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Research article

ENTOMOLOGICAL AND VIROLOGICAL METHODS FOR THE IDENTIFICATION OF POTENTIAL VECTORS OF LUMPY SKIN DISEASE VIRUS IN THE SOUTH-EASTERN PART OF NORTHERN CAUCASUS, RUSSIA

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The article provides assessment of field and laboratory methods for the collection and evaluation of potential vectors of lumpy skin disease virus (LSDV) in one of the districts of Krasnodarskiy Kray in southern Russia. In this study, we tested several methods of vector collection and a PCR protocol for the detection of the LSDV genome in insects. Descriptive data on samples were collected using a free web-based application Epicollect5.

Potential LSDV vectors are quite widely spread insects in this region. We identified 15 insect species, including *Musca domestica*, *Musca autumnalis* and *Stomoxys calcitrans*. Analysis of the insect population showed an increase in species diversity and a decrease in abundance of the insect population by the end of the flight season.

PCR tests did not detect LSDV genome in the collected samples. All the methods tested were found suitable for large-scale monitoring of lumpy skin disease (LSD). Further studies on potential risk factors of LSD spread are necessary to improve measures on preventing and eliminating the disease.

Key words: lumpy skin disease, LSD virus, Russia, cattle, insects, vectors, Krasnodarskiy Kray

INTRODUCTION

Lumpy skin disease (LSD) is one of the transboundary diseases of cattle that has emerged in Europe recently with a serious economic impact. It was introduced in the

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South of the Russian Federation several years ago, and despite taken measures it is still present in the country causing severe economic losses in livestock.

This viral infection causes fever, lymphatic dysfunction, visceral and hypodermal edema with specific skin nodules and lesions of the eyes and mucosa [1,2].

LSD is a transmissible disease, which requires restrictive measures [3]. The disease has high priority due to the spreading speed and economic losses associated with culling and cost of therapeutic and preventive measures.

The main sources of the virus are infected animals and animal skins affected by nodules. The virus has been isolated from the saliva, semen, milk, nasal and eye discharge, exudates, affected skin and mucosa [4,5]. However, direct contact rarely results in virus transmission. The virus spreads between animals mainly by mechanical vectors - blood-sucking insects. Their presence defines the speed and scale of LSD spread in the region.

At the same time, the introduction of the virus into previously free (remote) regions is generally caused by the movement (mainly illegal) of infected animals.

The following insects are potential mechanical vectors of the lumpy skin disease virus (LSDV): *Aedes aegypti*, *Culex mirificus, Aedimorphus (Aedes) natronius*, *Culex quinquefasciatus*, *Anopheles stephensi*, *Culicoides nubeculosus, Stomoxys calcitrans*, *Musca confiscata*, *Biomyia fasciata*, *Musca domestica, M. autumnalis*, *Haematobia irritans*, tabanids, and ticks *Rhipicephalus appendiculatus* [4,5].

First LSD outbreaks in Russia were reported in 2015 in the North Caucasian federal district: in Dagestan, Chechnya, and the republic of North Ossetia-Alania, where the disease has been registered for several years [1]. In 2016, LSD spread north and eastward along the border with Kazakhstan.

The first outbreak of the disease in the Krasnodarskiy Kray was officially registered in May 2016. However, LSD introduction routes and spreading ways in these outbreaks have not been studied.

These outbreaks were quickly eliminated, and every cattle was immunized with the heterologous dry vaccine produced by Federal Centre for Animal Health "Arriah" (Vladimir, Russia). Since then, preventive vaccination was annually carried out and the disease was not notified in the Krasnodarskiy Kray.

Krasnodarskiy Kray is a region of developed animal husbandry, with an intensive movement of animals throughout the region and between neighboring regions. That makes study of LSDV risk factors important for risk assessment and preventive measures in case of its re-introduction.

One of the critical factors of LSDV spread are climate characteristics that affect number and diversity of vectors. The climate in Kray is similar to the Mediterranean, where LSD extensively spread in 2016.

However, there are no available entomological data on the presence and a number of potential vectors in the Krasnodarskiy Kray.

LSD outbreaks were also reported in 2016 in the neighboring regions (Republic of Adygea, Karachay-Cherkess Republic, Rostov region, Stavropol Territory), but monitoring of the virus circulation and its possible overwintering in adjacent and previously affected areas were not carried out.

This research was conducted as a part of the project, performed at Federal Research Center for Virology and Microbiology (FRCVM) to develop an integrated analysis of epizootic data on emerging animal diseases, based on monitoring studies.

