2024 SCIENCELINE

Online Journal of Animal and Feed Research

Volume 14, Issue 2: 157-164; March 25, 2024

ISSN 2228-7701

DOI: https://dx.doi.org/10.51227/ojafr.2024.19

PCR-BASED STUDY ON VIRAL PATHOGENS CIRCULATION AMONG CERVIDS IN THE MOSCOW REGION

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Supporting Information

RESEARCH ARTICLE

PII: S222877012400019-14

Received: December 08, 2023

Revised: March 21, 2024

Accepted: March 24, 2024

ABSTRACT: A molecular survey of selected viruses in free-ranging cervids was conducted in 15 different districts of Moscow region. Samples were collected from 178 game animals including 144 moose (*Alces alces*), 19 roe deer (*Capreolus capreolus*) and 15 deer without species information. Nasal swabs and tissue samples including parts of the nasal septum, upper tracheal rings, lung, heart, liver, kidneys and pooled organ samples were tested using polymerase chain reaction (PCR). Samples were studied for pestiviruses, herpesviruses, coronaviruses, group A rotaviruses, adenoviruses, hepatitis e and parainfluenza type 3 virus. None of the samples were positive for Bovine Coronavirus and SARS-COV-2, hepatitis E virus and parainfluenza type 3 virus. PCR results were positive for bovine herpesviruses (5.05%), pestiviruses (0.56%), rotaviruses (1.68%). DNA of a new adenovirus, presumably causing a mild course of animal respiratory disease, was detected in samples of 6 animals (3.37%). In conclusion, the conducted studies have shown that game animals of the Moscow region can be a natural reservoir of cattle viruses, and this must be taken into account when planning and organizing measures for the control and eradication of such notifiable diseases as bovine viral diarrhoea and infectious bovine rhinotracheitis. Monitoring studies and general disease surveillance of wild animal populations provide additional information on the epidemiology of infectious diseases in the region and allow timely measures to be taken to protect wild animals, domestic animals and the public.

Keywords: Cattle, Deer, Epidemiology, PCR, Viral infection.

INTRODUCTION

Domestic cattle (*Bos taurus taurus*) belong to the same superfamily Cervoidea as cervids (Fernández and Vrba, 2005; Kuznetsova et al., 2005). Also, a quantity of cervids inhabits the forests of the European part of the Russian Federation (Rumyantsev et al., 2018). Animals may enter public gardens, city parks, or highways, where the likelihood of them coming into contact with people increases. Infected ungulates entering livestock pasturelands pose a separate danger. In this case, there can introduce infectious diseases into livestock farms (Böhm et al., 2007; Yatsentyuk et al., 2022).

Scientific studies indicated that wild ruminant ungulates can carry viruses that are common to wild and farmed animals and also those that are dangerous to humans (Ros and Belák, 1999; Ricci et al., 2019; Althof et al., 2023; Feng et al., 2023).

Viruses from different groups can either cause high mortality or cause subclinical forms of infection in deer (Auer et al. 2022; Domshy et al. 2023; Feng et al., 2023). Recently, wild animals have also been considered as potential sources or reservoirs of new viral pathogens, where viruses can persist and change, and can be transmitted to farm animals (Cripps et al. 2019; Yatsentyuk et al., 2022; Feng et al., 2023).

Herpesviruses are the viral group mostly studied on wild ungulates, and in this field, representatives of Alphaherpesvirinae are studied more frequently (Ros and Belák 1999; Rola et al. 2017). However, the presence of closely related α-herpesviruses that can occur in ruminants makes it difficult to estimate the prevalence by serological methods (Besi et al., 2018; Bianchessi et al., 2022). Bovine herpesviruses type 1 (BoHV-1) and type 5 (BoHV-5), buffalo herpesvirus type 1 (BuHV-1), caprine herpesvirus type 1 (CpHV-1), deer herpesvirus type 1 (CvHV-1), deer herpesvirus type 2 (CvHV-2) and elk herpesvirus type 1 (ElkHV-1) can induce similar antibodies in animals (Thiry et al., 2006). This may cause misinterpretation of test results. The prevalence of another herpesviral group - Gammaherpesvirinae, including bovine herpesvirus types 4 (BoHV-4) and 6 (BoHV-6), elk gammaherpesvirus (Elk-LHV) and fallow deer lymphotropic herpesvirus (LHV) in cervids is almost not studied, widely (Kálmán and Egyed 2005; Yatsentyuk et al., 2022).

