Part 3. Emergent diseases and biosafety

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THE INTERLABORATORY TESTING OF THE TEST-SYSTEM FOR DETECTION OF THE LUMPY SKIN DISEASE VIRUS DNA USING REAL-TIME PCR 'BOVI-DNA-TEST-LSD VIRUS'

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Summary. Lumpy skin disease (LSD, nodular dermatitis of cattle) — is the contagious poxviral disease of cattle. It characterized with severe losses and different ranges of mortality and morbidity. The disease is endemic in many Asian and African countries.

National Scientific Center 'Institute for Experimental and Clinical Veterinary Medicine' in collaboration with State Scientific and Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise developed the in-house PCR-based protocol for LSDV detection that requires fast implementation. The evaluation of diagnostics kit has been performed under OIE requirements with determination of the sensitivity, specificity, repeatability and domain-specificity. Detection kit is recommended for practical application.

Keywords: lumpy skin disease, diagnostics, real-time PCR, test system, cattle

Introduction. Lumpy skin disease (LSD, nodular dermatitis of cattle) — is the contagious poxviral disease, occurred in the ruminant species, caused by the *Lumpy skin disease virus*. LSD is characterized with severe losses, and different ranges of mortality and morbidity. It is endemic in many Asian and African countries, and it is rapidly spreading throughout the Middle East. Turkey, Bulgaria, Russia and Caucasus countries are affected with the disease (AHAW, 2015; OIE, 2016).

The disease has the economic importance because of temporary reduction in milk production, temporary or permanent sterility in bulls and fertility of cows. LSD could be increased in the damage level in association with secondary bacterial infections (Coetzer, 2004).

Historically, the first cases of LSD were described in 1929 in Zambia, and later detected in multiple African and Middle Eastern countries (Al-Salihi, 2014). The disease has been reported to Turkey in October 2013, Iran and Iraq in 2014. The expectation of the spreading and invasion of the LSD to free neighbor countries was possible. LSD may invade north and west from Turkey into Europe and the Caucasus and East to Central and South Asia. Nowadays cases of the disease are registered in Russian Federation, Turkey, Caucasus countries and Bulgaria. Both areas represent the transboundary threat of introduction of the disease via the bite-insects vector-borne way.

The early diagnostics and eradication of the disease has extremely significant role for epizootic chain destruction. The key place in laboratory diagnostics belongs to express-diagnostics tests such as PCR and real-time PCR detection of viral genome.

PCR procedures using capripoxvirus-specific primers have been published for use on ocular swabs, blood, tissue and semen samples are described in OIE Manual (OIE, 2016). These procedures serve as a general guideline and a starting point for the PCR protocol. Optimal reaction conditions (incubation times and temperatures, models and suppliers of equipment, concentrations of assay reagents such as the primers and dNTPs) may vary so the described conditions should be evaluated primarily.

Application of the Real-time PCR allows providing an effective detection of the virus in multiple type and ranges of samples with parallel reduction of the cross-contamination risks in laboratory conditions (Stegniy and Gerilovych, 2014).

The National Scientific Center 'Institute for Experimental and Clinical Veterinary Medicine' and the State Scientific and Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise were

developed the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' due to the reason of enhancement of LSD control measures in Ukraine.

The aim of the presented research is to study sensitivity, specificity, repeatability and reproducibility of the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus'.

Materials and methods. Appearance, activity, specificity, sensitivity of components of the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' and reproducibility were evaluated during commission trials.

The kit samples from batch 1, control eval. 1 produced in the National Scientific Center 'Institute for Experimental and Clinical Veterinary Medicine' in July 2016 were used.

The following samples panel has been used for specificity and sensitivity study of the test-system:

— reference panel DNAs (n = 3) of the LSDV, received from Animal Production and Health Laboratory of the Joint FAO/IAEA Division, International Atomic Energy Agent (Vienna, Austria), and its 1:10, 1:100, 1:1,000 and 1:10,000 dilutions;

- DNA samples isolated from the stabilized cattle blood from the intact animals (n = 10).
- positive control of the amlification (PC);
- negative control of the amplification (NC);
- non-template control (NTC).

Following heterological templates were used to demonstrate the kit's species-specific specificity (genetic material of other cattle viruses):

- *Bluetongue virus* cDNA (vaccine);
- *Bovine herpesvirus* type 1 DNA (IBR virus);
- *Bovine herpesvirus* type 5 DNA;
- cDNA of *Bovine viral diarrhea virus* (BVDV).

