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LUMPY SKIN DISEASES VIRUS ISOLATED IN 2015 IN RUSSIA FROM CATTLE IS PATHOGENIC FOR SHEEP AT EXPERIMENTAL INFECTION

T.R. USADOV, Yu.P. MORGUNOV, S.P. ZHIVODEROV, V.I. BALYSHEVA,
E.Yu. PIVOVA, A.Yu. KOLTSOV, D.V. YANZHIEVA, M.M. SUKHER,
A.V. LUNITSYN, N.I. SALNIKOV

Federal Research Center for Virology and Microbiology, Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail usadov.tr@mail.ru, morgunovvv@mail.ru, zhivoderov-serg@mail.ru, vbalysheva@vniivvm.ru, epivova@vniivvm.ru, akoltsov@vniivvm.ru, suhermail@mail.ru, darima.yanzhieva.90@mail.ru, lunicyn@mail.ru, nikolai.salnikov2010@yandex.ru (✉ corresponding author)

ORCID:

Usadov T.R. orcid.org/0000-0003-3102-1931
Morgunov Yu.P. orcid.org/0000-0003-4980-8302
Zhivoderov S.P. orcid.org/0000-0002-4919-3080
Balysheva V.I. orcid.org/0000-0003-0687-2734
Pivova E.Yu. orcid.org/0000-0003-4831-0852

Koltsov A.Yu. orcid.org/0000-0003-3294-6602
Yanzhieva D.V. orcid.org/0000-0001-7390-3874
Sukher M.M. orcid.org/0000-0002-1335-310X
Lunitsyn A.V. orcid.org/0000-0002-5043-446X
Salnikov N.I. orcid.org/0000-0002-0481-3872

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Abstract

Lumpy skin disease is an economically significant transmissible infectious disease with mortality rate from 4 to 95 %. Purebred animals are more susceptible to this infection, most seriously the disease occurs in young animals, not enough well-fed individuals, lactating cows. In Russia, the disease is registered since 2015. To eradicate this infection, it is necessary to study all components of the epizootic process. Currently, the studies on the pathogenicity of lumpy skin disease virus for sheep and goats and wild ruminants are insufficient to assess the role of such animals in the transmission of the virus. We estimated for the first time that lumpy skin disease virus isolated from cattle in the Republic of North Ossetia-Alania in 2015 is pathogenic for sheep. The causative agent was identified by sequencing the GPCR gene. In the experiment with 1.5-month-old lambs ($n = 4$), intravenous and intradermal administration of the suspension of the biopsy samples from sick cows caused the formation of nodules on the skin at the sites of virus inoculation. Nodules were benign in nature, after two weeks it formed the scabs and separated from the skin. On the skin in places of formation nodules there were small scars. The genome of lumpy skin disease virus was detected by real-time PCR in blood samples collected from 9 to 17 days post infection, and in the oral swabs collected from 17 to 27 days post infection. The duration of viremia in lambs ranged from 3 to 8 days. The presence of infectious virus was confirmed by isolation of virus on continuous cell culture of sheep kidney. The clinical signs of the disease corresponded to 2 points calculated in accordance with clinical scoring system within the range from 0 («no visible response») to 10 points («severe generalization, requiring slaughter»). After euthanasia the samples of the liver, popliteal lymph node, lungs and spleen were collected to test for the presence of the viral genome. The genome of the virus was detected only in the lung and lymph nodes. So, our results confirm literature data about pathogenicity of lumpy skin disease virus for sheep. Potentially, sheep can be involved in the epizootic process of lumpy skin disease as source of virus transmitted by blood feeding arthropods.

Keywords: lumpy skin disease, lumpy skin disease virus, sheep, experimental infection, viremia, PCR, genome, cell culture

Nodular dermatitis in cattle (infectious nodular dermatitis, malignant nodular dermatitis, lumpy skin disease, Dermatitits nodularis bovum) is a transmissible viral disease in cattle manifested by numerous nodules (nods) in skin, epithelium of mouth and nasal musoca, esophagus, trachea, and bronchus. Disease is characteristic of cattle, African buffalos (*Syncerus caffer*), springbucks (*Antidorcas marsupialis*), and gemsbucks (*Oryx leucoryx*, *O. gazelle*) [1, 2].