The purpose of this work was to test different methods for collection and monitoring of potential LSDV vectors and detection of the LSDV genome in insects.

To achieve the goal the following tasks were assigned:

- determination of climate characteristics during the insect flight period;
- optimization of methods for collecting insects, determination of species composition and identification of potential LSDV vectors and their seasonal dynamics in the Krasnodar region;
- optimization of a PCR protocol for LSDV genome detection in insect samples.

MATERIAL AND METHODS

The study area was the Vyselkovsky district of the Krasnodarskiy Kray, since LSD outbreaks were reported in the adjacent territories (Tbilissky and Gulkevichsky districts) in 2016.

Affected districts of Krasnodarskiy Kray and the LSD epizootic situation in 2016 are presented in Figure 1.

The Vyselkovsky district is located in the center of the Krasnodar Territory, the central part of the Prikubanskaya Plain, and borders with Pavlovsky, Tikhoretsky, Tbilissky, Ust-Labinsky, Korenovsky, and Bryukhovetsky districts. The hydrographic network of the region consists of Zhuravka and Beysug rivers with their confluents. Beysug flows into the Sea of Azov, which connects to the Black Sea via the Kerch Strait [6].

The climate in the region is similar to the Mediterranean, with a mild winter and torrid summer. Eastern and northeastern winds dominate. The total number of strong wind days is up to 25 per year. The winds cause drought in the summer and strong cooling, soil and seed shifting in the winter and spring. Forest belts protect some territories [6]. The summary of average climatic data in the Vyselkovsky district is presented in Table 1.

Climate characteristics at the site of insect collection were obtained from public sources, particularly Gismeteo website (https://www.gismeteo.ru), and were set into Epicollect5 form along with other information.

Figure 1. LSD epizootic situation in 2016 in the Krasnodar Territory and the district where the study was performed.

Table 1. Climate temperatures and average annual rainfall (https://ru.wikipedia.org/wiki. Climate from 1981 to 2018)

Month	Average day temperature	Average night temperature	Humidity of air
May	24.8	19.8	70%
June	30.2	23.5	57%
July	31.2	26.1	48%
August	32.1	25.5	50%
September	25.4	20	60%

Entomological studies were carried out in two stages: the insects were collected at the beginning (May, 2017) and at the end (September, 2017) of the flight period in a cattle holding in the Vyselkovsky district (with 300 heifers housed during the period of the study).

According to the guidelines [7], 3 types of traps were used to collect insects.

1. ***Fly strips*** were placed at the loafing area and several points in the barn, 2 strips per point.
2. ***UV traps*** were placed in the barn above the animals, opposite to the entrance.
3. ***Liquid traps*** for horseflies were placed near the loafing area.

The time of exposition for these traps was 12 hours.

We also used sweep net for capturing flies and midges on the fly; the net was periodically inverted with the removal of its content into a killing jar.

We used Epicollect5 (© 2019 Imperial College London, https://five.epicollect.net) to collect entomologic data. This application allows downloading images and linking data to geographic location, making it very useful for quick map visualization. The following information was recorded: insect collection date, district name, holding name and type, coordinates of collection site, air temperature, humidity and pressure, type and exposure time for traps.

Identification of insects was performed using a 5-fold magnifying glass and a Key to Insects [8] on the base of Samara Research Veterinary Institute - branch of FRCVM.

Data was statistically evaluated with the MS Office Excel 2010 software.

We used real-time PCR to detect LSD genome in insect samples. All insects were pooled according to their taxonomical group with 5-7 species in each pool. For each species at least 2 pools were formed, with additional pools in prevalent species. Each pool was analyzed as a separate sample. Overall, we tested 36 samples.

Isolation of nucleic acids was performed using Ribo-sorb kit (InterLabService, Russia). We performed a detection of viral genomic DNA according to the protocol proposed by T.R. Bowden et al. in 2008 [9, 10] using 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') oligonucleotide primers and CaPV-074P1 hybridization probe (5'-FAM-tgg ctc ata gat ttc ct-MGB -NFQ-3').

DNA was amplified on a CFX96 Touch Real-Time PCR Detection System (BioRad, USA). The following program for PCR was used: pre-denaturation for 3 min at 95 °C, followed by 45 amplification cycles, each consisting of 15 s at 95 °C and 30 s at 60 °C.

LSDV DNA isolated from an infected culture with 10^2 TCID50/cm3 activity was used as the positive control. Negative PCR control consisted of redistilled water.