Pestiviruses are the viral group that is an interesting object of research; Bovine viral diarrhea virus (BVDV), which causes the OIE-notified viral diarrhea disease, is widespread throughout the world (Scharnböck et al., 2018). BVDV is

Citation: Yatsentyuk S, Krasnikova M, Dolinskaya K and Pchelnikov A (2024). PCR-based study on viral pathogens circulation among cervids in the Moscow region. *Online J. Anim. Feed Res.*, 14(2): 157-164. DOI: https://dx.doi.org/10.51227/ojafr.2024.19

often detected in Russia in cattle (Glotov et al., 2016 a, b). The prevalence of this virus in wild ruminants in Russia is not well documented (Pchelnikov et al., 2023).

Some researchers believe that wild ruminants become infected exclusively from livestock, and not vice versa (Fernández-Aguilar et al., 2016). Others have suggested that cervids are a reservoir for BVDV (Rodríguez-Prieto et al. 2016). Bovine coronavirus (BCoV) and parainfluenza virus-3 (PIV-3) are also quite common among cattle in different countries (Burimuah et al., 2020; Vlasova and Saif, 2021). Coronavirus studies conducted in Canada, USA, Japan and South Korea have identified bovine-like CoVs in 6 deer species (Amer, 2018), and in recent years, information has appeared about the identification of another coronavirus in deer - SARS-COV-2, which caused the COVID-19 pandemic (Feng et al., 2023).

Parainfluenza virus-3 (PIV-3) is also can infect different species of ungulates. It has been detected in camels, buffaloes and different deer. PIV-3 is thought to increase an animal susceptibility to other respiratory pathogens. Moreover, PIV-3 itself can either cause subclinical or cause acute manifestations in animals. Serological and PCR studies confirm the prevalence of this respiratory infection in livestock. But reports of detection of PIV-3 in wild animals are few (Dastjerdi et al., 2022). There are even less reports of Bovine respiratory syncytial virus (BRSV) circulating in wild ungulates (Bergmann et al., 1990).

Rotavirus infections caused by group A rotaviruses (RVA) are a problem in young cattle (Steele et al. 2004). RVA are widespread in many countries, but information on the occurrence of rotaviruses in wild ungulates has only begun to accumulate in recent years. The viruses were found in cervids in Germany, Slovenia, and South Korea (Althof et al., 2023). The researches note that the relationship of animal RVA strains with human strains indicates the zoonotic potential of RVA and requires study (Jamnikar-Ciglenecki et al., 2016; Althof et al., 2023).

Hepatitis E virus (HEV) is a zoonotic virus that can also be detected in wild ungulates. HEV RNA was detected in wild boars and different deer samples from Lithuania (Spancerniene et al., 2018), Germany (Anheyer-Behmenburg et al., 2017), Spain (Boadella et al., 2010), Japan (Takahashi et al., 2022).

Adenoviruses are a group of viral pathogens that are often found in various vertebrates. Mastadenoviruses and Atadenoviruses are pathogens of various ungulates, occurring in cattle and sheep, as well as in various cervids. Different adenoviruses were described in wild ungulate populations. White-tailed deer (*Odocoileus virginiana*), red deer (*Cervus canadensis nelsoni*), mule deer (*Odocoileus hemionus*), and moose (*Alces alces*) have been shown to be susceptible to the Atadenovirus Odocoileus adenovirus 1, which causes adenoviral hemorrhagic disease with high mortality (Kauffman et al., 2021). Mastadenovirus Odocoileus adenovirus 2, isolated in 2017 from white-tailed deer, unlike Odocoileus adenovirus 1, does not cause an acute course of the disease and epizootic outbreaks. It is manifesting in mild respiratory lesions and slight thinning of immune tissues (Ridpath et al., 2017).

