AGRICULTURAL BIOLOGY,
2018, V. 53, № 2, pp. 422-429
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Virology

UDC 636.2:619:57.083.2:577.2

doi: 10.15389/agrobiology.2018.2.422eng
doi: 10.15389/agrobiology.2018.2.422rus

REAL TIME PCR FOR THE DETECTION OF FIELD ISOLATES OF LUMPY SKIN DISEASE VIRUS IN CLINICAL SAMPLES FROM CATTLE

Ya.E. PESTOVA, E.E. ARTYUKHOVA, E.E. KOSTROVA, I.N. SHUMOLIVA, A.V. KONONOV, A.V. SPRYGIN

Federal Center for Animal Health Control, FGBU VNIIZZh, mkr. Yurievets, Vladimir, 600901 Russia, e-mail kononov@arriah.ru, spriginav@mail.ru (✉ corresponding author);
ORCID:
Pestova Ya.E. orcid.org/0000-0002-7974-623X
Artyukhova E.E. orcid.org/0000-0003-2963-1415
Kostrova E.E. orcid.org/0000-0001-7150-7632
Shumoliva I.N. orcid.org/0000-0002-4663-3845
Sprygin A.V. orcid.org/0000-0001-5982-3675
The authors declare no conflict of interests
Received July 6, 2017

Abstract

Lumpy skin disease caused by lumpy skin disease virus (LSDV, *Capripoxvirus*, *Poxviridae*) is a capripoxviral disease with significant morbidity in cattle, which necessitates the development of reliable diagnostic tools in the context of live vaccine administration. OIE-recommended PCR assays target not only LSDV but also sheep pox virus and goat pox virus. Conventional PCR is prone to carry-over contamination, whereas real-time PCR offers more advantages, including prevention of amplicon carryover contamination post amplification. In this paper we report the development of a PCR real time assay for the detection of field isolates of lumpy skin disease virus in clinical samples from cattle. The specificity was validated against a panel of homologous and heterologous viruses retrieved from the strain depository of FGBI ARRIAH. The PCR assay was shown to be highly specific toward field LSDV. When tested in the presence of vaccine strain DNA and related capripoxviruses, no false-positive results were obtained. Using a series of 10-fold dilutions the assay proved to be highly sensitive with a detection limit of 0.21 lg TCD_{50}/ml. The calculated efficiency of amplification was 98.6 %, with SD ranging from 0.11 to 0.33 over five orders of magnitude. The PCR assay was also validated on samples from experimentally inoculated bulls. The animals received a subcutaneous injection of a field LSDV and were tested for the presence of LSDV DNA in blood and nasal swab in comparison to PCR by D.C. Ireland и Y.S. Binepal (1998) (data not shown). Overall, the presented assay demonstrated high specificity and sensitivity and can be recommended as a diagnostic tool for the detection of field isolated of LSDV.

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

Capripoxviruses of *Capripoxvirus* genus (infectious virus of nodular dermatitis in bovine animals, capripoxvirus in sheep and goats) are agents of cross-border zoonotic diseases in large and small cattle, which represent serious threat to cattle breeding industry, causing significant losses to farming and country economies [1, 2]. During the past few years, due to intensive trading and, possibly, natural factors, capripoxviruses had started massive dissemination northwards, including countries of Near East, Europe, Turkey, and Russia [3-7]. Today, lumpy skin disease virus (LSDV) in large cattle is deemed the most dangerous. Massive spread of LSDV in 2015-2016 at south of Europe, Balkan Peninsula, and in the Russian Federation, has reinforced the need to use highly sensitive methods for monitoring and accurate diagnosis of the disease as soon as possible for timely and adequate preventative and protective measures.

LSDV (*Capripoxvirus* genus, *Poxviridae* family) genome consists of double-stranded DNA [8]. LSDV transmission occurs mechanically through bites of insects or through contaminated feed, water, and sperm at fertilization [1, 8-11]. Recent studies established the role of mites in spreading LSDV [12, 13].

