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PERMISSIVITY OF VARIOUS CELL CULTURES TO LUMPY SKIN DISEASE VIRUS

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Abstract

Lumpy skin disease (LSD) is a transmissible and highly contagious transboundary emergent bovine viral disease that has become especially important for the Russian Federation since 2015 when it entered the Republic of Dagestan from Azerbaijan. In 2016, the infection was found in the Krasnodar Territory and later on in six more regions of the Russian Federation. The infection causes up to 50 % drops in milk productivity, body weight loss, abortions or stillbirths, skin damage, and reproductive disorders in affected livestock up to including a complete loss of bovine fertility and animal deaths due to secondary infections. LSD is caused by a DNA virus of family *Poxviridae*, genus *Capripoxvirus*. The virus isolation, identification and vaccine or diagnostic preparation construction largely depends on the adequate culture system used. This research was aimed at characterization of the cultural properties of an LSD virus isolate detected in internal organ (lung, spleen and lymph nodes) or affected subcutaneous tissue samples from Volgograd region of Russia. In order to isolate the virus, a goatling testicle primary culture (GT), a calf kidney (MDBK) and a rabbit kidney (RK-13/2-03) continuous cell lines were used. In passage 3, the virus titer obtained in cells MDBK and RK-13/2-03 was 4.67 to 5.00 lg $TCID_{50}/cm^3$. Using PCR analysis, a LSD virus genome was detected in the virus-containing culture medium. The obtained LSD virus strain was deposited to the State Collection of Microorganisms of the Federal Research Center for Virology and Microbiology, # 3161. Also, the permissivity of some other cell lines including elk embryo skin (KEL/07), African green monkey kidney (CV-1) and VERO cells, a hybrid line of porcine embryo kidney cells (SPEV TK^-) × porcine spleen splenocytes (A_4C_2/9k), and sheep kidney (ShK), rabbit kidney (RK-13/2-03) and calf kidney (Taurus-1) cells to this LSD virus strain were determined. We found that some continuous cell lines of both homologous (MDBK, Taurus-1, KEL/07, ShK) and heterologous (RK-13/2-03, VERO, CV-1, A_4C_2/9k, SPEV) origin were sensitive to the LSD virus. This work has revealed for the first time ever that LSD virus can proliferate in cells of wildlife species like elk. Also, permissivity of some heterologous continuous cells, RK-13/2-03 and A4C2/9k, to LSD virus was revealed for the first time. The virus culture period until 95 to 100 % CPE depended on the cell substrate selected and the multiplicity of infection. Thus, for MDBK or VERO cells it was 48 hours, and for Taurus-1, SkK, RK-13/2-03 or CV-1 the maximal destructive alterations in the cell monolayers were observed within 48 to 96 hours post infection. With an optimal multiplicity of infection of 0.001-0.00001 $TCID_{50}$ per cell and 2-5 % cattle serum in the maintenance medium the LSDV titers were 6.0 to 6.8 lg $TCID_{50}/cm^3$in the ShK and VERO cells, and 5.8 to 6.6 lg $TCID_{50}/cm^3$ for RK-13/2-03.

Keywords: lumpy skin disease virus, continuous cell lines, embryonic elk skin cell culture, CV-1, VERO, MDBK, Taurus-1, cytopathic effects

Lumpy skin disease (LSD) is an infectious pathology of cattle which is still a cause for significant economic damage to livestock in many countries. In the Russian Federation, the disease was entered the Republic of Dagestan from Azerbaijan in 2015 [1, 2]. At the same time, disease outbreaks were registered in

the Chechen Republic and the Republic of North Ossetia-Alania. In 2016, LSD was found in the Krasnodar Krai, then in six other Russian regions [3, 4]. Lumpy skin disease (infectious nodular dermatitis, pearl disease, vesicular exanthem) is a transmissible, highly contagious transboundary emergent viral disease characterized by fever, skin nodules, the nodular lesions on mucous membranes and viscera, depletion, lymphadenopathy and cutaneous dropsy. LSD is caused by a DNA virus, LSDV, of the *Capripoxvirus* genus (*Chordopoxvirinae* subfamily, *Poxviridae* family) [5-7]. The disease often reduces milk production up to 50 % and leads to body weight loss, abortions and stillbirths, skin damage, reproductive disorders in affected animals, down to total loss of maly fertility, and animal death from secondary infections [8-10]. Immunization is the only effective method to control LSDV infection in endemic regions [11, 12]. A virus vaccine from homologous attenuated Neethling strain or vaccines from heterologous live attenuated strains of sheep or goat pox viruses are used for specific prophylaxis of nodular dermatitis [13-15].