Nodular dermatitis causative agent is DNA-containing virus of *Poxviridae* family (*Capripoxvirus* genus) [3, 4]. Source of infection is ill animals, as well

as asymptomatic carriers [5]. Nodular dermatitis virus is mainly transmitted by inoculation. C.M. Chihota [6] had shown that nodular dermatitis virus may be transmitted by mosquitoes *Aedes aegypti* within 2-6 days after the agent enters the insect organism with blood of the infected animal. Ability to mechanically transmit virus to sensitive animals was also established in ixodid ticks of *Rhipicephalus*, *Amblyomma*, and *Hyalomma* genera from South Africa [7, 8]. There are data on a relationship between the nodular dermatitis outbreaks and activity of *Stomoxys calcitrans* fly [9]. Contact mode of transmission of nodular dermatitis virus is not proved. As per V.M. Carn and R.P. Kitching [10], intact animals located within 1 month in one box with infected ones remained clinically healthy during the entire surveillance period.

Protection measures at first drifts of the disease to healthy regions are stamping out and radical quarantine [11].

Along with skin nodes, nodular dermatitis virus causes fever, lymphadenopathy, swellings of subcutaneous tissue and organs, conjunctivitis, reduction of milk yields, sexual malfunctions and sterility in cattle. Purebred animals are more sensitive to the infection, most hardly the disease flows in young animals, animals deficient in weight, and lactating cows. Mortality varies from 4 to 95 % [12-14].

Incubation period at experimental infection of the cattle by nodular dermatitis virus is 2-5 days. Viremia is registered in infected animals during 1-2 weeks. Virus dissemination results in injury of mucosa, udder, salivary glands, testis, and other organs. Within 6-9 days following the experimental infection of cattle, nodes of nearly 1 cm in diameter appear in virus inoculation points, and in 12-14 days body temperature goes high up to 40.5 °C. Generalized form of disease is characterized by appearance of nodules on the skin in all parts of the animal body. Formation of nodules is accompanied by inflammation of subcutaneous tissue, and sometimes muscular tissue. Secondary bacterial infection leads to inflammation of lymphatic nodes and skin sores [15, 16].

At pathoanatomical examination, nodules are found in mucosa of intestines, trachea, ventricle, and in udder tissues of lactating cows [17]. Appearance of nodules is accompanied by skin swelling. In generalized form, nodules appear in mucosa of mouth and nasal cavities, vulva and preputial skin with further necrosis and purulent inflammation. In respiratory tract, pathogen causes heavy swelling with possible death from asphyxia or lung swell [18-20].

First cases of nodular dermatitis in Russia were registered in 2015 in cattle in settlements of Tlyaratinsk Region of the Republic of Dagestan bordering with Azerbaijan and Georgia. Later, the diseases occurred in cattle of Naursk Region of Chechen Republic and Kirov Region of the Republic of North Ossetia-Alania [21-23]. According to data of the Information Analytic Department of the Russian Service for Veterinary and Phytosanitary Surveillance, outbreaks of nodular dermatitis in cattle were registered in 2016-2017 in the Russian Federation in the Republic of Dagestan, Bashkortostan, Volgograd, Saratov, Samara, and Orenburg Regions [24].

Effective protection against nodular dermatitis requires deep studies of all components of epizootic process. However, the pathogenicity of the lumpy skin disease virus (LSDV) in small domestic and wild ruminants is still poorly understood, which hampers comprehension of their role in LSDV transmission. There are only few publications on the issue. M.S Kukushkina. et al. [25] report on low pathogenicity of LSDV strain 95 manifested as nodules in the injection point and fever of experimentally infected adult sheep. LSDV pathogenicity for wild impalas (*Aepyceros melampus*) and giraffe (*Giraffe camelopardalis*) is experimentally shown (animals of both sexes died 6-15 day post infection) [26].