LSD is cross-border viral disease in large cattle that is accompanied by fever, reduction of live weight, injury of lymphatic system, swelling of internal organs, formation of nodes (nodules, bumps) on skin and internal organs [14]. Recently, disease is included in the list of World Organization for Animal Health (International epizootic bureau, OIE — IEB, Paris) [15]. Infection of large cattle by nodular dermatitis is subject to compulsory notification. Subject to Decree Nr. 62 of the Ministry of Agriculture of the Russian Federation dated March 09, 2011, infectious nodular dermatitis in large cattle is included in the List of infectious and other diseases in animals, but is not included in the List of infectious, including highly dangerous diseases, for quarantine, approved by the Ministry of Agriculture of the Russian Federation Nr. 476 dated December 19, 2011.

Earlier, LSD seasonally emerged in large cattle in African countries [16]. Unprecedented spread of LSD causative agent at south of Russia in 2015-2016 [5, 17] emphasizes the need for highly sensitive and specific diagnostic tests. IEB recommends detecting LSDV genome by PCR [15], with both classic PCR [18] and real-time PCR used [19]. The problem is that the IEB tests also detect DNA of sheep and goat capripoxviruses and not discriminate these viruses from cattle capripoxvirus. Besides, in classic PCR there is a risk of contamination of amplification products as electrophoretic detection is needed. C.E. Lamien et al. [20] suggest PCR amplification with high-resolution melting analysis of DNA fragments to detect of LSDV and capripoxvirus of sheep and goats. However, this method is unusable in routine diagnostics because of high dependence on DNA quality and concentration. Besides, use of live Neethling strain-based vaccines against LSD requires tests able to identify and differentiate field isolates.

We propose rapid, reliable, sensitive, and specific real-time PCR method to identify DNA of LSDV field isolates in tissues and organs of experimentally and naturally infected animals.

Purpose of this research was development and validation of real-time PCR test for identification of LSDV DNA in biomaterial.

Techniques. Total DNA was extracted from 100 μl analyzed specimen suspension with RIBO-sorb kit (Central Research Institute of Epidemiology, Moscow) subject to the producer's instruction.

Primers and probes were developed with Primer3 Engine Software (available at http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and synthesized (Beagle Biotechnology, Saint Petersburg). For detection of virus, we used primers f2 TAGAAAATGGATGTACCACAAATACAG and r33 TTGTTACAACTCAAATCGTTAGGTG, and probe Taqman ACCACCTAATGATAGTGTTTATGATTTAC 5′-end labeled with fluorescent dye carboxyfluorescein (6FAM), and 3′-end labeled with fluorescence quencher BHQ-1.

For PCR, we used reagent kit GoTaq Flexi DNA Polymerase, 5× Colorless GoTaq PCR Buffer, $MgCl_2$ (Promega Corp., USA) and 100 mM dNTPs (Invitrogen, USA). Ultimate 25 μl reaction mixture contained 5 μl 10× PCR buffer, 3 μl of 25 мМ $MgCl_2$, 0.5 μl 10 nmol dNTP, 12.5 pmol of each primer (forward and reverse), and 7.5 pmol probe. Real-time PCR (qPCR) was carried out with the use of a Rotor Gene instrument (Qiagen N.V., Germany). PCR protocol was as follows: 10 minute activation at 95 °C; 40 cycles including 15 seconds at 95 °C and 1 minute at 60 °C.

The results were interpreted based on intersection of fluorescence curve and threshold line that correlates to presence or absence of threshold cycle value Ct in relevant column in the table of results at machine-based analysis. Results were deemed valid provided positive ($Ct < 30$) and negative (Ct not identified) amplification controls. Specimen was positive (presence of LSDV DNA) if Ct did not exceed 35, and negative if Ct was absent or exceeded 37.

