AGRICULTURAL BIOLOGY,
2019, V. 54, № 2, pp. 347-358
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 619:578:57.083.2:577.2

doi: 10.15389/agrobiology.2019.2.347eng
doi: 10.15389/agrobiology.2019.2.347rus

ONE-RUN REAL TIME PCR ASSAYS FOR THE DETECTION OF CAPRIPOXVIRUSES, FIELD ISOLATES AND VACCINE STRAINS OF LUMPY SKIN DISEASE VIRUS

A.V. SPRYGIN, Ya.E. PESTOVA, E.S. KOSTROVA, S.V. KONONOVA, O.P. BYADOVSKAYA, T.V. ZHBANOVA, A.V. KONONOV

Federal Center for Animal Health Control, FGBU VNIIZZh, mkr. Yurievets, Vladimir, 600901 Russia, e-mail sprig-inav@mail.ru (✉ corresponding author), pestova@arriah.ru, kostrova@arriah.ru, kononova@arriah.ru, bjadov-skaya@arriah.ru, zhbanova@arriah.ru, kononov@arriah.ru
ORCID:
Sprygin A.V. orcid.org/0000-0001-5982-3675
Pestova Ya.E. orcid.org/0000-0002-7974-623X
Kostrova E.S. orcid.org/0000-0001-7150-7632
Kononova S.V. orcid.org/0000-0002-3932-2416
Byadovskaya O.P. orcid.org/0000-0002-8326-7151
Zhbanova T.V. orcid.org/0000-0002-9857-5915
Kononov A.V. orcid.org/0000-0002-5523-3261
The authors declare no conflict of interests
Received September 25, 2018

Abstract

The cattle and sheep industry is economically important for sustainable growth. However, the increasing demand for livestock products drives animal population growth and risks for infection diseases. Lumpy skin disease (LSD) has recently expanded its historical range northward reaching countries that were never affected before. Prior to 2015 the territory of the Russian Federation was free of lumpy skin disease, whereas by 2017 Turkey, Serbia, Greece, Azerbaijan have reported incursions of this virus. Not only lumpy skin disease but also sheep pox has increased in incidence. Given this scenario, timely detection of these pathogens is key towards successful control and eradication. Moreover, diagnostic tools should detect both LSDV genome in the face of the use of live vaccine LSD virus strains and distinguish between the two. In this paper we report the development of a set of one-run real-time PCR assays to detect and differentiate between *Capripoxvirus* genome, field and vaccine LSD virus genomes. The assay for field LSD virus targets the 27 bp deletion in ORF126, the assay for vaccine LSD virus targets genetic signatures unique to Neethling vaccine strains, and the capripoxvirus assay targets the conserved *P32* gene. The assays proved highly sensitive and specific. The set of PCRs was validated against a panel of 596 samples collected in the field, including whole blood, serum, skin lesions, nasal and ocular discharge, milk, lymph nodes, lungs, trachea, spleen and aborted calves. Using the assays reported here some samples obtained as part of national surveillance for LSD virus from animals exhibiting clinical signs consistent with LSDV turned out to be positive for vaccine LSD virus genome in 2017. This vaccine strain is highly likely to have derived from commercial live-attenuated vaccines against LSD virus. The way of introduction of a vaccine LSD virus strain into Russian cattle remains to be investigated.

Keywords: lumpy skin disease, vaccine, diagnostics, real-time PCR, genome, virus

Bovine lumpy skin disease (LSD) (nodular dermatitis) is a transborder bovine infection manifested by fever and skin nodes (tubercles); generalized infection demonstrates lymphadenitis, as well as conjunctival disorders and respiratory/digestive mucosa conditions [1-4]. LSD causative agent is a DNA-containing enveloped virus (family *Poxviridae*, genus *Capripoxvirus*) involving related sheep and goat pox causative agents [5]. The viral genome represents a double-stranded DNA of 151 kb in length [6].

Cattle and buffaloes are susceptible to the disease [7]. Among them, dairy cows are the most vulnerable. Along with this, morbidity can be 3-80% [3, 8-10] indicating contribution of other potential non-investigated factors that influence on clinical sign severity. According to reported data, blood-sucking insect [11, 12] and tick [13, 14] bites are considered the most common route of infection. Nevertheless, a well-defined vector is not determined yet. All the LSD

outbreaks associated with the infection clinical signs must be notified in the International Epizootic Bureau (IEB, Office International des Epizooties, OIE, France). Nowadays, according to IEB, Turkey, Serbia, Greece, Albania, Bulgaria and other countries have reported incursions of the disease [15-19]. For the first time, Russia reported the infection in 2015 [20]. Then, 16 federal subjects (mainly, the Central Federal District) reported 313 outbreaks in 2016 [21]. In 2017-2018, the Volga Federal District started to report several incursions near the border of the Russian Federation [22].

Living homologous Neethling (i.e., an attenuated vaccine strain)-based LSD vaccines are in active use in near-border countries (e.g., the Republic of Kazakhstan, EU). Therefore, a complex of methods is required to detect and to differentiate causative agents of capripoxvirus infections including identification of Neethling vaccine strain that can induce clinical presentations of the disease [23, 24]. Apart from LSD, sheep pox reported in the Far East sometimes also jeopardizes the Russian Federation. The North Caucasus District and the Central Federal District [22] reported several incursions of the disease in 2015 and 2016-2018, respectively. Considering unexampled LSD virus spread (including subclinical recovery [25, 26], we need highly sensitive diagnostic methods that enable monitoring of latent infected susceptible animals to detect the causative agent's genome rapidly, to combat the infection and to prevent its extension.