Ethical approval: The conducted research is not related to animal use.

RESULTS

Figure 2 shows the average temperatures in the daytime and evening during the flight period (from May to September 2018).

Study of species composition at the beginning of the flight period (May 2018) identified the following species: *Musca domestica* (330, 48%), *Musca autumnalis* (172, 25%), *Culicoides pulicaris* (35, 5%), *Phyllotreta cruciferae* (38, 5%), *Simulium morsitans* (29, 4%), *Cnephia intermedia* (21, 3%), *Culicoides metagonatus* (18, 3%), *Sepsis punctum* (24, 3%), *Muscina stabulans* (14, 2%), *Culex pipiens* (6, 1%), and *Maniola jurtina* (6, 1%).

At the end of insect flight period (September 2018), the following species were identified: *Musca domestica* (159, 37%), *Stomoxys calcitrans* (95,22%), *Muscina stabulans* (44,

10%), *Simulium morsitans* (24, 6%), *Sepsis punctum* (21, 5%), *Culex pipiens* (17, 4%), *Cnephia intermedia* (11, 3%), *Culicoides pulicaris* (14, 3%), *Musca autumnalis* (14, 3%), *Culicoides metagonatus* (7, 2%), *Limnaecia phragmitella* (6, 2%), *Tribolium destructor* (10, 2%), *Vespidae* (3, 1%), and *Maniola jurtina* (1, 0%).

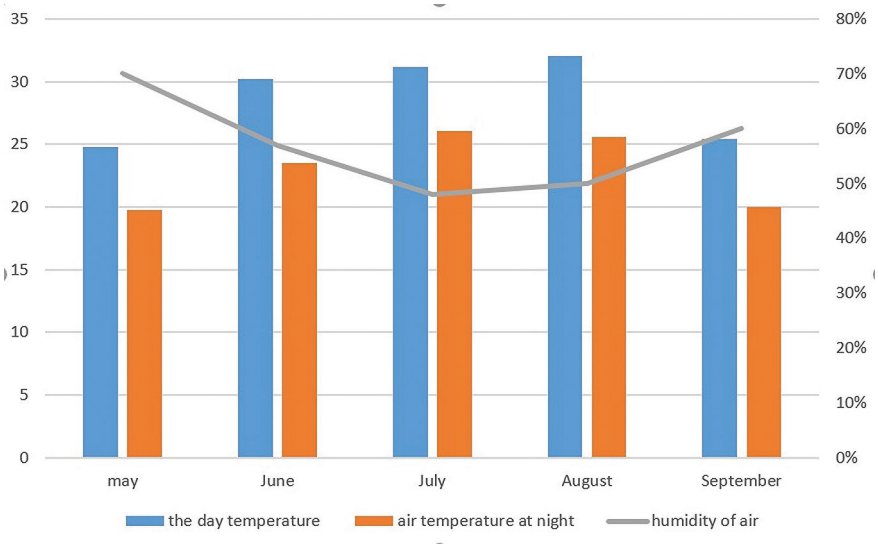

Figure 2. Average temperatures and humidity of air from May to September 2018.

PCR did not detect the LSDV genome in insect samples collected in Vyselkovsky settlement in both May and September 2018. Negative isolation and negative PCR controls had no Ct values on FAM channel while positive PCR control had a Ct value within 15-30 cycles.

DISCUSSION

Analysis of climatic parameters during the insect's flight period showed that the temperature in September 2018 was 3-4 °C higher than average values. It led to the extension of the breeding season for insects and an increase in their population.

According to the purpose of this study, we evaluated the effectiveness of different types of insect traps. Fly strips and UV traps were efficient for insect collection and provided approximately 93% of all insects caught. The sweep net was less efficient. It was efficient for catching flies but not *Simuliidae*. The liquid trap was inefficient due to being attractive for the cattle as a source of water. According to this, sweep net and liquid traps were found ineffective for the purpose of this study.

Epicollect5 notably facilitated data collection, storage, transfer, and processing of epizootological data.

Analysis of the insect composition has shown an increase in species diversity and a decrease in populations of most species by the end of the flight period (Figure 3). Species composition and quantitative analysis showed that *Musca domestica* was the predominant species throughout the entire study period, while *Musca autumnalis* was more common at the beginning and *Stomoxys calcitrans* at the end of the flight period. Role of *Stomoxys calcitrans* as a vector of lumpy skin disease was investigated in several studies, which showed high potential of *S.calcitrans* for virus transmission [11-13]. The role of *Musca domestica* as a vector of LSDV is not yet clear, but case of detection of virus in *M.domestica* samples indicates its potential for mechanical transmission [14].