Primer specificity was checked with BLAST online resource software (https://blast.ncbi.nlm.nih.gov/) and by testing genetic material of homologous and heterologous viruses. Specificity was assessed for DNA of each virus individually and also for several viral DNAs in a single reaction mixture. qPCR analytic sensitivity was determined with 10-fold dilutions (up to 10^{-5}, initial titer of 5.21 lg TCD_{50}/ml) of reference strain NDV/Dagestan/2015 genome DNA. For statistical verification, 3 repeated tests with 10-fold dilutions of genome DNA were carried out to assess result linearity.

Six bulls aged 1.5 years were experimentally infected by intravenous injection of 2 ml bovine virus NDV/Dagestan/2015 field strain (5.21 lg TCD_{50}/ml) according to common recommendations. One animal (control) was not infected. To detect LSDV on days 4 and 14 post inoculation, blood samples stabilized with EDTA and nasal washes were collected. To confirm qPCR results, animals were slaughtered at the end of tests to collect biomaterial for virus isolation in continuous lamb testis cells (collection of cell cultures of the Federal Center for Animal Health Control) as per description [21].

Linear regression was plotted with Statistica 10 software (StatSoft, Inc., USA). At assessment of repeatability, standard errors (±SD) and determination coefficient r^2 were calculated.

Results. The primers and probe were developed for amplification and detection of LSDV EEV (extracellular enveloped virions, ORF126) gene fragment with a 27 bps deletion characteristic of other *Capripoxviridae* members and also of Neethling vaccine strains but absent in field isolates [22, 23].

Specificity. For testing and optimization of the developed method, we used DNA of reference strains of heterologous viruses (deposited in collection of microorganisms of Federal Center for Animal Health Control), as well as DNA of LSDV strains isolated in different regions of Russia in 2015-2016 (Table 1). These results indicate absence of false positives and specific identification of field isolates both separately and at presence of DNA of heterologous viruses (viruses of sheep and goat pox), as well as vaccine strain.

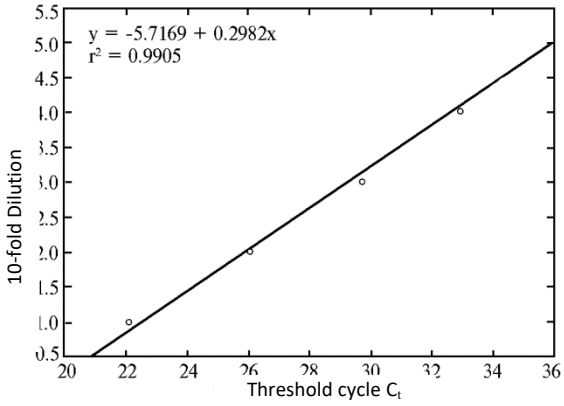
qPCR sensitivity. Sensitivity of the proposed method with diagnostic strain NDV/Dagestan/2015 is 0.21 lg TCD_{50}/ml at initial titer of 5.21 lg TCD_{50}/ml. To assess amplification effectiveness during repeated tests, Ct values were determined. Based on mean Ct values, we obtained linear regression with amplification effectiveness value (E) of 98.6 % (Fig.).

1. Specificity of the developed real-time PCR (qPCR) **method in detection of lumpy skin disease virus (LSDV) field isolates**

DNA source	Origin/collection	qPCR
NDV LC/Dagestan/2015 (reference strain)	Russia/FSBE VNIIZZH	
NDV LC E-95	Africa/ FSBE VNIIZZH	
Attenuated vaccine strain	SAR VRI Onderstepoort/ FSBE VNIIZZH	
Sheep pox virus, Afganian strain	Afganistan/ FSBE VNIIZZH	
Sheep pox virus, strain VNIIZZH	Russia/ FSBE VNIIZZH	
Sheep pox virus, field isolate	Russia (Yaroslavl Region)/–	
Goat pox virus, strain Primorskiy 2003	Russia / FSBE VNIIZZH	
Goat pox virus, strain VNIIZZH 2003	Russia / FSBE VNIIZZH	
Goat plague (PPR) virus plagues, strain VNIIZZH	Russia / FSBE VNIIZZH	
Vesicular stomatitis virus, strain VNIIZZH	Russia / FSBE VNIIZZH	
Sheep ecthyma virus, field isolate	Russia /–	
Cowpox viruss, strain VNIIZZH	Russia / FSBE VNIIZZH	