The paper proposes sensitive and specific RT-PCR test systems for a single-mode sample testing characterized by a similar temperature profile for capripoxvirus genome (PCR-CAPR), lumpy skin disease virus field isolates (PCR-LSDV) and vaccine strain (PCR-NEE) for the first time ever.

Our purpose was to develop a complex of real-time PCR methods to detect genomes and to differentiate all the *capripoxviruses*, LSDV field isolates and Neethling vaccine strain in various biomaterials.

Techniques. Total DNA was extracted from 100 μl of the biomaterial suspension with a DNA Nucleic Acid Extraction Mini Kit (Qiagen, Germany) as per the manufacturer's instructions.

To select amplification sites and probe annealing regions in PCR, we evaluated several whole-genome sequences of LSDV field isolates (KX683219, KSGP 0240, KY829023 Evros/GR/15, KY702007 SER-BIA/Bujanovac/2016, AF409137 Neethling Warmbaths LW, AF325528 Neethling 2490), vaccine strains (KX764643 SIS-Lumpyvax vaccine, KX764644 Neethling-Herbivac vaccine, AF409138 Neethling vaccine LW 1959, KX764645 Neethling-LSD vaccine-OBP), as well as those related to sheep and goat pox (KX576657 Gorgan, KC951854 FZ, AY077836 G20-LKV, AY077835.1 Pellor, AY077833 Sheeppox virus A, AY077832 Sheeppox virus 10700-99 strain TU-V02127, MG000156 Sheeppox virus strain Jaipur, AY077834 Sheeppox virus NISKHI) available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Primers and probes developed with Primer3 engine software (http://bioinfo.ut.ee/primer3-0.4.0/) were synthesized by Syntol (Moscow). FAM dye (5′-probe end) and BHQ1 (3′-probe end) were used as a source and a quencher of fluorescence, respectively. Primers and the probe used to detect viral field isolates in PCR-LSDV system we mentioned in the previous work [27].

Real-time PCR (RT-PCR, Rotor Gene Q 6 plex amplifier, Qiagen, Germany) was performed as per the appropriate procedure (activation at 95 °C for 10 min; 40 cycles: 95 °C 15 s, 60 °C 1 min). GoTaq® MDx Hot Start Polymerase reagent kit (Promega Corp., USA) was applied. Reagent mixture (25 μl) included 5 μl of 10× PCR buffer, 3 μl of 25 mM $MgCl_2$, 0.5 μl of 10 nmol dNTP, direct and reverse primers, 12.5 pmol each, and 7.5 pmol of the probe.

The results were interpreted depending on whether a fluorescence curve intercepts a threshold line or not. This corresponds to presence/absence of Ct threshold cycle value. If Ct ≤ 40, a sample was considered to be positive (i.e., a viral DNA was detected).

To assess RT-PCR test system specificity, it was tested with genetic material of homologous and heterologous viruses. Specificity tests were performed analyzing each viral DNA individually and in presence of several viral DNAs. To test and to improve developed methods, we used DNAs of reference strains of heterologous viruses (Microorganism Strain Collection, Federal State Budgetary Institution Federal Animal Health Care Center), as well as DNAs of homologous and heterologous viral isolates obtained from various Russian regions. PCR-LSDV specificity test we mentioned in the previous work [27].

Analytical sensitivity of the test system intended for detection of the vaccine strain genome (PCR-NEE) was evaluated by a series of 10-fold dilutions ($1:10^1$-$1:10^6$) of Neethling LSD vaccine virus genome DNA. Initial titer was 5.21 lg $TCID_{50}$/ml. To analyze the test system designed for capripoxvirus genome detection (PCR-CAPR), sheep pox virus (Afghan strain) was applied; titer was 6.17 lg $TCID_{50}$/ml. In view of statistical verification of the findings and linear regression plotting, 10-fold genome DNA dilution test was conducted in triplicate. Amplification efficiency was calculated as per the formula:

$$E = (10^{slope} - 1) \times 100\ \%,$$

where 10^{slope} is slope coefficient.

Using Ct values, reproducibility (*Cv*, %) of each dilution series was calculated considering standard deviation (±SD). To assess threshold cycle variability, each sample was tested for 3 times in pentaplicate (1 launch — 5 replicates) for 3 days. Mean Ct values, standard deviations (±SD) and coefficient of variation (*Cv*, %) were calculated for each launch (5 replicates) and all the launches (15 replicates).

Diagnostic sensitivity (DS) and diagnostic specificity (DSp) of test systems were tested on samples of blood and nasal discharge collected in naturally infected animals. The results were calculated as per formulae as follows:

$$DS = TP/(TP + FN),$$

where TP is true-positive results, FN is false-negative results;

$$DSp = TN/(TN + FP),$$

where TN is true-negative results, FP is false-positive results.

We analyzed 596 samples of biological materials, i.e. stabilized whole blood, blood serum, skin scrapings (nodules), nasal and ocular discharge, milk, lymph nodes, lungs, trachea, spleen and aborted calves, from animals with clinical signs of the disease. Analyses were hosted by the Disease Reference Laboratory (Federal Animal Health Care Center, 2016-2017).

To assess Ct values, Passing-Bablok Regression was used and Bland-Altman plots were constructed. Lin's concordance correlation coefficient was used to examine agreement between PCR assays [28].