This paper reports on the first estimate of pathogenicity of cattle LSDV field isolate (Republic of North Ossetia-Alania, Russia, 2015) for sheep. Our findings show 2-point severity score in infected animals as per a 10-point scale of V.M. Carn et al. [10]. Virus was detected in blood by PCR analysis and direct isolation in sheep kidneys cell culture (PO-VNIIVViM), as well as in lungs and spleen. These data indicate that sheep, despite weak pathogenicity of LSDV at infection, may be involved in LSDV transmission.

Purpose of this study was to estimate pathogenicity of cattle isolate of LSDV for experimentally infected sheep.

Techniques. Bioplates of skin nodes from cows with nodular dermatitis (Republic of North Ossetia-Alania) were collected by employees of the Republican Veterinary Service in 2015 and kept in thermal container (+4...+10 °C). For analysis, bioplates were grinded in porcelain jar with phosphate buffer. After centrifugation, the prepared 10 % suspensions were used to infect experimental animals and to extract viral DNA for identification.

DNA for sequencing and PCR analysis was extracted with RIBO-sorb kit (ILS CJSC, Moscow). After experimental infection, viral genome DNA was detected as per T.R. Bowden et al. [27] method with oligonucleotide primers CaPV 074 F₁ (5'-AAAACGGTATATGGAATAGAGTTGGAA-3'), CaPV 074 R₁ (5'-AAATGAAACCAATGGATGGGATA-3'') and hybridization probe CaPV-074P₁ (5'-FAM-TGGCTCATAGATTTTCCT-MGB-NFQ-3'). PCR mixture contained 10 pM of each primer, 3 pM fluorescent probe (Sintol CJSC, Russia), 2,5 µl 10× DNA buffer, 10 mM dNTPs and 1.5 IU recombinant Taq DNA polymerase (Thermo Fisher Scientific, USA). Real-time PCR was protocol was as follows: initial denaturation at 95 °C for 10 minutes; amplification at 95 °C for 15 sec, 60 °C for 1 min (45 cycles) (Rotor Gene 6000 amplification detection system, Corbette Research, Australia).

GPCR gene fragment PCR amplification (Gradient Palm Cycler, Corbett Research, Australia) and Sanger sequencing (3130xl Genetic Analyzer, Applied Biosystems, USA) were performed. Amplification was performed as per C. Le Goff et al. [28] with oligonucleotide primers (5'-TTAAGTAAAGCATAACT-CCAACAAAATG-3' and 5'-TTTTTTTATTTTTTATCCAATGCTAATACT-3') and Encyclo Plus PCR kit (Eurogen CJSC, Russia) according to following protocol: initial denaturation at 94 °C for 3 minutes; 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec (35 cycles).

Nucleotide sequences were analyzed with MEGA 7.0 software (<https://www.mega-software.net/>) and Neighbor-Joining tree clustering.

Infection activity of 10 % bioplate suspension was determined in 2 clinically healthy Kalmyk calves aged 6 months (210-220 kg weight) by subcutaneous injection of 0.25 cm³ aliquots of 10⁻¹-10⁻⁵ dilutions in four points along lines vertical to spinal column at 5-6 cm distance between dilutions. Skin injuries in 14-20 days post injection were and lumps were inspected visually.

To assess pathogenicity of LSDV for sheep, four Romanov lambs aged 1.5 months (7-8 kg weight) were inoculated with virus containing 10 % bioplate suspension (1.0 cm³ in jugular vein and 0.25 cm³ subcutaneously in each of four points in axilla). Prior to infecting, the control lamb was placed in separate housing and inoculated with physiological solution in the same mode. Animals were clinically inspected daily. Each 3 days, blood samples, mouth and nasal washes were collected for viral genome analysis.

Positive blood samples from infected lambs were used for LSDV identification in sheep kidney cell culture (PO VNIIBBiM) as per protocol of World Organization for Animal Health (International Epizootic Bureau, Paris, OIE — IEB) [29-31]. LSDV infection activity in specimens was determined by titration in

sheep kidney cell culture (48-well plates, Corning-Costar, USA) with Eagle MEM (Biolot LLC, Russia). Titers were calculated by Kerber's method.

