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TEST-SYSTEM

BASED ON INDIRECT "SANDWICH" ELISA TO DETECT THE LUMPY SKIN DISEASE VIRUS ANTIGEN

O. A. Ryabikina[1], V. I. Diev[2], O. P. Byadovskaya[3], A. V. Konstantinov[4], S. K. Starov[5], A. V. Kononov[6], V. Yu. Kulakov[7], O. N. Petrova[8]

[1] Junior Researcher, FGBI "ARRIAH", Vladimir, Russia, *e-mail: ryabikina@arriah.ru*

[2] Doctor of Science (Veterinary Medicine), Professor, FGBI "ARRIAH", Vladimir, Russia, *e-mail: diev@arriah.ru*

[3] Interim Head of the Laboratory, Candidate of Science (Biology), FGBI "ARRIAH", Vladimir, Russia, *e-mail: bjadovskaya@arriah.ru*

[4] Head of the Department, Candidate of Science (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: konstantinov@arriah.ru*

[5] Deputy Director, Candidate of Science (Veterinary Medicine), Leading Reseracher, FGBI "ARRIAH", Vladimir, Russia, *e-mail: starov@arriah.ru*

[6] Head of the Department, Candidate of Science (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: kononov@arriah.ru*

[7] Leading Researcher, Candidate of Science (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: kulakov@arriah.ru*

[8] Head of the Sector, Candidate of Science (Biology), FGBI "ARRIAH", Vladimir, Russia, *e-mail: petrova@arriah.ru*

SUMMARY

Lumpy skin disease is an economically significant disease as it results in decrease in weight gain and milk yield, abortions, mastitis, reproduction disorders, animal emaciation, lesions of respiratory organs and in some cases – death. Today the disease is included in the OIE list and is subject to obligatory notification. The emergence and spread of the disease in the Russian Federation necessitated performance of tests in the framework of laboratory diagnosis method improvement. The test-system based on the indirect "Sandwich" ELISA for diagnosis of lumpy skin disease allowing performance of biomaterial tests within 24 hours was for the first time developed in Russia. The test-system development included antibody preparation in laboratory animals (rabbits and guinea pigs), selection of optimal dilution of capture and detection antibodies, composition of a buffer solution and conditions of the reaction procedure. 50 samples of initial culture antigens of the lumpy skin disease virus as well virus-containing suspensions collected on different stages of purification and concentration were tested using this technique. To confirm the ELISA results all analyzed samples were tested using RT-PCR. Besides, the virus infectivity titer was determined by titration in YaDK-04 (Goat gonad cells). The test specificity was 100%, and analytical sensitivity – 3.5 lg TCD_{50}. The developed "Sandwich" ELISA allows performing tests of 24 antigen samples at 1:2–1:16 dilution simultaneously using 96-well plate and it can be used for lumpy skin disease diagnosis.

Key words: lumpy skin disease, ELISA, test-system, cattle.

INTRODUCTION

Lumpy skin disease is a disease of cattle characterized by fever, lesions of lymphatic system, subcutaneous oedema and oedema of internal organs, formation of nodules on the skin, lesions of eyes and mucous membranes of respiratory and gastrointestinal tract [2, 5].

Lumpy skin disease is caused by DNA-containing virus (*Dermatitis nodularis bovum*). According to classification of "International Committee on Taxonomy of Viruses" it belongs to *Capripoxvirus* genus of *Poxviridae*.

The disease is usually prevalent during the warm months, in the period of biological activity of blood-sucking insects, spreads rapidly and appears in an enzootic and epizootic form [2, 9].

Lumpy skin disease (LSD) causes significant losses in livestock production sector, as it results in decrease in weight gain and milk yield, animal emaciation, and in some cases – death.

LSD was registered in Israel, Lebanon, Bahrain, Kuwait, Oman, Yemen, Palestine, Iran, Iraq, Egypt, Syria, Turkey and Azerbaijan. In Europe, the occurrence of the disease was detected in Greece, Bulgaria, Macedonia, Kosovo. In 2017, 19 countries were LSD-infected (Fig. 1).