Continuous cell lines are promising for the LSDV culture. They provide production of large amount of uniform virus-containing material which is used in biological, molecular and genetic study of virus. Also continuous cell lines are effective laboratory models for studying how LSDV evolves and for development of disease diagnostics and specific prophylaxis [16, 17]. Success in developing vaccines and diagnostic methods depends largely on the proper choice of the culture system. Therefore, initially it is necessary to determine the sensitivity of cell cultures and their permissivity to a particular virus. In choosing cell systems, we relied on the species culture identity (*Bos taurus, Ovis aries, Capra hircus*), specific cell and tissue tropism of LSDV to dermis, as well as on using homologous cell cultures of lamb testicles LT, fetal bovine testis FBT, calf kidney cells MDBK, etc. [19-21] and heterologous cell lines [9] for these purposes.

A.V. Kononov et al. [22] identified that in cells of homologous origin, i.e. in the subculture of lamb testes (TL) and in continuous cell culture of goatling gonads (GT-04), LSDV isolated from the biomaterial, which was collected in the Dagestan Republic in 2015, was accumulated in titers of 4.5-5.5 lg $TCID_{50}/cm^3$. However, in some cases, there is a need for viruses produced in a heterologous cell system, particularly when the viral antigen is accumulated to produce specific sera. The use of heterologous cell culture systems make it possible to exclude the appearance of antibodies to homologous tissue antigens, which complicates using sera in diagnostic studies or requires additional procedures for antigen purification.

This research first identified that the LSDV can proliferate in cells of wild animals (i.e. of elk) and that heterologous continuous cell lines RK-13/2-03 and A4C2/9k are effective for LSDV culture.

Our aims were the study of cultural properties of a nodular dermatitis virus isolate and optimization of cell cultures the most perspective for its production.

Techniques. Organs (liver, lungs, spleen, lymph nodes) and affected subcutaneous tissue samples were collected from the forcibly killed bulls of the Kalmyk breed (farms of the Volgograd Region, 2016) with typical manifestations of clinical ND symptoms. A 10 % tissue suspensions were prepared in Eagle's medium (MEM, Sigma, USA, HyClone, USA) supplemented with antibiotics (penicillin and streptomycin of 200-1000 IU/ml each, and nystatin of 20 IU/ml). After clarification by centrifugation at 2000 rpm, the suspension was introduced into culture vessels with a pre-formed cell monolayer. At this stage, we used primary goatling testicle cells (TG), as well as continuous cell lines of the bovine kidney (MDBK) and the rabbit kidney (RK-13/2-03) from FRCV&M collection of cell cultures [23]. An hour after adsorption, the suspension was removed, supporting 2 % bovine serum was infused and then it was incubated for 5-6 days at 37±0.5 °C. Ves-

sels with cell cultures were frozen and stored at –40±0.5 °C, then the culture medium was thawed out at room temperature. A 1 cm^3 aliquot of culture liquid was placed on a cell monolayer, with the next reseeding prior to the signs of characteristic viral cytopathic effect (CPE) developed. The state of cells monolayer in CPE tests was evaluated by viewing the culture vessels under the inverted microscope Olympus CKX31 (Olympus Co., Japan).