Results. See a brief description of used primers and probes in the Table 1. See description of heterologous and homologous viruses (reference strains, Microorganism Strain Collection, Federal Animal Health Care Center), as well as homologous and heterologous viral isolates obtained from various Russian regions used to assess specificity of RT-PCR test systems in the Table 2.

In virtue of sequence alignment, we identified several sites, which were the most conserved in field isolates only, in vaccine strains only and in all the capripoxviruses. To amplify field isolate genome region, we selected a LSDV126 reading frame for *EEV* gene of nodular dermatitis virus where 27 bp region is deleted in other *Capripoxviridae* and Neethling vaccine viruses, whereas the in-

sertion is observed in field isolates (Fig. 1, A). To amplify a vaccine strain genome fragment, we selected LSDV008 region where unique strain-specific substitutions are available (Fig. 1, B). To amplify the capripoxvirus genome, we selected *P32* gene, which is conserved for all the *Capripoxviridae* (Fig. 1, C).

1. Primers and probes used to detect *capripoxviruses* and to differentiate a genome of the LSDV vaccine strain and field isolates in RT-PCR

Agent (test system)	Nucleotide sequence (5′→3′)	Primers, probes	Gene	Amplicon, bp	Reference
BLSD vaccine virus (NEE)	TGTTTCCATTCTCCACTGCT TACTTACTAAAAAATGGGCGCA TCGCTGACATCGTTAGTCCACTC	fnee3 rnee3 Probe	*LSDV008*	185	The study
Capripoxviruses (CAPR)	ATGAAACCAATGGATGGGATA CGAAATGAAAAACGGTATATGGA ATGAGCCATCCATTTTCCAA	Capr_f Capr_r Probe	*P32*	92	The study
BLSD field isolate (LSDV)	AGAAAATGGATGTACCACAAATACAG TTGTTACAACTCAAATCGTTAGGTG ACCACCTAATGATAGTGTTTATGATTTACC	f2 r33 lsdv probe	*EEV*	96	[27]

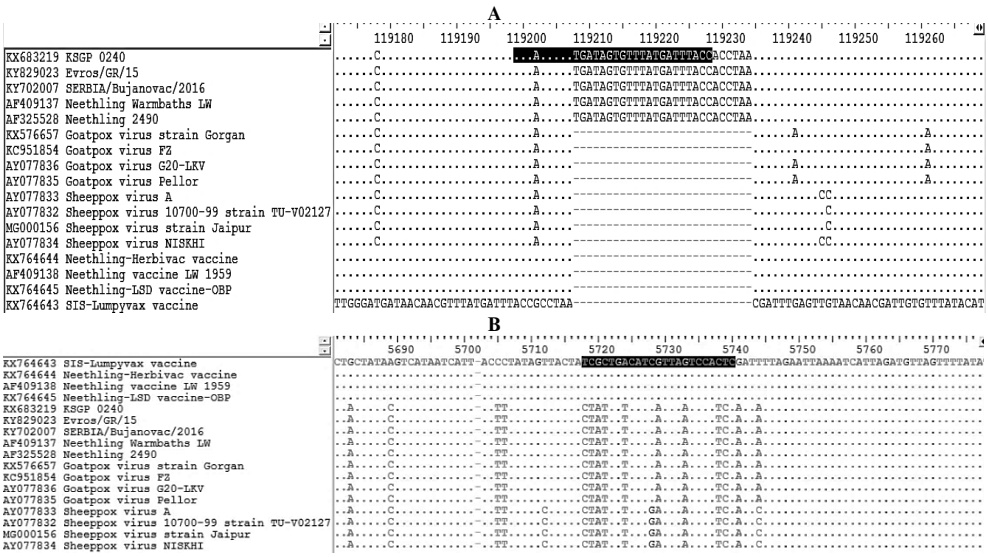
N o t e. BLSD is bovine lumpy skin disease.

2. Studied viral strains and isolates

Virus	Strain/isolate	Origin/collection	PCR	
			CAPR	NEE
BLSD virus	CNDV/Dagestan/2015 (diagnostic strain)	Russia/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
BLSD virus	CNDV E-95 (D)	Africa/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
Attenuated LSD vaccine strain	Neethling	RSA VRI Onderstepoort/Federal State Budgetary Institution Federal Animal Health Care Center	+	+
Sheep pox virus	Afghan strain	Afghanistan/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
Sheep pox virus field isolate		Russia (Yaroslavl' Region)/NA	+	–
Goat pox virus	Primorye 2003	Russia/Federal State Budgetary Institution Federal Animal Health Care Center	+	–
Sheep ecthyma virus field isolate		Russia/NA	–	–

N o t e. BLSD is bovine lumpy skin disease, “+” and “–” are positive and negative RT-PCR.

Evaluating the method specificity, we studied material containing DNA of heterologous viruses (PPR, vesicular stomatitis, sheep ecthyma and cow pox). Finally, we did not reveal any false-positive results of the diagnostic system testing using individual DNA of each virus or mixture of several viral DNAs.