In 2015, LSD was registered in the territory of the Russian Federation in the Republic of Dagestan. Later it became spread among cattle of various regions (Fig. 2).

Today the disease is included in the OIE list and is subject to obligatory notification [2]. Lumpy skin disease is not included in the List of dangerous and quarantine animal diseases, approved by Order No. 476 of the Ministry of Agriculture of the Russian Federation of December 19, 2011.

The diagnosis is made on the basis of analysis of epidemiological and clinical data, postmortem and histological changes, as well as results of laboratory studies. Detection

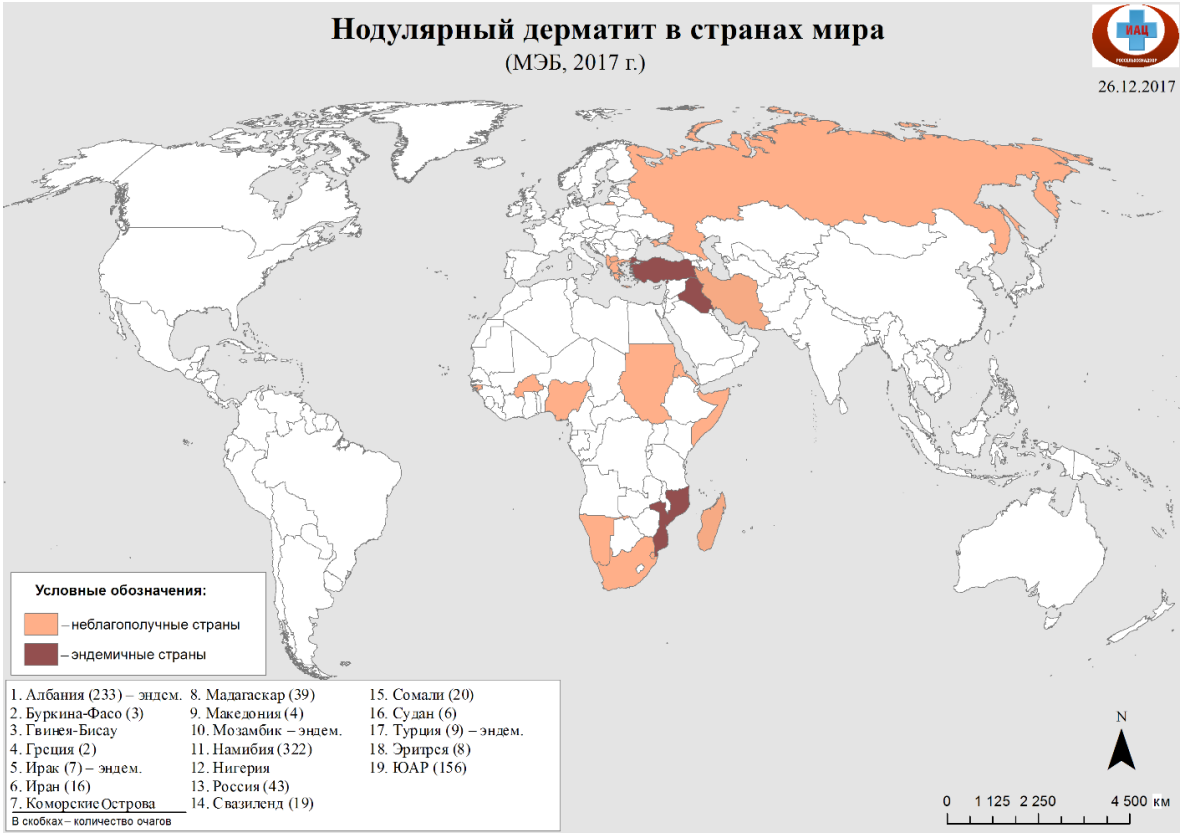


Fig. 1. LSD spread in the world

of LSD agent in samples of biomaterial is carried out using a number of methods: immunofluorescence test, agar gel immunodiffusion test, western blotting, virus isolation using sensitive cell culture, etc., which are rather labor- and time-consuming [1, 2, 6–8].

LSD occurrence and spread in the territory of the Russian Federation made it necessary to conduct research aimed at improving laboratory diagnostic methods.