The purpose of this study was to find out whether there is data on the presence of viruses of different groups in the population of free-living cervids in the Moscow region using molecular methods. The first goal of the study was to screen samples for bovine viruses common to cattle and cervids, including bovine herpes viruses, bovine coronavirus, bovine viral diarrhea virus, and parainfluenza virus-3. The second objective was to assess the presence of viruses with zoonotic potential - hepatitis E virus, rotavirus and SARS-COV-2. The third task is to investigate the presence of viruses specific to cervids.

MATERIALS AND METHODS

Ethical approval

This study used samples obtained from hunted animals. All licences for hunting were issued by the Ministry of Ecology and Natural Resources of the Moscow Region. The study protocol was approved by the Ethics Committee of the Federal State Budgetary Institution “All-Russian State Center for Quality and Standardization of Medicines for Animals and Feed” (Protocol No. 125 of April 16, 2020).

Sample collection

Samples used in the study were obtained from wild free-living cervids, which were shot during the winter hunting seasons 2019-2023 in 15 different districts Moscow region (Figures 1-3). Samples were taken from the following animal species: from 144 moose (*Alces alces*); 19 roe deer (*Capreolus capreolus*); 15 animals without species information.

Total 78 nasal swabs and 371 tissue samples, including parts of the nasal septum, upper tracheal rings, lung, heart, liver, kidneys and pooled organ samples were tested.

Detection of viral nucleic acids

Nasal swabs and frozen organ samples from all animals were subject to DNA and RNA isolation. Total nucleic acids were extracted from 100 µL of the 10% suspension of tissue or swab sample using the RIBO-prep kit (AmpliSens, Russia).

Previously published PCR protocols were used to detect BVDV, BoHV-1 and other α-herpesviruses (Pchelnikov et al., 2023), BoHV-4 and BoHV-6 (Yatsentyuk et al., 2022), SARS-COV-2 (Krasnikova et al., 2022). For RNA of BCOV, RVA and

PIV-3 detection were used RT-PCR kits "BOVINE CORONAVIRUS-FACTOR", "ROTAVIRUS-FACTOR", and "PIV-3-FACTOR" (VetFaktor, Russia). PCR and RT-PCR for the nucleic acids of adenoviruses, HEV and BRSV were made with oligonucleotides and conditions shown in the Table 1.

Conventional PCR was carried on "Tercyk" Multi-block Thermocycler (DNA-technology, Russia) in a 25 µL reaction mixture containing 2.5× PCR-mix2 blue (AmpliSens), 10 mM of dNTPs, 0.6 µM of both forward and reverse primers. RT-PCR amplification mixture contains 5X One-Step RT-PCR Mastermix (Belbiolab, Russia), 0.6 µM forward and reverse primers, 0.3 µM fluorescent probe. Real-time PCR was carried on a RotorGene Q (Qiagen, Germany). Conventional PCR products from all samples were analyzed by electrophoresis in 1.8% agarose gel and visualized under ultra violet light. The selected PCR products were submitted for sequencing.

Sequencing of purified amplicons was carried out using the BrilliantDye V3.1 reagent kit on an Applied Biosystems 3100 Genetic Analyzer (Life Technologies, USA). The obtained nucleotide sequences were analyzed using the BLAST algorithm on the Internet search resource of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Maps were constructed using ArcMap10.8 program (Esri, Redlands, California, USA).