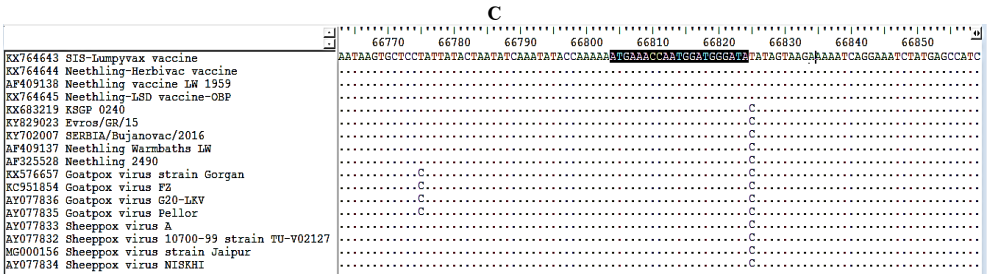


Fig. 1. Probe annealing area to detect bovine lumpy skin disease field isolates (A)**, vaccine strains of the virus** (B) **and capripoxviruses** (C) **in RT-PCR** (also see the journal website: http://www.agrobiology.ru).

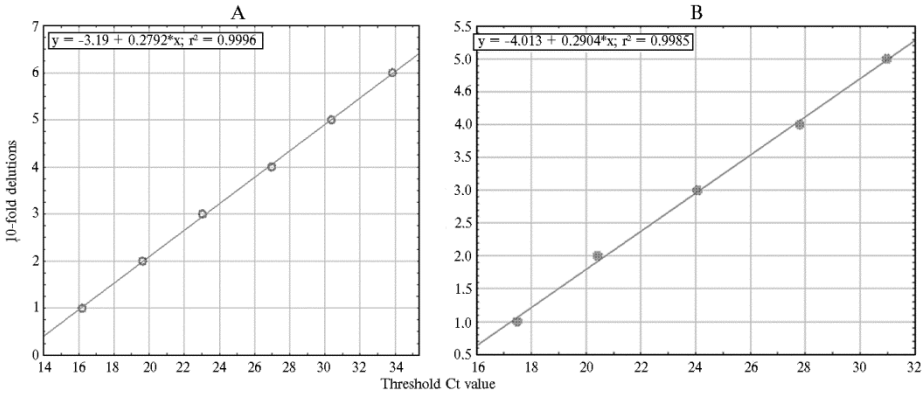


Fig. 2. Linear regressions of Ct values based on RT-PCR testing of 10-fold dilutions of capripoxvirus DNA (A) **and lumpy skin disease vaccine strain** (B) **confirm linearity of the results.**

To evaluate sensitivity of the PCR-CAPR test system, we used DNA of sheep pox virus (Afghan strain) whose infectious activity titer is 6.17 lg $TCID_{50}$/ml. The PCR-CAPR test system (Fig. 2, A) detected viral DNA at 0.17 lg $TCID_{50}$/ml. To assess amplification efficiency, we conducted three replicate tests and obtained Ct values applicable to the efficiency calculation. In virtue of the slope coefficient value resulted from linearity regression (Fig. 2, A), amplification efficiency is E = 90.2%. Reproducibility test (6 successive 10-fold dilutions) demonstrated a standard deviation (SD) of 0.12-0.32.

PCR-NEE test system sensitivity was assessed using DNA of Neethling LSDV vaccine strain whose infectious activity titer is 5.21 lg $TCID_{50}$/ml. The test system detected viral DNA at 0.21 lg $TCID_{50}$/ml. Amplification efficiency (Fig. 2, B) was E = 95.16%. The PCR-NEE test system (5 successive 10-fold dilutions) demonstrated a standard deviation (SD) of 0.03-0.60.

Primary quantitative characteristics of developed test systems are summarized in the Table 3.

3. Efficiency, standard deviation (SD) **and determination coefficient** (r^2) **of RT-PCR test systems to detect and to differentiate bovine lumpy skin disease field isolates, the virus vaccine strains and capripoxviruses**

Test system	Efficiency, %	SD (min-max)	r^2
PCR-CAPR	90.20	0.12-0.32	0.999
PCR-LSDV	98.60	0.11-0.33	0.990
PCR-NEE	95.16	0.03-0.60	0.998

N o t e. Test systems are described in the Techniques section. Reported data of prior PCR-LSDV study are presented [27].

See Ct value variations associated with parameter measurements in the Table 4.

See PCR-CAPR and PCR-LSDV DS and DSp comparison results on

the Figure 3. As per the reported data, there were no any significant differences between Ct values of PCR-CAPR and PCR-LSDV ($p > 0.05$). In virtue of Ct values of two test systems, Lin's concordance correlation coefficient between PCR CAPR and PCR-LSDV tests was 91.3%.

4. Ct variability associated with 1-3 launches of a RT-PCR test system

Test system, launch	Mean Ct value	±SD	*Cv*, %
PCR-CAPR (*n* = 5):			
1st	28.80	0.23	0.79
2nd	29.05	0.54	1.85
3d	29.15	0.32	1.09
PCR-LSDV (*n* = 5):			
1st	30.01	0.43	1.43
2nd	30.76	0.41	1.33
3d	29.93	0.16	0.53
PCR-NEE (*n* = 5):			
1st	29.56	0.37	1.20
2nd	29.53	0.36	1.20
3d	30.15	0.25	0.80
Three launches, mean (*n* = 15):			
PCR-CAPR	29.00	0.39	1.34
PCR-LSDV	30.27	0.52	1.71
PCR-NEE	29.76	0.50	1.60

N o t e. Test systems are described in the Techniques section. Each launch was performed in pentaplicate.