The aim of the study was the development of an indirect "sandwich" ELISA to detect LSD virus antigen.

MATERIALS AND METHODS

ELISA test system consists of: culture positive LSD virus antigen of strain "E-95", adapted in goat gonad continuous cell culture (YaDK-04), with the infectivity titer of 5.5 lg TCD_{50}/cm^3; culture negative normal antigen; capture antibodies – specific hyperimmune polyclonal rabbit serum; detection antibodies – specific hyperimmune polyclonal guinea pig serum; anti-species conjugate – anti-guinea pig IgG conjugated to horseradish peroxidase; 0.05 M carbonate-bicarbonate buffer (pH 9.5–9.6) for dilution of the antigen; Tris-buffered solution (pH 7.4–7.6); wash buffer with Tween-20, Tween-20 (0.1% solution); blocking buffer (pH 7.4–7.6), based on a 10% dry milk solution; buffer for diluting samples and conjugate; a solution of 2,2-azino-bis- (Ethyl 3-aminobenzoate sulfonate); stop solution - 1% solution of sodium dodecyl sulfate.

RESULTS AND DISCUSSION

Indirect "sandwich" ELISA is based on the interaction between the studied antigen with capture antibodies adsorbed on the surface (specific polyclonal rabbit serum), and then with detection antibodies (specific polyclonal guinea pig serum). The resulting immune complex was detected using horseradish peroxidase conjugated anti-guinea pig IgG antibodies and a chromogenic substrate.

"Sandwich" ELISA development included specific antibody preparation in laboratory animals (rabbits and guinea pigs), selection of optimal dilution of capture and detection antibodies, composition of a buffer solution and conditions of the reaction procedure.

Purified and concentrated LSD virus antigen was used to obtain specific blood sera of laboratory animals. The culture fluid containing the virus was purified from ballast proteins and cell fragments by low-speed centrifugation at 4,800 g for 40 min, and then the resulting supernatant was centrifuged for 1 h 40 min at 45,000 g. The precipitate formed after centrifugation was dissolved in 10 mM Tris-HCl with 100 mM NaCl and 1 mM EDTA (TNE) buffer solution and further centrifuged through a layer of 30% sucrose at 106,000 g for 2.5 h. The resulting precipitate was resuspended in TNE buffer solution in 1/250 of the original volume, thus obtaining 250 times diluted solution. The antigen was filled in cryovials and stored until use at –80 °C.

To assess the specificity of the purified viral antigen, a real-time polymerase chain reaction (RT-PCR) was performed. Nucleic acid was isolated from 100 µl of the studied biological material using the "RIBO-sorb" kit (NextBio, Moscow) according to the manufacturer's instructions. RT-PCR was performed in accordance with the guidelines [4].

All RT-PCR-tested virus antigen samples had a cut-off value Ct<35 and ranged from 17 to 23, which indicates a high virus content in the preparations (Fig. 3).

The concentration of protein in LSD virus antigen preparations was measured according to Bradford method

