutinin gene fragment by reverse transcription Real-Time PCR. Sensitivity of the test system is 0.83 ± 0.22 lg $TCID_{50}/cm^3$. It can differentiate PPR viral genome from genomes of contagious bovine typhus and bluetongue that cause similar symptoms in sheep. The proposed test system suitability for practical diagnosis of PPR was verified experimentally.

Our purpose was to develop a reverse transcription Real-Time PCR-based test system to detect genome of PPR virus and to validate it using blood samples of experimentally infected sheep.

Techniques. We used several strains of PPRV (Nigeria 75/1, Epizootichesky and $45G_{37}$/35-K), contagious bovine typhus (Nakamura and LT 67 vaccine strains) and bluetongue (serotype VIII, NET 2007 strain) stored in the National Microorganism Collection of the Federal State Budgetary Scientific Institution Federal Research Virology and Microbiology Center. Cell cultures (Cell Culture Collection, Federal Research Center for Virology and Microbiology) were used as negative samples to assess analytical specificity of the test system.

Viral RNA was extracted with a RNA-Sorb kit (Interlabservis OOO, Russia). Reverse transcription was carried out with a Tertsikl thermocycler (DNK-Tekhnologii ZAO, Russia) and Real-Time PCR was carried out with a DT-Prime detecting amplifier (DNK-Tekhnologii ZAO, Russia). To synthesize a cDNA on the matrix of extracted RNA samples, we used a reaction mixture (20 μl) consisted of a test sample (10 μl), a reverse primer (1 μl; 10 pmol/μl, Evrogen ZAO, Russia), 5× reverse transcription buffer (4 μl; Alfa-ferment OOO, Russia), deoxynucleoside triphosphate mixture (0.3 μl; 10 mmol/ml, Syntol ZAO, Russia), reverse transcriptase (0.2 μl; 200 units/μl; Alfa-ferment OOO, Russia) and bidistilled water (4.5 μl). The reaction mixture was incubated at 42 °C for 30 min. Using obtained cDNA, we conducted Real-Time PCR in the 25 μl reaction mixture containing matrix (5 μl), direct and reverse primers (1 μl each), Taq-man probe (0.3 μl; 10 pmol/μl), 10× PCR buffer (2.5 μl; Alfa-ferment OOO, Russia), deoxynucleoside triphosphate mixture (0.3 μl; 10 mmol/ml), $MgCl_2$ (0.5 μl; 25 mmol/ml, Syntol ZAO, Russia), Taq MS polymerase (0.1 μl; 5 units/μl, Alfa-ferment OOO, Russia) and bidistilled water (14.3 μl). We applied following PCR schedule: pre-denaturation at 94 °C for 2 min and 40 cycles of amplification (94 °C — 15 s, 60 °C — 15 s, 72 °C — 15 s). Fluorescence (FAM) was detected at 60 °C.

To design a recombinant amplification control, PCR products were extracted from the reaction mixture with a Cleanup Standard kit (Evrogen ZAO, Russia). As a part of pTZ57 R/T plasmid vector (Thermo Fisher Scientific, USA), extracted PCR products were cloned in *Escherichia coli* strain Top10 (Invitrogen, USA). A plasmid DNA was extracted with a Plasmid Miniprep kit (Evrogen ZAO, Russia) and its concentration was determined with a NanoDrop Lite microspectrophotometer (Thermo Fisher Scientific, USA).

Three Romanov sheep weighed 45-50 kg were experimentally PPR-

infected. Sheep nos. 1 and 2 were infected with intravenously administered culture containing PPR virus (Epizooticheskiy strain) (3.5 lg $TCID_{50}$). Sheep no. 3 was used as an intact control. All the animals underwent a clinical examination (including daily thermometry and blood sampling). Animal experiments were observed by the Bioethics Committee of Federal Research Center for Virology and Microbiology.

For "dry" blood preparations, we used 0.05 cm^3 of PPRV-contaminated blood stabilized with EDTA on circles ($\varnothing \approx$ 4-5 cm) cut out of Whatman DE-81 filter paper (Whatman, Great Britain). Blood was contaminated with cultural material containing PPRV (Nigeria 75/1 strain) with a titer of 4.83±0.22 lg $TCID_{50}/cm^3$ (1000:1). To extract viral RNA, we placed these preparations into microcentrifuge tubes with 800 μl of lysis buffer containing 4 M guanidine isothiocyanate, and incubated at 56 °C for 10 min. Then, they were centrifugated at 10,000 g for 10 min. Supernatant was used to extract RNA by a nucleosorption method as per instructions of RIBO-Sorb kit (Interlabservis ZAO, Russia).