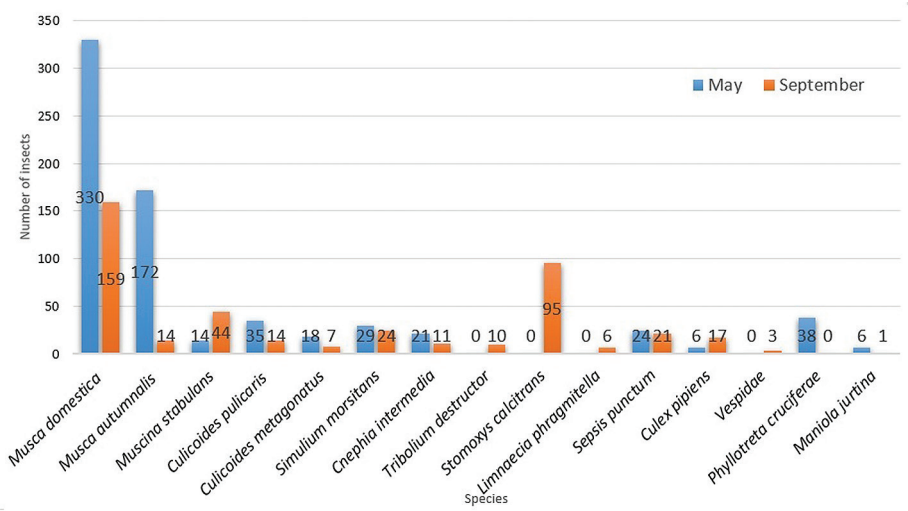

Figure 3. Insect dynamics at the beginning and at the end of the flight period

PCR protocol was effective for detection of LSDV genome in infected tissue cultures. LSDV genome was not detected in the insects pools.

These results show a significant risk of LSD spreading through vectors in the case of re-introduction to this region. Annual control of density and species composition of pathogen vectors is important for comprehensive analysis of risk factors conducive for LSDV.

The study will be continued to provide further information on the LSD related risk factors critical for the prevention of LSD outbreaks.

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Authors' contributions

GAA and UTR carried out the sample collection. UTR and KOV performed molecular studies and participated in writing. GAA, DSG and DGA performed the statistical analysis of data and helped to draft the manuscript. GAA and STA prepared the manuscript. DZ and LDA participated in the design of the study and critically revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ENTOMOLOŠKE I VIRUSOLOŠKE METODE ZA IDENTIFIKACIJU POTENCIJALNIH VEKTORA VIRUSA IZAZIVAČA NODULARNOG (*LUMPY*) DERMATITISA U JUGOISTOČNOM REGIONU SEVERNOG KAVKAZA, RUSIJA

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Rad se odnosi na procenu terenskih i laboratorijskih metoda za sakupljanje i evaluaciju potencijalnih vektora virusa izazivača nodularnog (*Lumpy*) dermatitisa u jednom od distrikta Krasnodarskiy Kray, u južnom delu Rusije. U ovoj studiji, testirano je nekoliko metoda sakupljanja vektora i PCR protokola za dokazivanje prisustva genoma virusa nodularnog dermatitisa u insektima. Deskriptivni podaci u vezi uzoraka su sakupljeni korišćenjem web aplikacije Epicollect5.

Potencijalni vektori virusa nodularnog (*Lumpy*) dermatitisa su insekti koji su široko rasprostranjeni u navedenom regionu. Identifikovano je 15 vrsta insekata, uključujući *Musca domestica*, *Musca autumnalis* i *Stomoxys calcitrans*. Analiza populacije insekata je ukazala na povećanje diverziteta vrsta kao i na smanjenje gustine populacije insekata pri kraju sezone njihovog leta.

U sakupljenim uzorcima, metodom PCR, nije mogao da se ustanovi genom virusa izazivača nodularnog (*Lumpy*) dermatitisa - LSDV. Sve testirane metode bile su podesne za sprovođenje nadzora nodularnog (*Lumpy*) dermatitisa, velikog obima. U budućnosti, neophodno je da se obave studije potencijalnih faktora širenja nodularnog (*Lumpy*) dermatitisa, a radi poboljšanja mera prevencije i iskorenjivanja ovog zaraznog oboljenja.