The polymerase chain reaction was managed under the recommended conditions described in the manual to the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus'. All testing was done without changes of the recommended features of reaction. To estimate the repeatability of testing it has been performed three times (Table 1).

Table 1 – Amplification cycle for LSD virus detection

Stage	Temperature	Length	Step	Number of cycles
1	95 °C	3 min	Activation of the DNA-polymerase	1
2	95 °C	10 sec	DNA denaturation	45
3	60 °C	30 sec	Primer annealing/ elongation	

Results. Tests were conducted to verify the commission sensitivity and specificity of the test system and reproducibility obtained when using the results.

At the beginning of the commission trials, PCR test-systems were checked for completeness. All components and instruction necessary for operation were available.

In assessing the appearance, it was found that the test system consists of the following components:

DNA extraction kit (kit # 1):

- 'extraction buffer' (lysis buffer) — 1 flack — 15 (30) ml, transparent non-colored liquid;
- sorbent solution — 1 (2) tube — 1.5 ml, opalescentic fluid with white color;
- 'washing buffer' — 1 flack — 50 (100) ml, transparent non-colored liquid;
- 'washing buffer ethanol' — 1 flack — 50 (100) ml, transparent non-colored liquid;
- 'solution № 4' for the final washing — 1 flack — 30 (60) ml, transparent non-colored liquid;
- TE-buffer — 1 flack — 20 (40) ml, transparent non-colored liquid.

Kit for PCR amplification (kit # 2):

- 'RT-PCR MasterMix' — 1 (2) tubes — 0.5 ml — transparent non-colored liquid;
- probe solution (10 pM/μl) — 1 tube — 0.02 or 0.04 ml — transparent rose liquid;
- primer solution (20 pM/μl) — 1 tube of each — 0.125 (0.25) ml — transparent non-colored liquid;
- deionized water — 1 (2) tubes — 1.25 ml — transparent non-colored liquid;
- positive control template (for 5 or 10 reactions) — 1 tube — 0.05–0.1 ml — transparent non-colored liquid.

The PCR master mix was prepared using number of samples amount plus one under proportion per sample:

No.	Component	Final concentration	1× for reaction (µl)
1.	Water for PCR		6.7
2.	RT-PCR Master Mix-Path ID	**1×**	10
3.	Probe **CaPV074P1** (10 pM/µl)	**250 nM**	0.5
4.	Primer **CaPV074F1** (20 pM/µl)	400 nM	0.4
5.	Primer **CaPV074R1U** (20 pM/µl)	**400 nM**	0.4
	DNA (template or control)		2

The results of the sensitivity testing is the ability to identify all obviously encrypted positive samples. It was found that the test system is able to detect DNA of the LSD virus in both positive reference material samples, including their dilutions 1:10–1:10,000 with Ct value 18.6–27.2. This is equal to the minimum titer of virus in the cattle organism, we registered an infected animals (in the skin scrabs and blood samples).

The specificity of the test system proved amplicon in the absence of any size of Ct value or Ct value over 38 in samples of cattle origin tissues from non-infected animals.

In addition, primers designed didn't hybrydizated with DNA samples from other viruses, including DNA extracts from *Bovine herpesvirus* of types 1 and 5, and cDNAs of bluetongue virus and BVDV virus (Table 2).

Table 2 – Testing results for the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' with the panels of positive, negative and heterogenic samples