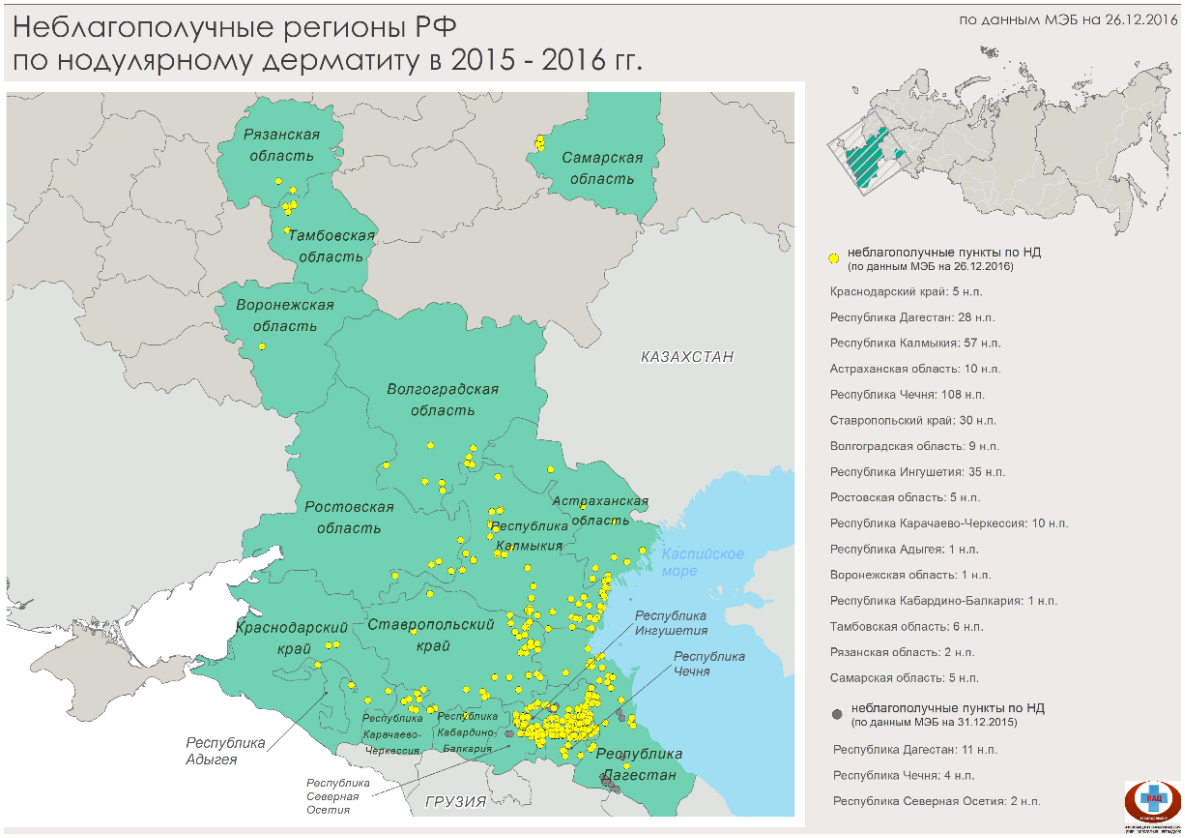


Fig. 2. LSD spread in the Russian Federation

using standard solutions of bovine serum albumin [2]. The concentration was 0.5–1.0 mg/ml. Purified and concentrated virus antigen was used to obtain specific components: hyperimmune rabbit sera and hyperimmune guinea pig sera used as capture and detection antibodies in ELISA.

The working dilution of the components was determined in preliminary experiments using indirect and direct ELISA according to the flowchart ("chessboard" ELISA). The method of serial dilutions was used, starting with a dilution of 1:2, adding 100 µl of the corresponding antigen dilutions into the wells of a polystyrene plate (Nunc MaxiSorp, Denmark) with pre-adsorbed capture antibodies. Then, the bound antigen was detected using detector antibodies. All components of the reaction were added in a volume of 100 µl, incubated at 37 °C. 0.05 M carbonate-bicarbonate buffer (pH 9.5–9.6) was used to immobilize the capture antibodies. Test and control samples, detection antibodies, antispecies conjugate were diluted in 1 M Tris-HCl with 0.15 M NaCl buffer solution containing 0.05% Tween-20 and 1% dry milk. The same buffer, but without the addition of dry milk, was used for interstage washings. A ready ABTC solution (Sigma) was used as a substrate. The reaction was stopped by adding 1% sodium dodecyl sulfate solution. The reaction was read using spectrophotometric method at a wavelength of 405 nm, using ELISA reader Sunrise (Tecan, Austria). Optical density (OD) was measured at 405 nm wavelength.

The last dilution of the tested sample was considered as the antigen titer, with the OD value of this last dilution being twice as high as the OD of the negative control (reaction with a normal antigen).

The optimal dilutions of the detection antibodies and anti-species conjugate, determined in direct ELISA, were 1:3000 and 1:500, respectively; the working dilution of the capture antibodies, determined by indirect sandwich ELISA, was 1:4000.