Note. FSBE VNIIZZH — Federal Center of Animal Health; dashes mean that sample was not included into the collection.

Repeatability was determined by standard deviation (±SD) for each series of dilutions, with the use of Ct values. SD for five 10-fold dilutions varied from 0.11 to 0.33. Determination coefficient r^2 is 0.9905.



Linearity of real-time PCR estimates at 10-fold DNA dilutions (reference strain NDV LC/Dagestan 2015 of lumpy skin disease virus; linear regression is plotted with Statistica 10 software, StatSoft, Inc., USA).

qPCR assay upon experimental infection of animals. Prior to virus inoculation and on days 4 and 14 post infection, we collected nasal washes from all animals using sterile prewetted cotton applicator. Blood samples were collected too.

Obtained results show (Table 2) that all animals prior to inoculation were LSDV negative. On day 4 blood samples of bulls No 1, 2 and 3 were positive in qPCR while in washes LSDV genome was found in bulls No 2, 5 and 6. On day 14, LSDV genome was found in all bulls, except for animal No 3, in washes — in all animals, except for bulls No 1 and No 3. All probes from control animal were negative in qPCR. In cell culture, virus was isolated from animals No 2, 3 and 4.

2. Identification of lumpy skin disease virus genome (LSDV) in experimentally infected bull by real-time PCR method (qPCR) and isolation in lamb testis cell culture

Animal No	qPCR (Ct values)					Virus isolation in cell culture
	before infection	day 4		day 14		
		blood	nasal wash	blood	nasal wash	
1	0/–	25.01	nf	24.07	nf	–
2	0/–	26.11	29.87	26.30	29.00	+
3	0/–	29.50	0	nf	nf	+
4	0/–	nf	nf	29.09	29.40	+
5	0/–	nf	29.21	21.00	25.54	–
6	0/–	nf	29.44	19.30	29.22	–
Control	0/–	nf	nf	nf	nf	–

N o t e. «–»/«+» mean absence/presence of virus at isolation in cell culture, nf — genome not found.

When compared two methods, i.e. the qPCR that we developed and PCR recommended by IEB [15], we failed to detect viral DNA by classical PCR if Ct exceeded 26.4 (data not shown).

Main purpose of our research is development of real-time PCR effective for practical identification of LSDV field isolate DNA in situation when live homological vaccines against infectious nodular dermatitis in cattle are widely used. Developed primers and probe were tested for specificity by BLAST and experimentally with DNA of all members of capripoxviruses of different origin deposited to strain collection of the Federal Center for Animal Health Control (see Table 1), as well as related sheep and goat capripoxviruses. Positive results obtained in all the cases indicate high analytical specificity of developed test-system. Importantly, that this test system allows identification of only field LSDV isolates with negatives for vaccine virus and other related capripoxviruses (see Table 1).

Analysis of scholarly publications shows that qPCR for LSDV field isolate identification described herein is of interest as a routine diagnostic tool. In Russia, papers on the topic are few and those dealing with diagnostic tests are practically absent. Validation of commercial test systems known at the Russian market are also not reported that makes objective comparison reasonable.

Recently, we know the only Russian report [22] on detection and differentiation of capripoxviruses by classic PCR method. Multiplex PCR described

therein has a number of advantages. However it is qPCR that avoids the risk of cross contamination at identification of LSDV genome due to lowering probability of false positives and shortening analysis time since amplification products are not studied in agarose gel. Also, qPCR method is more sensitive and allows quantitation. Assessment of viral particle copy number at experimental infection is outside the scope of this research and will be subject of our further study of LSDV biological properties.