After blood-free euthanasia (Adilin-super medicine, Federal Center of Toxicology, Radiation, and Biological Safety, Kazan), samples of hepatic tissue, popliteal lymph nodes, lungs, and spleen were collected to detect viral genome.

Tests on animals were carried out according to approval of Federal Agency for Scientific Organizations of Russia (No 33-11-0132/16.06.2016) under supervision of Bioethics Commission of Federal Research Center of Virology and Microbiology.

At calculation of virus titers, mean (M) and standard errors of the mean (\pm SEM) were determined.

Results. Pathogen which caused clinical signs of nodular dermatitis in cows in 2015 in the Republic of North Ossetia-Alania was identified by GPCR (G-protein-coupled chemokine receptor) gene sequencing. *GPCR* is a host-range gene suitable for discrimination of capripoxviruses [28]. Analysis of the sequence we obtained and deposited in GenBank database (accession No KY595106) with other LSDV full-size *GPCR* gene sequences from GenBank identifies this Russian isolate as nodular dermatitis virus (Fig. 1).

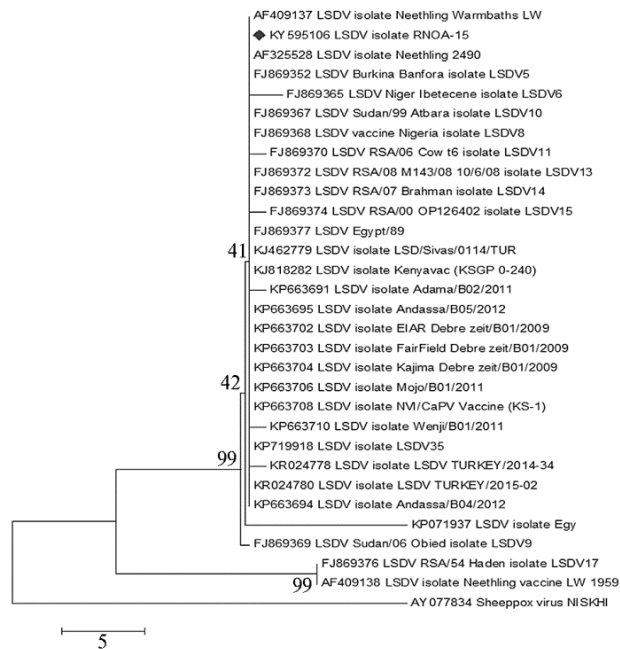


Fig. 1. *GPCR* gene-based phylogenetic tree of LSDV isolates. *GPCR* gene sequence of sheep pox virus strain NISHI is external comparison group. Rhomb labels the studied isolate. Tree branches are scaled in number of nucleotide replacements for sequence.

In preliminary test, the bioptate suspension diluted up to 10^{-4} causes local skin nodes in calves on day 20. Hence, the LSDV titer is $10^{5.1}$ ID₅₀/cm³ (given the used volume of 0.25 cm³) and in experimental infection, total (intravenous and subcutaneous) infectious dose is $10^{5.4}$ ID₅₀. Nodules in the points of virus entering (Fig. 2) occurred in lambs on day 10 to day 13 post inoculation. Skin

indurations were constantly growing in diameter from 2 to 5 cm during 7 days. The injured skin healed and scabs appeared since week 2 after infection with full healing on week 3. Temperature reaction in all infected lambs during 25 day surveillance was within the norm (38.8-40.5 °C).