We performed a statistical processing of the findings as per standard methods. Statistical significance between means was determined as per the Student-Fischer difference method. In this view, in most of bioassays $p > 0.05$ indicates absence of differences between compared values; $p < 0.05$, $p < 0.1$ and $p < 0.001$ mean statistically significant differences. Arithmetic means (M) of n number of tests and standard deviation (σ) of arithmetic mean were calculated with Microsoft Excel 365 software.

Results. A nucleotide sequence of hemagglutinin (H) gene was selected as a primer annealing target after analysis of nucleotide sequences related to genomes of various PPRV strains and isolates available in the GenBank database (https://www.ncbi.nlm.nih.gov/). Using BioEdit 7.0 and Oligo 6.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; http://www.oligo.net), we aligned nucleotide sequences, searched for conserved regions and calculated structure of oligonucleotide primers. Finally, we chose several oligonucleotide primers (PPRHf (5′- TCAAGATCGGGTCCAACATG-3′) and PPRHr (5′-CAATCGGACTGGGTAGAAGTAAG-3′) flanking a hemagglutinin gene 148 bp fragment. To detect amplification products, we applied a PPRHz 5′-(FAM)TCGCTCCTGGAAACATCATAAGTGGC(BHQ1)-3′ hybridization fluorescent-labeled probe.

Reverse transcription Real-Time PCR conditions were optimized by RNA preparations extracted from PPRV-infected (Nigeria 75/1 strain) Vero cell culture. Since primer melting points determined by Oligo 6.0 software were 58-62 °C, 60 °C was set as an annealing temperature in the temperature-time profile of the reaction.

To evaluate analytical sensitivity of the test system, we applied successive 10-fold dilutions of cultural PPR virus (Nigeria 75/1 strain) whose infectious activity was 4.83±0.22 lg $TCID_{50}/cm^3$.

According to Figure 1 and Table 1, the last dilution of the virus-containing material led to a positive result was 10^{-4}. Thus, analytical sensitivity of reverse transcription Real-Time PCR was 0.83±0.22 lg $TCID_{50}/cm^3$ during detection of PPRV genome. Amplification efficiency was 87%, and approximation accuracy (R^2) was ≈ 0.99. Intratest repetition of PCR results (threshold cycle values) characterized by mean standard deviations was 0.12-0.41. Coefficients of threshold cycle value variation resulted from re-tests of similar threshold samples were < 1.5% indicating high repetition of reverse transcription PCR analytical sensitivity test results.

Analytical specificity of the test system was assessed while testing samples

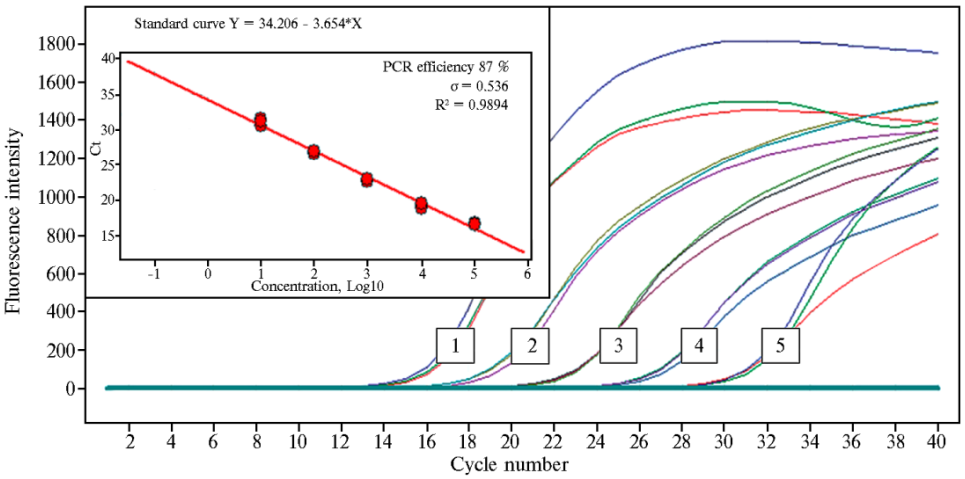

Fig. 1. Evaluation of successive 10-fold dilutions of cultural material containing virus of peste des petits ruminants (PPRV) (Nigeria 75/1 strain) of 4.83±0,22 lg $TCID_{50}/cm^3$ initial activity with the proposed test system: 1 — initial material; 2-5 — dilutions from 10^{-1} to 10^{-4}.