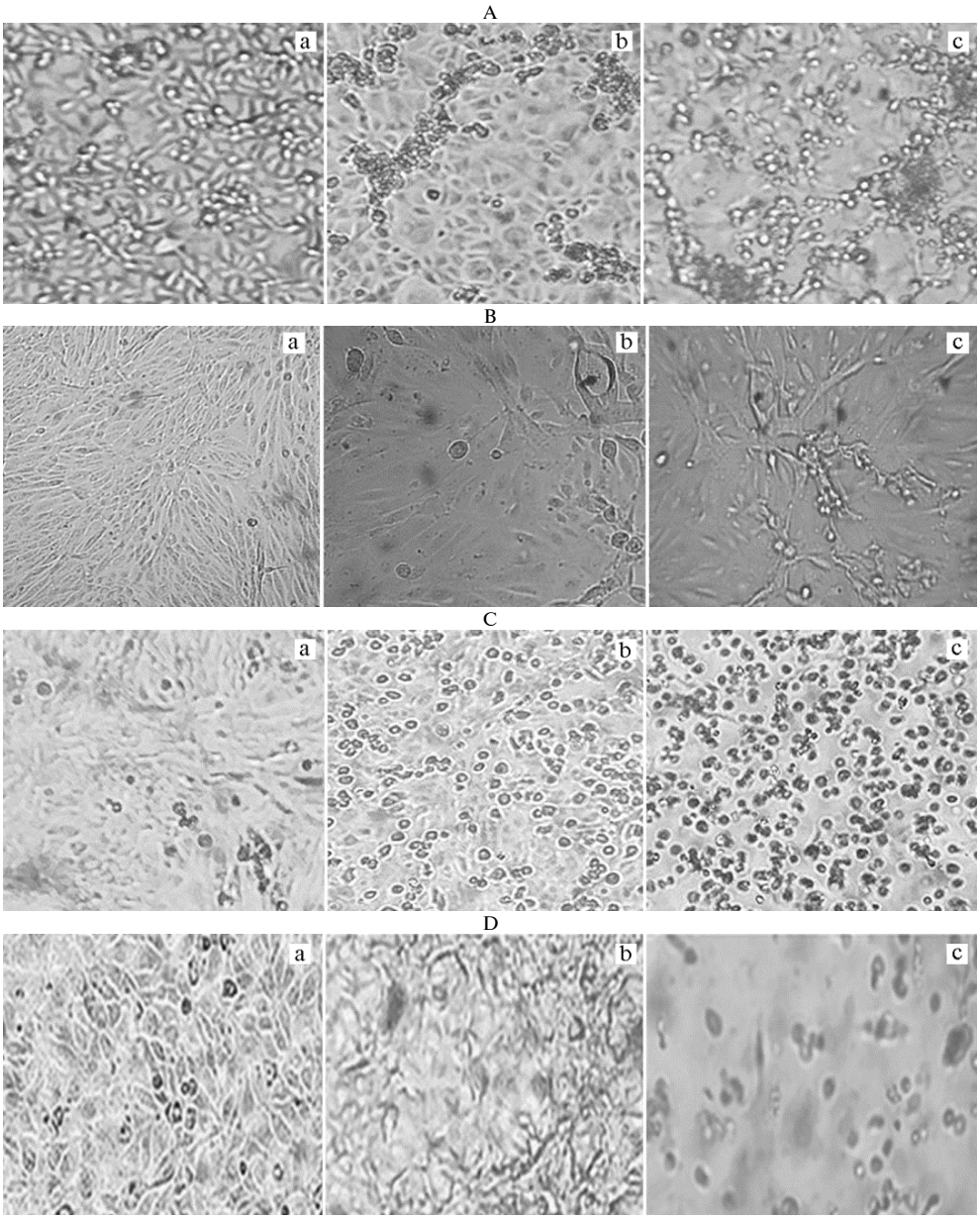
For virus adaptation to homologous and heterologous cell lines, serial passages were used. Cell cultures of elk (*Alces alces*, order *Artiodactyla*) embryo skin (KEL/07), African green monkey kidney (CV-1, VERO), porcine embryo kidney (PEK-66b), hybrid line of porcine embryo kidney cells (РЕКСЫПЭ ТК$^-$) × porcine spleen splenocytes (A_4C_2/9k), sheep kidney (ShK), calf kidney (Taurus-1) were grown in Eagle's medium supplemented with 10 % fetal bovine serum. After formation of a continuous monolayer (24 hours), the growth medium was removed from the vessels and the virus was inoculated at a multiplicity of 0.1-0.00001 $TCID_{50}$ per cell. The virus was allowed for adsorption for an hour at 37.0±0.5 °C. Next, maintenance medium supplemented with 2 % fetal bovine serum was added. The infected cell culture was incubated at 37.0±0.5 °C for 5 days or until 90-100 % cytopathic effect (CPE) developed. Then, the cell culture and the liquid culture were frozen at –50.0 ± 0.5 °C. In the next culture passage, the cells were infected with a thawed virus-containing suspension. Presence of cytopathic changes in the monolayer and a change in the viral titer during the passage indicate permissivity of cells culture to the virus. Viral infectious activity was determined by titration in 1-2-day continuous VERO or ShK cell lines grown in 96-well microplates. Virus titer was estimated by Reed and Muench methods and expressed in lg $TCID_{50}/cm^3$ [24].