We have not found any available information on experimental inoculation of sensitive animals with LSDV in Russian academic periodicals. So this paper is the first such report. However, data we obtained are insufficient to make statistically reliable conclusions because of small sample.

Proposed qPCR method had shown higher sensitivity compared to the classical method [18] as the latter did not identify virus DNA at threshold values of more than 26.4. Nevertheless, virus isolation resulted in three positives, of which two positives were in animals LSDV negative in classical PCR [18]. Interestingly, at the end of experiment virus commenced to appears in nasal washings [24, 25]. In identification of LSDV genome by proposed qPCR method, Ct did not practically differ in blood samples and nasal washings, whereas M. Sevik et al. [24] noted lesser Ct values for nasal washing specimens than for blood specimens. Possibly, the reason is that in our study the bulls were infected experimentally, whereas M. Sevik et al. [24] reported on naturally infected animals.

The qPCR test system had already been successfully validated on field specimens from cattle during LSD outbreaks in 2015-2016. These data will be a scope of our next report.

Therefore, we suggest rapid, reliable, sensitive, and specific real-time PCR method to identify genetic material of LSDV (lumpy skin disease virus) field isolates in cattle. This method can be recommended for diagnostic laboratories to control nodular dermatitis virus.

REFERENCES

1. 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. *Transbound. Emerg. Dis.*, 2017, 64: 729-745 (doi: 10.1111/tbed.12444).
2. Beard P.M. Lumpy skin disease: a direct threat to Europe. *Vet. Rec.*, 2016, 28: 557-558 (doi: 10.1136/vr.i2800).
3. Şevik M., Doğan M. Epidemiological and molecular studies on lumpy skin disease outbreaks in Turkey during 2014-2015. *Transbound. Emerg. Dis.*, 2016, 64(4): 1268-1279 (doi: 10.1111/tbed.12501).
4. 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. *Transbound. Emerg. Dis.*, 2015, 62(5): 549-554 (doi: 10.1111/tbed.12177).
5. Mishchenko A.V., Karaulov A.K. Mishchenko V.A. *Veterinariya*, 2016, 4: 3-6 (in Russ.).
6. 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. *Transbound. Emerg. Dis.*, 2016, 63(3): 260-265 (doi: 10.1111/tbed.12497).
7. 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. *Transbound. Emerg. Dis.*, 2017, 00: 1-5 (doi: 10.1111/tbed.12624).
8. Tulman E.R., Afonso C.L., Lu Z., Zsak L., Kutish G.F., Rock D.L. Genome of lumpy skin disease virus. *J. Virol.*, 2001, 75(15): 7122-7130 (doi: 10.1128/JVI.75.15.7122-7130.2001).
9. Chihota C.M., Rennie L.F., Kitching R.P., Mellor P.S. Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (*Diptera*: *Culicidae*). *Epidemiol. Infect.*, 2001, 126: 317-321 (doi: 10.1017/S0950268801005179).
10. Irons P.C., Tuppurainen E.S., Venter E.H. Excretion of lumpy skin disease virus in bull semen. *Theriogenology*, 2005, 63: 1290-1297 (doi: 10.1016/j.theriogenology.2004.06.013).
11. Annandale C.H., Holm D.E., Ebersohn K., Venter E. H. Seminal transmission of lumpy skin disease virus in heifers. *Transbound. Emerg. Dis.*, 2013, 61: 443-448 (doi: 10.1111/tbed.12045).