50 samples of initial culture antigens of the lumpy skin disease virus as well virus-containing suspensions collected on different stages of purification and concentration were tested using this technique. To confirm the ELISA results all analyzed samples were tested using RT-PCR. Besides, the virus infectivity titer for all the tested materials was determined by titration in YaDK-04 cells (Goat gonad cells).

The results are partially presented in Table 1.

The study of specific activity demonstrated that virus-containing materials (LSD) were positive in ELISA and their titer was 1:4–1:32 (Table 1). All samples with a virus infectivity titer of 3.5–6.5 lg TCD_{50}/cm^3 were positive in RT-PCR. A positive-negative cut-off value for the reaction results

Fig. 3. Results of LSD viral genome detection in RT-PCR

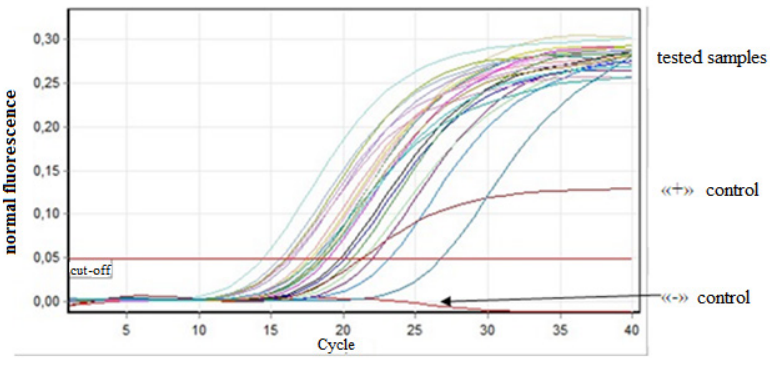


Table 1
Results of LSD antigen detection in virus-containing suspensions using ELISA
n = 3

Tested material	Virus infectivity titer TCD_{50}/cm^3	RT-PCR	Indirect "sandwich" ELISA	
			titer	result
Virus-containing culture suspension, 1st pass.	4.75	pos.	1:8	pos.
Virus-containing culture suspension, 1st pass.	5.5	pos.	1:32	pos.
Virus-containing culture suspension, 2nd pass.	6.5	pos.	1:32	pos.
Virus-containing culture suspension, 3rd pass.	5.92	pos.	1:16	pos.
Virus-containing culture suspension, 4th pass.	4.75	pos.	1:8	pos.
Virus-containing culture suspension, 6th pass.	6.5	pos.	1:32	pos.
Virus-containing culture suspension, 12th pass.	4.5	pos.	1:8	pos.
Virus-containing culture suspension, 13th pass.	4.5	pos.	1:16	pos.
Suspension from testicular nodules	6.5	pos.	1:32	pos.
Suspension from dewlap nodules	4.83	pos.	1:4	pos.
Suspension from skin nodules	3.5	pos.	1:16	pos.
Suspension from skin nodules	4.5	pos.	1:32	pos.
Suspension from skin nodules	3.5	pos.	1:2	doubtful
Normal antigen (uninfected cell culture)	neg.	neg.	< 1:2	neg.
Bovine rotavirus antigen	neg.	neg.	< 1:2	neg.
Bovine viral diarrhea antigen	neg.	neg.	< 1:2	neg.
Bovine parainfluenza-3 virus antigen	neg.	neg.	< 1:2	neg.

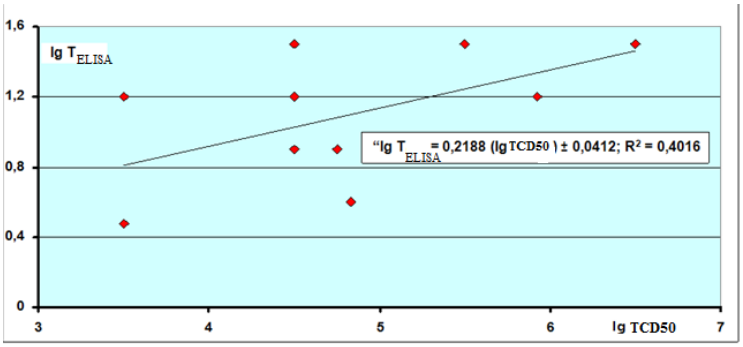
pos. – positive; neg. – negative.

was established: <1:2 – negative, 1:2 – doubtful, and ≥1:4 – positive.