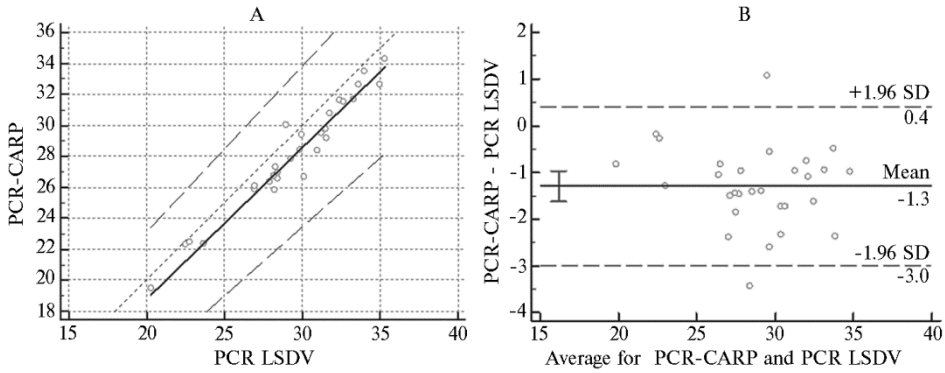


Fig. 3. Passing-Bablok regression (A) **and Bland-Altman scatter diagram** (B) **for Ct values (PCR-CAPR and PCR-LSDV test systems) at 95% CI.** Test systems are described in the Techniques section.

5. Detection of LSD virus genome in field isolates from cattle biomaterial (2015-2017)

Biomaterial	Samples		
	total	positive	
		n	%
Nodules	95	74	78
Whole blood	235	31	13
Blood serum	117	17	14.5
Nasal discharge	104	26	25

We applied three RT-PCR test systems to study 596 bovine biological material samples collected in 2015-2017. LSD virus genome was detected in 155 samples (26.0%) (Table 5). Bovine LSD virus was the most common in a nasal discharge (25.0%), serum (14.5%) and whole blood (13.0%). As a result of pathological material testing, LSDV genome was detected in 78% of affected skin (nodule) samples. LSDV genome was not mentioned in trachea, spleen and aborted calves. Additionally, 3 milk samples (2 LSDV-positive ones) from cattle with clinical signs of LSD, 5 lymph node samples (2 LSDV-positive ones) and 4 lung samples (3 LSDV-positive ones) were tested. PCR-CAPR and PCR-LSDV demonstrated 100% correspondent positive/negative results.

LSD laboratory diagnosis is of importance to confirm a suggested diagnosis and, in turn, to perform timely actions to prevent the viral spread. Also, we note that LSDV biological and epizootological properties are still understudied,

and more comprehensive understanding is required. Lumpy skin disease and sheep pox viruses are urgent threat to global livestock production (including the Russian Federation) [21]. Since LSD was historically restricted by the Africa and the Middle East, Russia was considered to be a LSD-free country before 2015 [25]. As for sheep pox, it was typically reported by the Far East and the North Caucasus. Vaccination is the only way to combat capripoxviruses. In terms of LSD, this is a vaccination with heterologous and homologous (attenuated) vaccines [25]. Sheep pox virus-based heterologous vaccine is safe for cattle whereas living attenuated vaccines, e.g., Lumpyvax (RSA) and equivalent, can induce clinical signs of the disease [26]. That is why the development of differential molecular diagnostic methods is a hot issue.

The paper presents a developed complex of RT-PCR test systems providing simultaneous detection of capripoxvirus genomes, LSDV field isolates and Neethling vaccine strain for the first time ever. The advantage of the approach is that reagent concentrations and temperature profile are similar in all the tests. These test systems are validated successfully with a great number of samples collected in experimentally infected animals or during the disease outbreak. The results are confirmed by sequencing and virus extraction in a cell culture (data are not available). All the samples collected during notified LSD outbreaks in the Russian Federation since 2015 were tested in PCR-CAPR and PCR-LSDV test systems. As a result, 100% similar findings were reported.

PCR-CAPR and PCR-NEE test systems were developed as a part of the trial, and PCR-LSDV test system dealing with field isolates was described [27]. To provide high specificity, all the amplification loci and probe annealing sites were selected considering conservation (all the isolates whose data are available in GenBank) and unicity (see Fig. 1). Unique genetic signatures provided detection of mentioned viruses by each developed RT-PCR test system.

During a launch amplification efficiency of all the test systems is > 90% in a series of 5 10-fold dilutions of the viral material; sensitivity is 0.3 lg $TCID_{50}/cm^3$ (see the Table 3). Moreover, highly sensitive and specific DNA panel testing of homologous/heterologous viruses make the complex irreplaceable to establish a diagnosis while dealing with field material samples. Comparison between PCR-CAPR and PCR-LSDV demonstrated no significant differences between Ct values while testing the same samples ($p > 0.05$); mean difference between the values was 1.3 cycles (Lin's concordance correlation coefficient is 91.3%) (see the Table 4). Unfortunately, it is impossible to compare PCR-CAPR and PCR-NEE test systems because of too small sampling of biomaterial containing vaccine LSDV genome.

Several data on methods to detect genomes of capripoxviruses, field or vaccine LSDV strains are reported. Although the common Ireland and Binepal's method [29] based on a conventional PCR was used previously, it is nonspecific for a given virus detecting all the capripoxviruses [27]. At the same time, a standard PCR associated with a cross-contamination risk is less sensitive. As for R-T PCR types, there are several reported papers dealing with duplex studies of capripoxvirus genome [30], field isolates [31], vaccine and field strains (Duplex PCR is a simultaneous detection of two target genes) [32]. It is significant that our PCR-LSDV and PCR-NEE test systems were more efficient (95.16% and 98.60%, respectively) than mentioned [32] duplex PCR (91.3% and 90.7%, respectively).