No.	Material	1st repeat result	2nd repeat result	3rd repeat result
1.	Sample 1	Ct 18.6	Ct 18.8	Ct 18.6
2.	Sample 1 1:10	Ct 21.2	Ct 20.9	Ct 20.9
3.	Sample 1 1:100	Ct 22.4	Ct 22.1	Ct 22.2
4.	Sample 1 1:1000	Ct 26.3	Ct 26.4	Ct 25.9
5.	Sample 1 1:10000	Ct 27.2	Ct 27.1	Ct 27.0
6.	Sample 2	Ct 19.2	Ct 19.4	Ct 19.2
7.	Sample 2 1:10	Ct 20.7	Ct 20.8	Ct 20.7
8.	Sample 2 1:100	Ct 21.2	Ct 22.4	Ct 22.2
9.	Sample 2 1:1000	Ct 24.5	Ct 24.6	Ct 24.5
10.	Sample 2 1:10000	Ct 27.2	Ct 27.2	Ct 27.1
11.	Sample 3	Ct 18.6	Ct 18.8	Ct 18.9
12.	Sample 3 1:10	Ct 19.7	Ct 19.6	Ct 19.8
13.	Sample 3 1:100	Ct 22.4	Ct 22.3	Ct 22.6
14.	Sample 3 1:1000	Ct 25.3	Ct 25.2	Ct 24.9
15.	Sample 3 1:10000	Ct 27.2	Ct 26.7	Ct 26.7
16.	Bovine blood 1	N/d	N/d	N/d
17.	Bovine blood 2	N/d	N/d	N/d
18.	Bovine blood 3	N/d	N/d	N/d
19.	Bovine blood 4	N/d	N/d	N/d
20.	Bovine blood 5	N/d	N/d	N/d

21.	Bovine blood 6	N/d	N/d	N/d
22.	Bovine blood 7	N/d	N/d	N/d
23.	Bovine blood 8	N/d	N/d	N/d
24.	Bovine blood 9	N/d	N/d	N/d
25.	Bovine blood 10	N/d	N/d	N/d
26.	IBRV	N/d	N/d	N/d
27.	BHV-5	N/d	Ct 42.0/ N/d	N/d
28.	BTV	N/d	N/d	N/d
29.	BVDV	N/d	N/d	N/d

It was also marked that complete coincidence test results of the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' in three repetitions under similar conditions and using different Thermocyclers of the same type.

Conclusions. 1. The specificity of the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' developed by the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' and the State Scientific-Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise has the appropriate level of sensitivity and specificity, aligned with the detection of LSD virus DNA in clinical samples and their 1:10 to 1:10,000 dilutions, in Ct values of 18.6–27.2, with its absence of Ct value over 38 in the 'negative samples'. The developed kit does not demonstrate the false positive reactions with heterogenic DNA and cDNA samples, such as.

2. The sensitivity of the system can be considered satisfactory, since positive results were positive for samples with low concentrations of specific DNA.

3. Primers, probe and other components of the proposed test kits are highly sensitive, specific and comply with TU.

4. Reproducibility and repeatability of Lumpy skin disease virus DNA detection method using the LSD virus target gene amplification for that is the basis of the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' has the appropriate level, in all three repetitions differences were observed results.

The perspectives for further application of the results. As the commission trials of the Test-System for Detection of the Lumpy Skin Disease Virus DNA Using Real-Time PCR 'Bovi-DNA-Test-LSD virus' developed by the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' and the State Scientific-Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise indicators of quality, such as sensitivity, specificity and reproducibility fit the requirements. In this regard, this test system can be recommended for interestablishmental testing and using in practice of the State veterinary laboratories for the detection of DNA of Lumpy skin disease virus.

References

AHAW (EFSA Panel on Animal Health and Welfare) (2015) 'Scientific opinion on lumpy skin disease', *EFSA Journal*, 13(1), p. 3986. doi: 10.2903/j.efsa.2015.3986.

Al-Salihi, L. (2014) 'Lumpy Skin disease: Review of literature', *Mirror of Research in Veterinary Sciences and Animals*, 3(3), pp. 6–23. Available at: http://mrvsa.com/upload/3-3-2-2014%20Lumpy%20Skin%20disease%20%20Review%20of%20literature.pdf.

Coetzer, J. A. W. (2004) 'Lumpy skin disease', in Coetzer, J. A. W. and Tustin, R. C. (eds.) *Infectious diseases of livestock*. 2nd ed. Cape Town, South Africa: Oxford University Press, pp. 1268–1276. ISBN 9780195761719.

OIE (World Organisation for Animal Health) (2016) 'Chapter 2.4.13. Lumpy skin disease', in: *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees)*. Paris: OIE. Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.13_LSD.pdf.

Stegniy, B. T. and Gerilovych, A. P. (eds.) (2014) *Molecular genetic methods of diagnostics in veterinary medicine and biotechnology: A tutorial [Molekuliarno-henetychni metody diahnostyky u veterynarnii medytsyni ta biotekhnolohii: navchalnyi posibnyk]*. Kyiv: ST Druk. ISBN 9789662717143. [in Ukrainian].