In this experiment we used 10-point scale proposed by V.M. Carn et al. [10], with 0 for absence of visual reactions, 1 for transitive local response, 2 for local moderate response (nodules of less than 5 cm in diameter, no lymphadenopatia), 3 for moderate local response with nodules of less than 5 cm in diameter and moderate lymphdenopatia, and 10 for generalized infection with numerous secondary nodules of 0.5-5.0 cm in diameter, swelling, hyperemia, severe lymphadenopatia, conjunctivitis, rhinitis, apathy, loss of appetite and deaths, which requires slaughter. As per the scale, clinical severity in lambs infected with LSDV from cattle scores 2 points that means low pathogenicity for sheep. Skin of a control

lamb had no visual changes. Body temperature was 38.5-39.0 °C during the entire surveillance period.



Fig. 2. Skin nodules in lamb at inoculation with LSDV containing biobtate collected in 2015 in the Republic of North Ossetia-Alania (day 13 post injection).

On day 15, the lamb No 2 was subjected to euthanasia for autopsy of internal organs. No pathologic changes typical for bovine nodular dermatitis were visually found. Of liver, popliteal lymph node, lungs and spleen specimens, only in lungs and lymph nodes LSDV genome was detected by qPCR method (Ct values of 36.48 and 32.94, respectively).

qPCR test revealed LSDV genome in blood of experimentally infected lambs on days 9-17 and in mouth washes on days 20-27 post inoculation (Table 1), unlike the control lamb.

1. qPCR identification of cattle LSDV genome (Ct) in experimentally infected lambs

Days post experimental inoculation	Lamb No 1		Lamb No 2		Lamb No 1		Lamb No 4	
	blood	washes	blood	washes	blood	washes	blood	washes
3	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	37.45	-
13	-	-	-	-	-	-	36.23	-
15	35.99	-	-	-	-	-	-	-
17	38.38	-	-	-	39.08	-	37.23	-
20	-	36.65	-	-	-	-	-	35.71
23	-	35.51	-	-	-	-	-	36.43
27	-	38.34	-	-	-	-	-	33.19
29	-	-	-	-	-	-	-	-

Note. Virus containing biopate for inoculation was collected in 2015 in the Republic of North Ossetia-Alania. Samples with Ct ≤ 40 are positive; «-» means negative samples with no Ct. Lamb No 2 was subjected to euthanasia on day 15 for pathoanatomic and PCR study.

2. LSDV genome isolation from blood of experimentally infected lambs in sheep kidneys cell culture PO VNIIVViM

Days post experimental inoculation	Virus titer, lg TCD ₅₀ /cm ³			
	control lamb	lamb No 1	lamb No 3	lamb No 4
3	Not tested	Not tested	Not tested	Not tested
6	Not tested	Not tested	Not tested	Not tested
9	-	-	-	-
13	-	-	-	-
15	-	1,0±0,12 (+)	-	1,5±0,14 (+)
17	-	1,5±0,24 (+)	1,6±0,15 (+)	1,7±0,18 (+)
20	-	-	-	-
23	-	-	-	-
27	Not tested	Not tested	Not tested	Not tested
29	Not tested	Not tested	Not tested	Not tested

Note. «-» — virus not found (no cytopathic effect for 5 consecutive passages); «+» — presence of specific cytopathic action of virus on the 2nd passage.

Isolation from blood of the experimentally infected lambs in sheep cell culture confirms viremia after day 13 till day 23 post inoculation. Note that the LSDV-specific cytopathic action occurs only in passage 2 in 5-7 days after culture inoculation at viral titer of 1.5-1.7 lg TCD₅₀/cm³ (Table 2).

Thus, our studies confirm the available data on pathogenicity of nodular dermatitis virus for sheep during experimental infection [25].

Therefore, field LSDV isolate from cattle (the Republic of North Ossetia-Alania, 2015) injected to sheep subcutaneously and intravenously causes skin nod-

ules only the points of injections. Observed clinical signs correspond to 2 points score of 10-point scale of severity. qPCR test detects LSDV genome in blood, mouth washes, lungs and popliteal lymph nodes of experimentally infected lambs. LSDV isolation from qPCR positive blood samples in sheep cell culture also confirms presence of the pathogen. These facts bring to assumption that sheep can serve a source of nodular dermatitis virus. More studies, including those on the carrier role, are required to ultimately understand whether LSDV transmission by sheep may naturally occur.

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