Table 1 - List of oligonucleotides for HEV, BRSV and Bovine adenovirus-3 PCR detection

Virus	Target region	Primer Sequence 5'-3'	Size (bp)	Tm	Reference
ADV	E2A	Forward: GAGATGGATGTGAACAGCGA Reverse: ACATTCTGATGCTGGTACTG	644	95°- 5 min, 45 cycles (95°- 20 c, 55°- 20 s, 72°- 40 s), 72°- 5 min	Zhu et al. (2011)
BRSV	gene N	Forward: GCAATGCTGCAGGACTAGGTATAAT Reverse: ACACTGTAATTGATGACCCCATTCT TaqMan probe: R6GACCAAGACTTGTATGATGCTGCCAAAGCABHQ	124	50°- 30 min 95°- 15 min, 10 cycles (95°- 10 s, 60°- 20 s, 72°- 10 s), 35 cycles (95°- 10 s, 55°- 20 s detection, 72°- 10 s), 72°- 5 min	Boxus et al. (2005)
HEV	ORF3	Forward: GGTGGTTTCTGGGGTGAC Reverse: CGAAGGGGTTGGTTGGATG TaqMan probe: Cy5GGGTTGATTCTCAGCCCTTCGCBHQ	73	50°- 30 min 95°- 15 min, 5 cycles (95°- 10 s, 60°- 20 s, 72°- 10 s), 40 cycles (95°- 10 s, 55°- 20 s detection, 72°- 10 s), 72°- 5 min	Jothikumar et al. (2006)

ADV: Bovine adenovirus-3; BRSV: bovine respiratory syncytial virus, HEV: Hepatititis E virus

RESULTS

DNA of herpesviruses BoHV-4, BoHV-6, CvHV-1, CvHV-2 and ElkHV-1 was not found by conventional PCR in all 178 animals. Also, all samples were negative for BCoV, SARS-COV-2, PIV-3, HEV and BRSV. The PCR results for RVA, BVDV, BoHV and Adenoviruses are shown in Table 2. The location of the shooting sites of positive animals is displayed on the map of the Moscow region (Figure 2)

Adenoviral DNA was founded in swabs and respiratory samples (parts of the nasal septum and trachea) of 4 moose, 1 roe deer and 1 animal without information about species. Animals were hunted in Yegoryevsk (N=1), Stupino (N=1) and Pavlovsky-Posad districts (N=4).

Analysis of the nucleotide sequences of the 644 bp amplicons, obtained by conventional PCR with primers to Bovine adenovirus 3, showed only 83.97% identity with E2A region of Bovine adenovirus 3. All sequences of 6 positive PCR samples from different cervids were identical. A partial DNA sequence of the virus named Roe deer adenovirus 1 from a sample of a roe deer hunted in 2022 has been deposited in the GenBank database (GenBank: ON936732.1).

Group A Rotavirus RNA was detected in pooled organ samples from 3 moose hunted in Stupino, Lukhovitsy and Rusa districts of Moscow region. BVDV RNA was founded in nasal swab of moose from Serpukhov district. Viral concentration in samples was low, the average Ct value during real-time PCR for BVDV was 28.33, for RVA -33.10.

Bovine herpesvirus DNA was detected in samples from 9 animals. It was found in the nasal swabs of 2 roe deer hunted in 2019 in Lukhovitsy district. In 2022-2023 hunting season BoHV DNA was detected in organ samples of 2 moose (in a lung sample of one moose, as well as in samples of the kidney and heart of another animal) from Stupino; in the nasal swab of 1 roe deer from Serpukhov; in the pooled organ samples of 2 roe deer from Lukhovitsy and 2 moose from Klin and Orekhovo-Zuevo districts. The average Ct value during real-time PCR was 26.8.

Analysis of the nucleotide sequences of the herpesviral gB gene fragment, obtained as a result of sequencing PCR products with primers common to α-herpesviruses, confirmed the presence of BoHV-5 DNA in a nasal swab sample from a roe deer and BoHV-1 DNA in two moose samples. Due to the low quality of the samples, it was not possible to identify the types of bovine herpesviruses in the remaining animal samples.