1. Analytical sensitivity parameters of reverse transcription Real-Time PCR in detection of virus of peste des petits ruminants (n = 3)

Dilution	Viral titer, lg $TCID_{50}/cm^3$	Reverse transcription Real-Time PCR result		
		mean Ct value	mean σ value	*Cv*, %
Initial material	4.83±0.22	16.53	0.12	0.75
10^{-1}	3.83±0.22	19.13	0.29	1.50
10^{-2}	2.83±0.22	22.83	0.17	0.74
10^{-3}	1.83±0.22	26.70	0.14	0.53
10^{-4}	0.83±0.22	31.07	0.41	1.35
10^{-5}	–0.17±0.22	Not available		

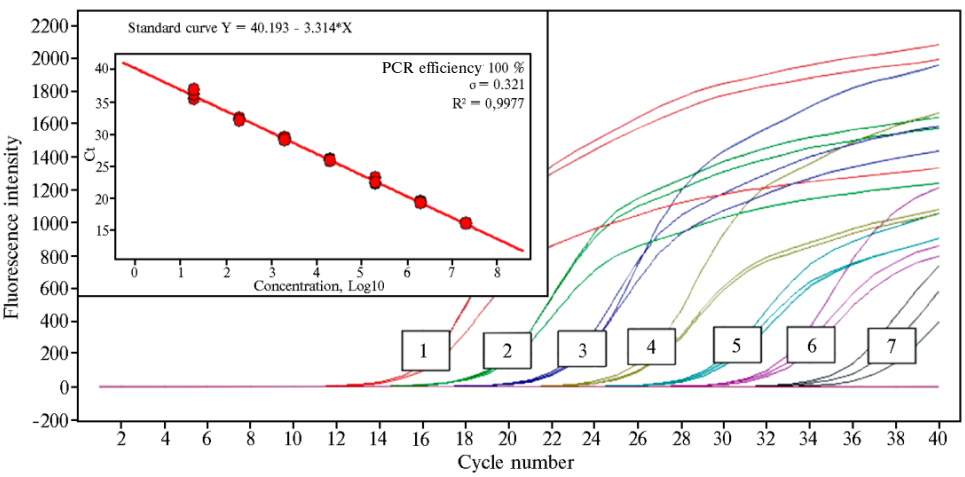

Fig. 2. Determination of working concentrations of positive recombinant amplification control (copies/μl) **to the test system intended for detection of PPRV genome by reverse transcription Real-Time PCR:** 1 — $2{,}4\times10^7$, 2 — 2×10^6, 3 — $2{,}4\times10^5$, 4 — $2{,}4\times10^4$, 5 — $2{,}4\times10^3$, 6 —$2{,}4\times10^2$, 7 — 24.

containing PPR, CBT and bluetongue viruses, blood samples of healthy sheep, as well as intact Vero and VNK-21 cell cultures (Cell Culture Collection, Federal Research Center for Virology and Microbiology). In this view, the test system specificity was confirmed by positive results demonstrated by PPRV-containing samples only.

After construction of a positive amplification control of the test system based on cloning of PPRV hemagglutinin gene 148 bp fragment as a part of

pTZ57 R/T vector and synthesis by reverse transcription PCR we obtained a plasmid DNA preparation whose concentration, according to spectrophotometry, was 80 ng/μl (equivalent to 2.4×10^{10} molecules per 1 μl). To improve quantity of plasmid DNA used as a positive control, 8 10-fold dilutions of the preparation were studied (starting with 10^{-3}) by reverse transcription PCR method. Plasmid DNA concentration range associated with a linear amplification was from 2.4×10^{7} to 24 DNA molecules/μl, with 100% amplification efficiency, 0.99 approximation accuracy (R^2) and 0.32 standard Ct deviation (σ) (Fig. 2). Recommended working plasmid concentrations in the test system correspondent with an analytical sensitivity range were 2.4×10^{7}-2.4×10^{4} DNA molecules/μl.