To assess specificity, we used national isolates, whereas our foreign colleagues evaluated test specificity in foreign strains. To consider a method to be an all-purpose one, a cross-validation must be conducted involving all the strains currently detected worldwide.

Potential differentiation of a field isolate from a vaccine strain in the same tube is both an advantage and disadvantage of the method proposed by Agianniotaki et al. [32]. As distinct from a duplex testing where simultaneous presence of both viruses decreases sensitivity of the response, our test systems can function independently in different tubes. The feature of a duplex PCR is very critical in the beginning of a living vaccine immunization in areas of active spread of field viral strains.

The developed complex of PCR test systems was tested on 596 biomaterial samples (i.e., various tissues and organ samples) collected in naturally LSD-infected cattle in the Russian Federation in 2015-2017 [21] (see the Table 5). We emphasize that correlation between test results in PCR-LSDV and PCR-CAPR is 100%. So, this confirms reliability of these diagnostic methods. It must be noted that suspected LSD-associated sampling should consider clinically healthy animals because they can be latent virus carriers without evident symptoms [24]. Due to the fact that bovine LSD epizootic situation is observed mainly intravitally, this has a special practical importance.

LSDV genome is the most common in nodules (78% samples). The fact complies with other study findings and confirms an evident viral tropism to skin epithelium [33]. Further, it was shown that PCR can detect LSDV genome in blood after a nodule appearance [33]. In terms of our study, 13-25% of other biomaterial samples demonstrated LSDV genome. Although the viral genetic material was detected in lungs, lymph nodes and milk, a precise statistical processing is still impossible due to restricted number of samples. It's important to stress that samples were collected at the onset of clinical signs that can influence on efficiency of LSDV detection. For example, absence of the viral genome in most of discharge samples, serum and whole blood may be associated with insufficient accumulation of the virus and its concentration is lower than a test system sensitivity limit during excretion in biological fluids. Moreover, Babiuk et al. [33] found that after experimental infection mucosal excretion of the virus can be observed after a nodule appearance. Moreover, the low concentrated viral DNA is detected in discharge within a short period (i.e., several days); transient (9 days) viremia is associated with intermittent presence of the virus during the test [33]. These properties may cause weak transmission of the virus between animals without flying transmitters.

It is important to notice that the PCR-NEE test system to detect the vaccine strain validated with the same volume of material revealed several cases of potential illegal use of Neethling-based LSDV-vaccine in number of Russian regions (data are not shown) despite Neethling genome was firstly found in cows with clinical signs of LSD in the Bashkortostan [34]. Although living attenuated LSDV vaccines are banned in the Russian Federation, they are approved in other countries of the Customs Union (e.g., the Republic of Kazakhstan) that, in turn, can lead to LSDV spreading in near-border regions [34]. Sequencing showed 100% homology of the appropriate *RPO30* fragment (data are not available) in the detected vaccine virus and vaccine strains used in commercial attenuated living vaccines. Agreement of PCR-CAPR and PCR-NEE results was 100%.

All the results obtained with proposed test systems are confirmed by virus extraction in a susceptible cell culture (data are not available) indicating reliability of R-T PCR methods used to differentiate the vaccine strain and the field isolate. High-degree certification and validation of proposed test systems was confirmed by the Russian Federation patent application. This is also an evidence of the development novelty [35, 36].

Thus, we developed reliable RT-PCR test systems to perform a single-

mode testing for genome of capripoxviruses, lumpy skin disease virus field isolates and LSDV vaccine strain. Methods demonstrated high specificity and sensitivity of panel testing of biomaterial samples collected in naturally infected animals.