Nucleic acids were extracted using RIBO-sorb kit (InterLabService LLC, Russia). Viral genomic DNA was detected by T.R. Bowden et al. [25] with oligonucleotide primers CaPV 074 F1 (5′-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3′), CaPV 074 R1 (5′-AAA TGA AAC CAA TGG ATG GGA TA-3′), and hybridization probe CaPV-074P1 (5′-6FAM-TGG CTC ATA GAT TTC CT-MGB-NFQ-3′). The reaction mixture contained 10 pM of each primer, 3 pM of a fluorescent probe (CJSC Synthol, Russia), 2.5 μl of 10× DNA buffer, 10 mM dNTPs solution mix and 1.5 IU of recombinant Taq DNA polymerase (Thermo Fisher Scientific, USA). Real-time PCR was performed on a thermocycler Rotor Gene 6000 with detecting system (Corbette Research, Australia) as follows: preliminary denaturation at 95 °C for 10 min followed by 45 cycles of amplification (15 s at 95 °C, 1 min at 60 °C) [25].

The data were processed using variational statistics. The table shows the mean (*M*) and standard mean errors (±SEM).

Results. To extract LSDV, the virus-containing material was poured into culture vessels, 3 vessels for each culture, with a pre-formed monolayer of TK, MDBK or RK-13/2-03 cells. First passage did not lead to culture changes, in the second passage, slight changes in cell morphology and their rounding were revealed. In the third passage, the typical cytopathic effect was observed on MDBK and RK-13/2-03 cells (Fig., A) infected initially with a suspension of liver tissue. In this, on day 2 of incubation, in infected culture RK-13/2-03 the cells formed strands, and on day 3 the cells were rounded, while in the control culture such changes were not revealed. The typical changes were in MDBK cell culture. The virus titer in MDBK and RK-13/2-03 cell cultures was 4.67-5.00 lg $TCID_{50}/cm^3$. The isolated strain (deposited to FRCV&M State Collection under accession No. 3161) was further used in the work. While continuous cell lines EES/07 contamination, typical changes were observed (see Fig., B) (virus titer of 4.5-5.5 lg $TCID_{50}/cm^3$). In the TR primary cells culture

during the third passage, the infectious activity of the virus was lower (3.5 lg $TCID_{50}/cm^3$). Homologous and heterologous cell lines MDBK, Taurus-1, ShK, CV-1, VERO, RK-13/2-03, A_4C_2/9k, and SPEV were used to adapt the strain to the continuous cell lines, and to identify sensitive cultures.



Cytopathic effect of bovine lumpy skin disease on continuous cell lines of rabbit kidney RK-13/2-03 (A)**, elk embryo skin KEL/07** (B)**, African green monkey kidney VERO (C), sheep kidney SK** (D)**:** a — control cell culture, b and c — cell culture on day 2 and day 3 (for sheep kidney cells — on day 4) after inoculation (magnification ×150, microscope Olympus CKX31, Olympus Co., Japan).

The character of CPE in different cell cultures was not the same. So, in RK-13/2-03 (see Fig., A), the CPE was similar to that under virus reproduction in the ShK cell culture (see Fig., D). That is, 48 h after inoculation, spindle-shaped cells formed strands, and after 72 h, we observed rounding and detaching of infected cells from the substrate, with lysis and destroying the cell monolayer. In the infected VERO culture (see Fig., C), there was an increased cells rounding, the

formation of inclusions, which are not inherent for normal (uninfected) cells, with further lysis and detaching. The viral infectious activity in these culture systems also varied. The maximum titers were observed in cell cultures Taurus-1 and A_4C_2/9k-7.00, the lg $TCID_{50}/cm^3$, and also VERO and RK-13/2-03-6.67, the lg $TCID_{50}/cm^3$ (Table 1).

1. Propagation of the nodular dermatitis virus in different continuous cell cultures

Cell culture	Passage	Time, h	Titer, lg $TCID_{50}/sm^3$
		Homologous cells cultures	
MDBK	4-6	48	4.67-5.67
Taurus-1	4-6	48-72	6.00-7.00
KEL/07	4-6	72	4.5-5.50
TK	3	144	3.50-4.50
ShK	4	120	4.67
	5-10	72-96	6.0-6.33
	11	48	6.50
		Geterologous cells cultures	
RK-13/2-03	4-7	48-72	5.00-6.67
VERO	4-9	48	5.00-6.67
CV-1	4-11	48-72	5.00-6.67
PEKC	4-6	48-72	4.50-5.50
A_4C_2/9к	4-	48-72	6.00-7.00

Taxonomic attribution of the virus accumulated in cell cultures was confirmed by revealing LSDV genome by real-time PCR. The Ct values were 11.79 for RK-13 (passage 8), 11.91 for Taurus-1 (passage 4), 18.45 and 35.82 for A_4C_2/9K (passage 3) and A_4C_2/9K (passage 3, dilution 10^{-5}), respectively. Samples were considered positive at Ct ≤ 40).