Table 2 - Results of PCR detection of group A Rotaviruses, bovine herpesviruses 1 and 5, bovine viral diarrhea virus, and adenoviruses with species and number of animals sampled in brackets

Positive PCR results / Animal species tested	`RVA (%)	ADV (%)	BVDV (%)	BoHV (%)
Moose (Alces alces) (N=144)	0	1 (5.26)	0	5 (26.31)
Roe deer (*Capreolus capreolus*) (N=19)	0	1 (6.67)	0	0
Deer without species information (N=15)	3 (1.68)	6 (3.37)	1 (0.56)	9 (5.05)
Total (N=178)	3 (1.68)	6 (3.37)	1 (0.56)	9 (5,05)

E.P.C.<0.05

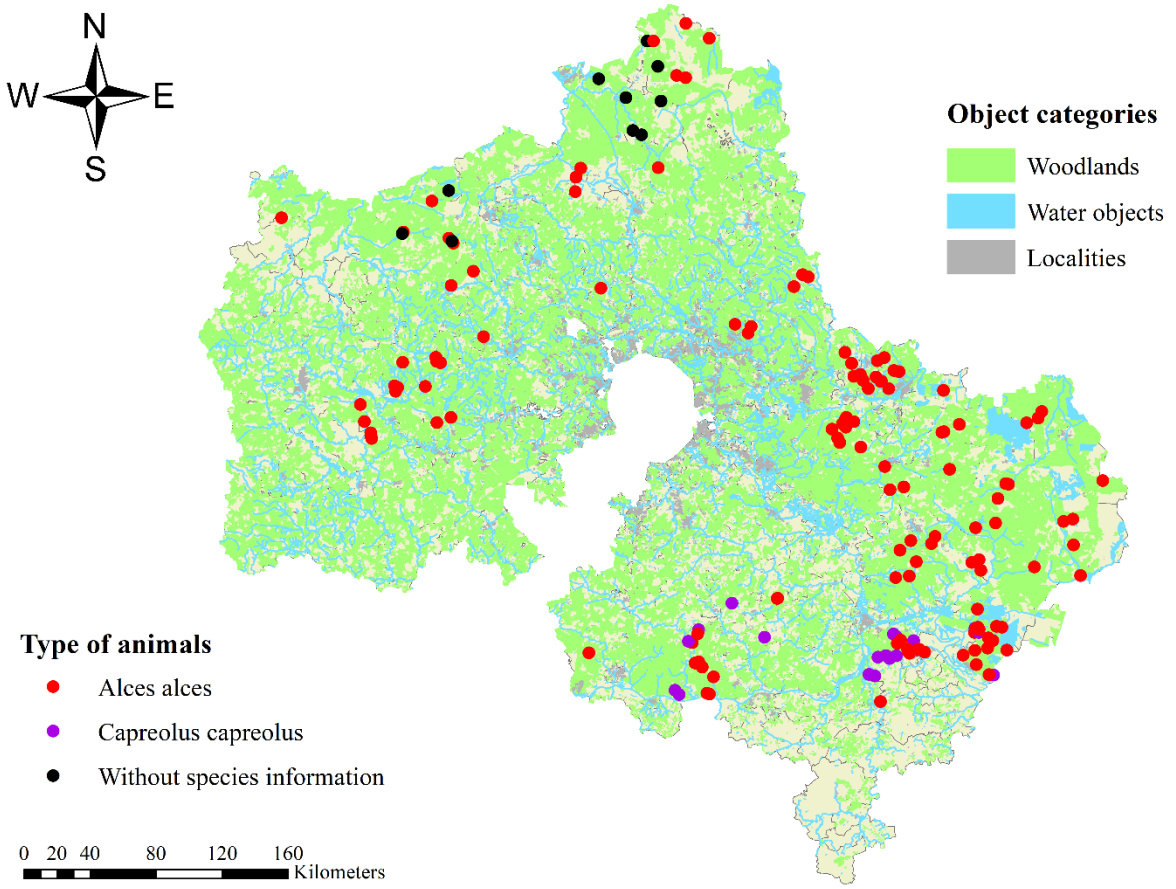

Figure 1- A map of Moscow region depicting sampling sites

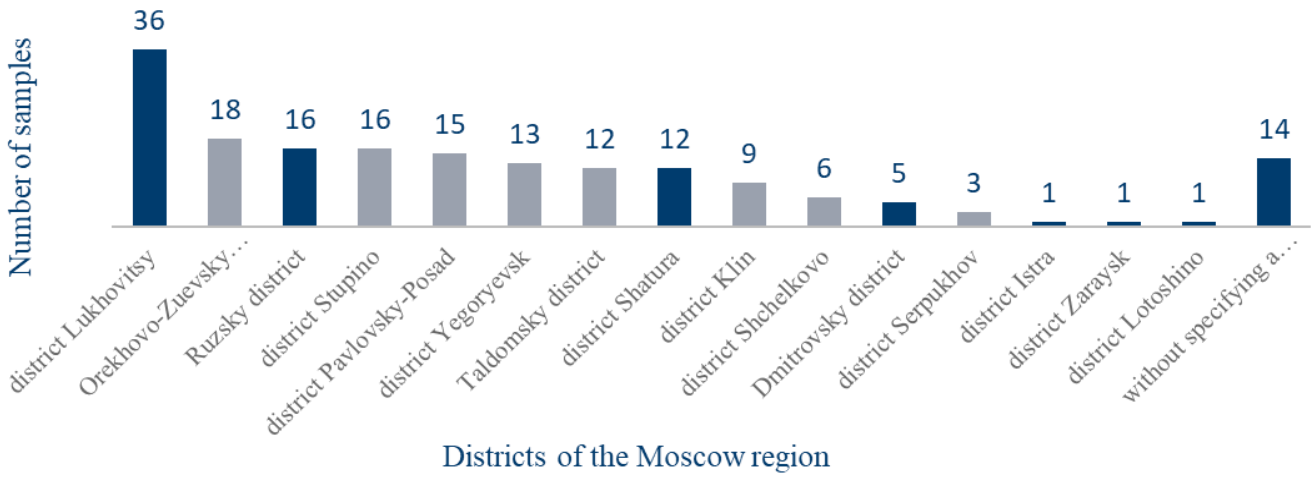

Figure 2 - Distribution of studied samples by Moscow region samples

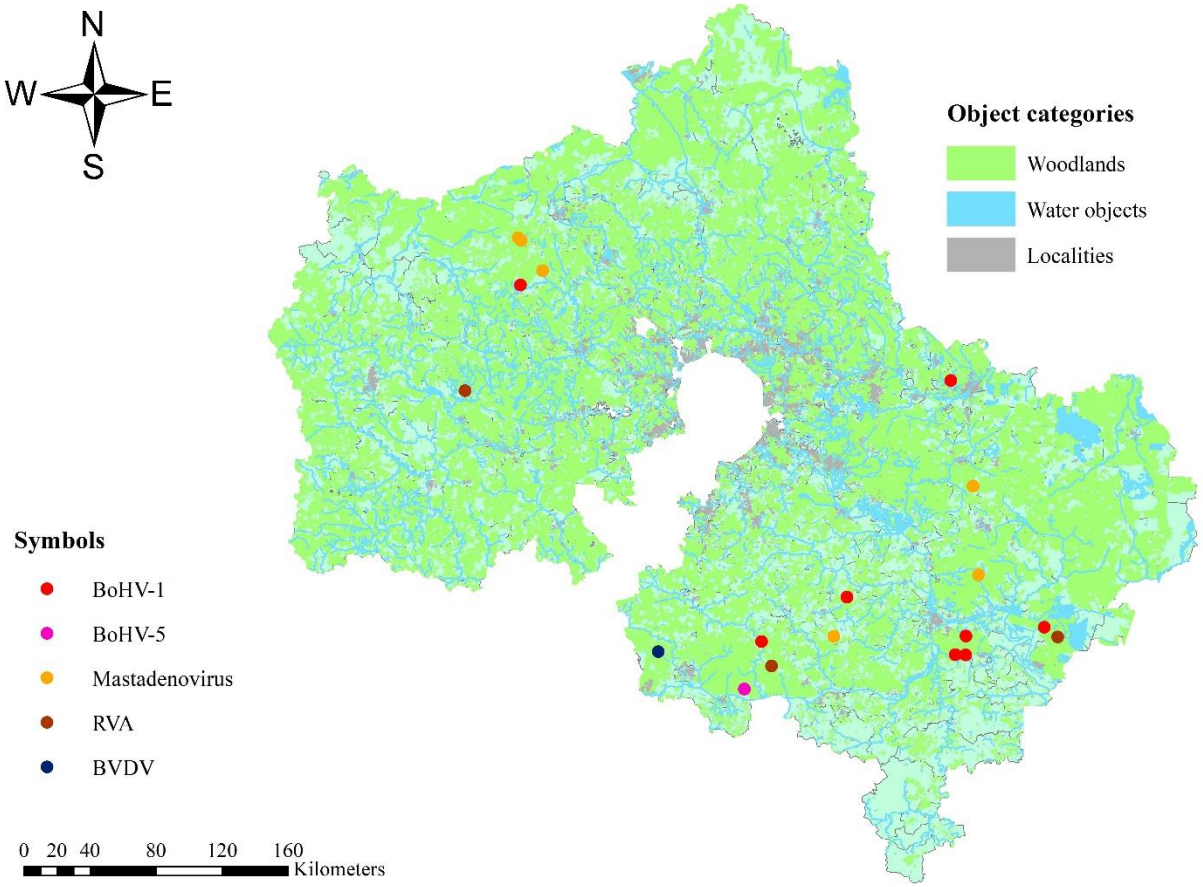

Figure 3 - The location of the shooting sites of positive animals on the map of the Moscow region

DISCUSSION

In the studies, was detected the DNA of bovine herpesviruses in various pathological material of 9 animals out of 178. The sampling was limited to districts of the Moscow region, and the sampling size for moose was significantly larger than the sampling for roe deer. In