REFERENCES

1. Coetzer J.A.W. Lumpy skin disease. In: *Infectious diseases of livestock*. J.A.W. Coetzer, R.C. Tustin (eds.). Oxford University Press, Cape Town, 2004.
2. Hunter P., Wallace D. Lumpy skin disease in southern Africa: a review of the disease and aspects of control. *Journal of the South African Veterinary Association,* 2001, 72: 68-71 (doi: 10.4102/jsava.v72i2.619).
3. Woods J.A. Lumpy skin disease: a review. *Tropical Animal Health and Production,* 1988, 20(1): 11-17.
4. Sameea Yousefi P., Mardani K., Dalir-Naghadeh B., Jalilzadeh-Amin G. Epidemiological study of lumpy skin disease outbreaks in North-western Iran. *Transboundary and Emerging Diseases*, 2016, 64(6): 1782-1789 (doi: 10.1111/tbed.12565).
5. Buller R.M., Arif B.M., Black D.N., Dumbell K.R., Esposito J.J., Lefkowitz E.J., McFadden G., Moss B., Mercer A.A., Moyer R.W., Skinner M.A., Tripathy D.N. Family *Poxviridae*. In: *Virus taxonomy: classification and nomenclature of viruses. Eighth Report of the International Committee on Taxonomy of Viruses*. C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (eds.). Elsevier Academic Press, San Diego, 2005.
6. Tulman E.R., Afonso C.L., Lu Z., Zsak L., Kutish G.F., Rock D.L. Genome of lumpy skin disease virus. *Journal of Virology*, 2001, 75(15): 7122-7130 (doi: 10.1128/JVI.75.15.7122-7130.2001).
7. Elhaig M.M., Selim A., Mahmoud M. Lumpy skin disease in cattle: frequency of occurrence in a dairy farm and a preliminary assessment of its possible impact on Egyptian buffaloes. *Onderstepoort Journal of Veterinary Research*, 2017, 84(1): e1-e6 (doi: 10.4102/ojvr.v84i1.1393).
8. Gomo C., Kanonhuwa K., Godobo F., Tada O., Makuza S.M. Temporal and spatial distribution of lumpy skin disease (LSD) outbreaks in Mashonaland West Province of Zimbabwe from 2000 to 2013. *Tropical Animal Health and Production*, 2017, 49(3): 509-514 (doi: 10.1007/s11250-017-1222-y).
9. Swiswa S., Masocha M., Pfukenyi D.M., Dhliwayo S., Chikerema S.M. Long-term changes in the spatial distribution of lumpy skin disease hotspots in Zimbabwe. *Tropical Animal Health and Production*, 2017, 49(1): 195-199 (doi: 10.1007/s11250-016-1180-9).
10. Zeynalova S., Asadov K., Guliyev F., Vatani M., Aliyev V. Epizootology and molecular diagnosis of lumpy skin disease among livestock in Azerbaijan. *Frontiers in Microbiology*, 2016, 7: 1022 (doi: 10.3389/fmicb.2016.01022).
11. Carn V.M., Kitching R.P. An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). *Epidemiology & Infection*, 1995, 114: 219-226 (doi: 10.1017/S0950268800052067).
12. Chihota C.M., Rennie L.F., Kitching R.P., Mellor P.S. Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (*Diptera*: *Culicidae*). *Epidemiology & Infection*, 2001, 126: 317-321 (doi: 10.1017/S0950268801005179).
13. Lubinga J.C., Tuppurainen E.S., Coetzer J.A., Stoltsz W.H., Venter E.H. Transovarial passage and transmission of LSDV by *Amblyomma hebraeum*, *Rhipicephalus appendiculatus* and *Rhipicephalus decoloratus* ticks. *Experimental and Applied Acarology*, 2014, 62(1): 67-75 (doi: 10.1007/s10493-013-9722-6).
14. Tuppurainen E.S., Venter E.H., Coetzer J.A., Bell-Sakyi L. Lumpy skin disease: attempted propagation in tick cell lines and presence of viral DNA in field ticks collected from naturally-infected cattle. *Ticks and Tick-borne Diseases*, 2015, 6(2): 134-140 (doi: 10.1016/j.ttbdis.2014.11.002).
15. Tasioudi K.E., Antoniou S.E., Iliadou P., Sachpatzidis A., Plevraki E., Agianniotaki E.I., Fouki C., Mangana-Vougiouka O., Chondrokouki E., Dile C. Emergence of lumpy skin disease in Greece, 2015. *Transboundary and Emerging Diseases*, 2016, 63(3): 260-265 (doi: 10.1111/tbed.12497).
16. Carn V.M., Kitching R.P. An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). *Epidemiology and Infection,* 1995, 114(1): 219-226.
17. Alkhamis M.A., Vander Waal K. Spatial and temporal epidemiology of lumpy skin disease in the Middle East, 2012-2015. *Frontiers in Veterinary Science*, 2016, 3: 19 (doi: 10.3389/fvets.2016.00019).
18. Mercier A., Arsevska E., Bournez L., Bronner A., Calavas D., Cauchard J., Falala S., Caufour P., Tisseuil C., Lefrançois T., Lancelot R. Spread rate of lumpy skin disease in the Balkans, 2015-2016. *Transboundary and Emerging Diseases*, 2017, 65: 240-243 (doi: 10.1111/tbed.12624).
19. Abutarbush S.M., Ababneh M.M., Al Zoubi I.G., Al Sheyab O.M., Al Zoubi M.G., Alekish M.O., Al Gharabat R.J. Lumpy skin disease in Jordan: disease emergence, clinical signs, complications and preliminary-associated economic losses. *Transboundary and Emerging Diseases*, 2015, 62(5): 549-554 (doi: 10.1111/tbed.12177).
20. Biryuchenkova M.V., Timina A.M, Zinyakov N.G., Shcherbakov A.V.. *Veterinariya segodnya*, 2015, 4(15): 43-45 (in Russ.).