During the experimental PPRV infection designed to evaluate efficiency of the developed test system infected animals did not demonstrate any specific signs of the disease for 15 days. However, hyperthermia (< 40.2-40.5 °C) was observed in sheep 6-8 days after the infection. Along with this, using the proposed reverse transcription Real-Time PCR test system, PPRV genome was detected in blood samples collected in 5-12 days after the infection (Table 2).

2. Results of thermometry and PPRV genome detection in blood samples of experimentally infected sheep with the proposed reverse transcription Real-Time PCR test system

Post-infection period, days	Sheep no. 1		Sheep no. 1 2		Sheep no. 1 3	
	body temperature, °C	Ct value	body temperature, °C	Ct value	body temperature, °C	Ct value
1	38.6	–	39,5	–	39,0	–
2	39.4	–	39,4	–	39,3	–
3	39.5	–	39,2	–	39,1	–
4	39.6	–	39,5	–	39,0	–
5	39.6	–	39,6	–	39,3	–
6	40.2	+ (20,15)	39,7	–	38,9	–
7	40.5	+ (20,30)	40,2	+ (20,38)	39,2	–
8	40.4	+ (21,45)	40,5	+ (20,57)	39,3	–
9	39.2	+ (21,89)	39,9	+ (21,74)	38,8	–
10	39.0	+ (22,12)	39,5	+ (21,90)	39,0	–
11	38.7	+ (22,90)	39,9	+ (22,25)	38,9	–
12	38.8	+ (23,12)	39,9	+ (22,85)	39,1	–
13	38.7	–	39,8	–	38,8	–
14	38.8	–	39,9	–	38,9	–
15	39.2	–	39,7	–	39,1	–

N o t e. "+" — positive test, "–" — negative test..

Observed viremia period complies with reported data [25, 26]. Absence of clear specific signs can be associated with low infection dose (3.5 lg $TCID_{50}$). For example, in 2012 El Harrak et al. [27] used 5.1 lg $TCID_{50}$ of PPR virus to simulate the infection.

3. Detection of peste des petits ruminants viral genome in "dry" blood preparations on the paper basis by reverse transcription Real-Time PCR

Preparation no.	Native preparation (virus-contaminated blood)	"Dry" blood preparation	
		freshly prepared	1-month storage
1	20.76	20.51	23.67
2	20.15	20.83	24.98
3	21.00	21.43	25.01
4	20.35	21.02	23.73
Average	20.64±0.64	20.99±0.38	24.55±0.62

Nowadays, a "dry" blood method is a common technique to sample and to ship biological fluids. We evaluated applicability of the method in our test system. During development of "dry" blood method to collect and to store samples (PPRV diagnosis) on the paper basis we conducted comparative studies of RNA samples extracted from initial virus-containing material and freshly prepared "dry" blood samples with our test system. The results demonstrated absence

of significant differences between threshold cycles (Ct). Reverse transcription PCR analysis of RNA samples extracted from "dry"' blood preparations stored on the paper basis in airtight polyethylene ZIP-bags at 20±2 °C for 1 month, and initial preparations tested in the beginning of the experiment resulted in differences of 3-5 cycles between Ct values (Table 3).

Thus, we developed the highly specific and sensitive test system based on hemagglutinin gene fragment amplification by reverse transcription Real-Time PCR method to detect a genome of peste des petits ruminants (PPR) virus. Due to high sensitivity (0.83±0.22 lg $TCID_{50}/cm^3$), the test system can differentiate PPRV genome from viruses causing similar clinical signs in sheep (i.e., contagious bovine typhus and bluetongue). Practical application of the test system intended for PPR diagnosis was confirmed by blood testings involving experimentally infected sheep. Also, we developed a method of collection and storage of blood samples on the paper basis (i.e., "dry" blood method) to reveal PPRV genome. It was found that "dry" blood preparations on the paper basis are stable at room temperature for a month. So, they are suitable for reverse transcription Real-Time PCR test.

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