12. Tuppurainen E.S., Lubinga J.C., Stoltsz W.H., Troskie M., Carpenter S.T., Coetzer J.A., Venter E.H., Oura C.A. Evidence of vertical transmission of lumpy skin disease virus in *Rhipicephalus decoloratus* ticks. *Ticks Tick Borne Dis.*, 2013, 4: 329-333 (doi: 10.1016/j.ttbdis.2013.01.006).
13. Lubinga J.C., Tuppurainen E.S., Mahlare R., Coetzer J.A., Stoltsz W.H., Venter E.H. Evidence of transstadial and mechanical transmission of lumpy skin disease virus *by Amblyomma hebraeum* ticks. *Transbound. Emerg. Dis.*, 2013, 62: 174-182 (doi: 10.1111/tbed.12102).
14. Makarov B.V., Grubyi V.A., Gruzdev K.N., Sukharev O.I. *Spisok MEB i transgranichnye infektsii zhivotnykh: monografiya* [OIE List and transboundary animal infections: monograph]. Moscow, 2012: 76-79 (in Russ.).
15. Beard P., Lubisi B.A. *Manual of diagnostic tests and vaccines for terrestrial animals 2017. Chapter 2.4.13. Lumpy skin disease.* Available http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.13_LSD.pdf. Accessed December 21, 2017.
16. Abera Z., Degefu H., Gari G., Ayana Z. Review on epidemiology and economic importance of lumpy skin disease. *International Journal of Basic and Applied Virology*, 2015, 4(1): 8-21 (doi: 10.5829/idosi.ijbav.2015.4.1.9117).
17. Mishchenko A.V., Mishchenko V.A., Kononov A.V., Shevkoplyas V.N., Dzhailidi G.A., Dresvyannikova S.G., Chernykh O.Yu. *Veterinariya Kubani*, 2015, 5: 3-6 (in Russ.).
18. Ireland D.C., Binepal Y.S. Improved detection of capripoxvirus in biopsy samples by PCR. *J. Virol. Methods*, 1998, 74: 1-7 (doi: 10.1016/S0166-0934(98)00035-4).
19. Bowden T.R., Babiuk S.L., Parkyn G.R., Copps J.S., Boyle D.B. Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats. *Virology*, 2008, 371(2): 380-393 (doi: 10.1016/j.virol.2007.10.002).
20. Lamien C.E., Lelenta M., Goger W., Silber R., Tuppurainen E., Matijevic M., Luckins A.G., Diallo A. Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses. *J. Virol. Methods*, 2011, 171: 134-140 (doi: 10.1016/j.jviromet.2010.10.014).
21. Kononova S.V., Shumilova I.N., B'yadovskaya O.P. *Metodicheskie rekomendatsii po vydeleniyu virusa nodulyarnogo dermatita krupnogo rogatogo skota v kul'ture kletok: metodicheskii material* [Guidelines on the isolation of the nodular dermatitis virus in cattle cell culture]. Moscow, 2017: 5-17 (in Russ.).
22. Orlova E.S., Shcherbakov A.V., Diev V.I., Zakharov V.M. *Molekulyarnaya biologiya*, 2006, 40(1): 158-164 (in Russ.).
23. Menasherow S., Rubinstein-Giuni M., Kovtunenko A., Eyngor Y., Fridgut O., Rotenberg D., Khinich Y., Stram Y. Development of an assay to differentiate between virulent and vaccine strains of lumpy skin disease virus (LSDV). *J. Virol. Methods*, 2014, 199: 95-101 (doi: 10.1016/j.jviromet.2013.12.013).
24. Şevik M., Avci O., Doğan M., İnce Ö.B. Serum biochemistry of lumpy skin disease virus-infected cattle. *Biomed. Res. Int.*, 2016, 2016: 6257984 (doi: 10.1155/2016/6257984).
25. Balinsky C.A., Delhon G., Smoliga G., Prarat M., French R.A., Geary S.J., Rock D.L., Rodriguez L.L. Rapid preclinical detection of sheep pox virus by a real-time PCR assay. *J. Clin. Microbiol.*, 2008, 46: 438-442 (doi: 10.1128/JCM.01953-07).