21. Sprygin A., Artyuchova E., Babin Y., Prutnikov P., Kostrova E., Byadovskaya O., Kononov A. Epidemiological characterization of lumpy skin disease outbreaks in Russia in 2016. *Transboundary and Emerging Diseases*, 2018, 65(6): 1514-1521 (doi: 10.1111/tbed.12889).
22. OIE. *Follow-up report No. 20. Report Date: 28/12/2018.* Available http://www.oie.int/wahis_2/temp/reports/en_fup_0000029055_20181228_142744.pdf. No date.
23. Bedeković T., Šimić I., Krešić N., Lojkić I. Detection of lumpy skin disease virus in skin lesions, blood, nasal swabs and milk following preventive vaccination. *Transboundary and Emerging Diseases*, 2018, 65(2): 491-496 (doi: 10.1111/tbed.12730).
24. Kononov A., Prutnikov P., Shumilova I., Kononova S., Nesterov A., Byadovskaya O., Pestova Y., Diev V., Sprygin A1. Determination of lumpy skin disease virus in bovine meat and offal products following experimental infection. *Transboundary and Emerging Diseases,* 2019, 00: 1-9 (doi: 10.1111/tbed.13158).
25. Tuppurainen E.S., Venter E.H., Shisler J.L., Gari G., Mekonnen G.A., Juleff N., Lyons N.A., De Clercq K., Upton C., Bowden T.R., Babiuk S., Babiuk L.A. Capripoxvirus diseases: current status and opportunities for control. *Transboundary and Emerging Diseases*, 2017, 64(3): 729-745 (doi: 10.1111/tbed.12444).
26. Bedeković T., Šimić I., Krešić N., Lojkić I. Detection of lumpy skin disease virus in skin lesions, blood, nasal swabs and milk following preventive vaccination. *Transboundary and Emerging Diseases*, 2018, 65(2): 491-496 (doi: 10.1111/tbed.12730).
27. Pestova Ya.E, Artyukhova E.E., Kostrova E.E, Shumilova I.N., Kononov A.V., Sprygin A.V. Real time PCR for the detection of field isolates of lumpy skin disease virus in clinical samples from cattle. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2018, 53(2): 422-429 (doi: 10.15389/agrobiology.2018.2.422eng).
28. Lin L.I. A concordance correlation coefficient to evaluate reproducibility. *Biometrics*, 1989, 45(1): 255-268 (doi: 10.2307/2532051).
29. Ireland D.C., Binepal Y.S. Improved detection of capripoxvirus in biopsy samples by PCR. *Journal of Virological Methods*, 1998, 74(1): 1-7 (doi: 10.1016/S0166-0934(98)00035-4).
30. Stubbs S., Oura C.A., Henstock M., Bowden T.R., King D.P., Tuppurainen E.S. Validation of a high-throughput real-time polymerase chain reaction assay for the detection of capripoxviral DNA. *Journal of Virological Methods*, 2012, 179(2): 419-422 (doi: 10.1016/j.jviromet.2011.11.015).
31. Vidanovic D., Sekler M., Petrovic T., Debekjak Z., Vaskovic N., Matovic K., Hoffmann B. Real-time PCR assays for the species detection of field Balkan strains of lumpy skin disease virus. *Acta Veterinaria Belgrade*, 2016, 66(4): 444-454 (doi: 10.1515/acve-2016-0038).
32. Agianniotaki E.I., Chaintoutis S.C., Haegeman A., Tasioudi K.E., De Leeuw I., Katsoulos P.D., Sachpatzidis A., De Clercq K., Alexandropoulos T., Polizopoulou Z.S., Chondrokouki E.D., Dovas C.I. Development and validation of a TaqMan probe-based real-time PCR method for the differentiation of wild type lumpy skin disease virus from vaccine virus strains. *Journal of Virological Methods*, 2017, 249: 48-57 (doi: 10.1016/j.jviromet.2017.08.011).
33. Babiuk S., Bowden T.R., Parkyn G., Dalman B., Manning L., Neufeld J., Embury-Hyatt C., Copps J., Boyle D.B. Quantification of lumpy skin disease virus following experimental infection in cattle. *Transboundary and Emerging Diseases*, 2008, 55(7): 299-307 (doi: 10.1111/j.1865-1682.2008.01024.x).
34. Sprygin A., Pestova Y., Prutnikov P., Kononov A. Detection of vaccine-like lumpy skin disease virus in cattle and *Musca domestica* L. flies in an outbreak of lumpy skin disease in Russia in 2017. *Transboundary and Emerging Diseases,* 2018, 65(5): 1137-1144 (doi: 10.1111/tbed.12897).
35. Sprygin A.V, Kononov A.V., B'yadovskaya O.P. et al. *Oligonukleotidnye praimery i fluorestsentno-mechenyi zond, sposob i test-sistema PTSR v rezhime real'nogo vremeni dlya vyyavleniya genoma kapripoksvirusov. Pat. 2658493, FGBU «Federal'nyi tsentr okhrany zdorov'ya zhivotnykh» (FGBU «VNIIZZH»). № 2017130118. Zayavl. 24.08.2017. Opubl. 21.06.2018. Byul. № 18* [Oligonucleotide primers and labeled fluorescent probe, method and real-time PCR test system for detecting the genome of capripoxviruses. No. 2017130118. Appl. 24.08.2017. Publ. 21.06.2018. Bul. No. 18] (in Russ.).
36. Sprygin A.V, Kononov A.V., B'yadovskaya O.P. et al. *Oligonukleotidnye praimery i fluorestsentno-mechenyi zond, sposob i test-sistema dlya vyyavleniya genoma polevykh izolyatov virusa zaraznogo uzelkovogo dermatita (nodulyarnogo dermatita) KRS v reaktsii polimeraznoi tsepnoi reaktsii v rezhime real'nogo vremeni. Pat. 2668398, FGBU «Federal'nyi tsentr okhrany zdorov'ya zhivotnykh» (FGBU «VNIIZZH»). № 2017124318. Zayavl. 07.07.2017. Opubl. 28.09.2018. Byul. № 28* [Oligonucleotide primers and labeled fluorescent probe, method and test system for detecting the genome of field isolates of infectious nodular dermatitis virus (nodular dermatitis) of cattle in real-time polymerase chain reaction. No. 2017124318. Appl. *07.07.2017.* Publ. *28.09.2018.* Bul. No. 6] (in Russ.).