Negative result was obtained during determination of test-system specificity with various heterologous antigens (rotavirus, viral diarrhea, parainfluenza-3 virus, bovine coronavirus, foot-and-mouth disease virus). This indicates the specificity of the test-system.

Then the analytical sensitivity of the solid-phase indirect "sandwich" ELISA was determined. The results are presented in Table 2.

Fig. 4. Relationship between virus infectivity titer (lg TCD_{50}) and virus antigen titer in ELISA (lg T_{ELISA})



The data given in Table 2 demonstrate that, in the logarithmic dimension, the contrast mean value was 3.910. Variation coefficient was 9.6%. The measurement error of the contrast mean was ± 0.240.

This means that there were 3.910 lg TCD_{50} per one lg T_{ELISA} as the average (i. e., 8,128 TCD_{50}). Taking into account the standard measurement error, this indicator was within 4,677 ÷14,125 (TCD_{50}). The determined values characterize the relative sensitivity of the developed test-system.

Figure 4 represents the scatter diagram of lg T_{ELISA} values (◆) against lg TCD_{50} values. Regression model is presented: lg T_{ELISA} = 0.219 (lg TCD_{50}) ± 0.041, where lg T_{ELISA} is the predictive titer in the ELISA for a given lg TCD_{50}.

The correlation coefficient between the lg T_{ELISA} and lg TCD_{50} values was R = 0.634, which according to the Chaddock's scale [3] demonstrates significant relationship.

The diagnostic specificity of the test was 100%, and the analytical sensitivity was 3.5 lg TCD_{50}.

The developed "sandwich" ELISA allows performing tests of 24 antigen samples at 1:2–1:16 dilution simultaneously using 96-well plate.

CONCLUSION

Sensitive and specific ELISA test-system for the detection of LSD virus antigen allowing performance of bioma-

Table 2
Study of relationship between lg TCD_{50} and lg T_{ELISA}
n = 3

Antigen characteristic	Titer, lg TCD_{50}/cm^3	Indirect "sandwich" ELISA lg T_{ELISA}	Contrast estimation [d = (lg TCD_{50}/cm^3) –(lg T_{ELISA})]
Culture antigen, 1st pass.	5.5 ± 0.1	1.505 ± 0.4	3.995 ± 0.12
Culture antigen, 1st pass.	4.75 ± 0.2	0.903 ± 0.1	3.847 ± 0.36
Culture antigen, 2nd pass.	6.5 ± 0.5	1.505 ± 0.2	4.995 ± 0.24
Culture antigen, 3rd pass.	5.92 ± 0.4	1.204 ± 0.2	4.716 ± 0.21
Culture antigen, 4th pass.	4.75 ± 0.5	0.903 ± 0.1	3.847 ± 0.26
Culture antigen, 6th pass.	6.5 ± 0.2	1.505 ± 0.2	4.995 ± 0.36
Culture antigen, 12th pass.	4.5 ± 0.1	0.903 ± 0.2	3.597 ± 0.23
Culture antigen, 13th pass.	4.5 ± 0.3	1.204 ± 0.3	3.296 ± 0.21
Suspension from testicular nodules	6.5 ± 0.2	1.505 ± 0.4	4.995 ± 0.36
Suspension from dewlap nodules	4.83 ± 0.1	0.602 ± 0.1	4.228 ± 0.45
Suspension from skin nodules of neck	3.5 ± 0.1	1.204 ± 0.4	2.296 ± 0.24
Suspension from skin nodules (Dagestan, s. Kamilukh)	3.5 ± 0.1	0.477 ± 0.3	3.023 ± 0.22
Suspension from skin nodules (Dagestan, s. Barnab)	4.5 ± 0.2	1.505 ± 0.2	2.995 ± 0.20
Contrast mean value (± *m*)			3.910 ± 0.240

terial tests within 24 hours was for the first time developed in Russia. The developed test system can be used by the personnel of research institutions and veterinary laboratories for LSD diagnosis.

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