The time required for the development of 95-100 % CPE depended on the cell culture. For MDBK and VERO cell lines, it took 48 hours (see Table 1). For Taurus-1, ShK, RK-13/2-03, and CV-1, the 100 % CPE time varied from 48 to 96 hours.

When determining the optimal virus/cell multiplicity for lines RK-13/2-03, VERO and ShK (Table 2), the growth medium was replaced by the maintenance medium and incubated until the destruction of the cell monolayer completes. With a multiplicity of 0.1-0.01 $TCID_{50}$ per cell, CPE appeared on day 2 of incubation. At 0.001-0.00001 $TCID_{50}$ per cell, the virus titer increased up to 1.50 lg $TCID_{50}/cm^3$, the time for the complete destruction of the monolayer was up to 3-5 days. The infectious titer of virus-containing material was 6.2-6.8 lg $TCID_{50}/cm^3$ when produced by ShK and VERO cells, and 5.8-6.6 lg $TCID_{50}/cm^3$ for RK-13 cells used. To optimize FBS concentration, virus-infected ShK cells were cultured in a maintenance medium without serum and with the addition of 2; 5 and 10 % FBS. The highest virus titer of 6.67 lg $TCID_{50}/cm^3$ was observed at 2-5 % FBS compared to 5.5 lg $TCID_{50}/cm^3$ and 6.0 lg $TCID_{50}/cm^3$ for serum-free medium and 10 % FBS, respectively.

2. Propagation of nodular dermatitis virus (lg $TCID_{50}/cm^3$) in continuous cell cultures depending on multiplicity of infection ($n = 3$, $M \pm m$)

Cell culture	Multiplicity of infection, $TCID_{50}$ per cell					
	0.1	0.01	0.001	0.0001	0.00001	0.000001
ShK	5.3±0.13	6.0±0.19	6.2±0.23	6.7±0.12	6.7±0.23	6.0±0.12
RK-13/2-03	4.7±0.21	4.7±0.00	5.8±0.20	6.0±0.00	6.6±0.18	3.5±0.17
VERO	5.5±0.15	6.0±0.17	6.5±0.26	6.7±0.15	6.8±0.26	6.3±0.17

The development of effective preparations for viral disease prevention and diagnostics depends on the quality of the virus-containing material, which is usually obtained using highly productive cell culture systems and effective methods for culturing virus-infected cells. In the literature, there is information about LSDV propagation in cell cultures of homologous origin, such as kidneys and testes of lambs and calves, as well as in the calves' dermis, with a charac-

teristic cytopathic effect, and in heterologous cell cultures, i.e. rabbit embryonal kidney and skin, VERO. The infectious activity of such cultural virus-containing materials is 4-6 lg $TCID_{50}/cm^3$ [9, 22, 26]. However, these are mostly primary cell cultures. In the present work, we have tested sensitive continuous cell lines which are more technologically suitable. In this, we have shown permissivity of KEL/07 cells of wild elk, a member of *Artiodactyla*, to LSDV. Also, there are reports on experimental infection of Asian buffalo, antelope and giraffe which resulted in clinical symptoms of nodular dermatitis [27, 28]. Given that and also the fact that LSDV is a transmissible virus, one should pay attention to possible LSDV circulation among wild fauna and the formation of enzootic foci in central Russia.

Thus, the continuous cell lines of both homologous and heterologous origin are susceptible to lumpy skin disease virus (LSDV). At optimal MOI of 0.001-0.00001 $TCID_{50}$ per cell and culturing in a maintenance medium with 2-5 % FBS, the virus titers were 6.2-6.8 lg $TCID_{50}/cm^3$ for ShK and VERO continuous cell culture, and 5.8-6,6 lg $TCID_{50}/cm^3$ for RK-13/2-03. As known, cell cultures of heterologous origin not susceptible to viral and prion pathogens of target animals, i.e. cattle and small ruminants, including slow infections, are preferably used in producing vaccine and diagnostic preparations. Our findings indicate that clonal continuous rabbit kidney cell culture RK-13/2-03 seems to be the most prospective for LSDV production.

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