general, this data corresponds to the results of BoHV-1 DNA detection in European roe deer (Kálmán and Egyed, 2005). In a study of cervids in Hungary, BoHV-1 DNA fragments were found in 21.4% roe deer samples. More than 12.5% of samples from roe deer contained BoHV-4, but BoHV-5 DNA was not detected (Kálmán and Egyed, 2005). In the study no BoHV-4 DNA were detected, but in nasal swab of one roe deer sample was detected BoHV-5 DNA. These differences are likely due to different animal tissues tested.

The PCR method used to detecting DNA of α-herpesviruses in swabs and upper respiratory tract during the period of active replication of viral DNA, which this method is commonly used in equine viral tests (Pusterla and Leutenegger, 2015). For BoHV-1, the period of active shedding is 5-14 days after infection. During the latent period, when herpes viruses remain in the nerve cells, there are not detected in biological fluids and upper respiratory tract by PCR. In the study nerve tissues were not tested. The positive PCR result obtained in the study indicates not only the presence of herpesviruses, but also its activation in the animal. It can be assumed that the activation of BoHV-1 in wild cervids may be caused by climate change, as well as stressful situations.

The results of RVA studying correlate with the data of Shulyak et al., who demonstrated the presence of rotavirus RNA in moose in the Moscow region (Shulyak et al., 2020). Although intestinal tissues associated with rotavirus replication were not examined in this assay, viral RNA was detected in the pooled samples. This may indicate that the tissues were contaminated during sampling and necropsy. Previous genotyping of RVA strains revealed unique types in roe deer and fallow deer (Althof et al., 2023). Unfortunately, the low content of RVA RNA in the samples did not allow us to determine the genotype of the virus in moose.

The detection of BVDV RNA in a moose sample indicates the circulation of BVDV in the population of moose in the Moscow region. The question of whether wild cervids can be a source of BoHV-1 and BVDV for cattle remains controversial. Although animals on small farms can graze freely on summer pastures, contact between wild and domestic ungulates for viral transmission is limited. Additional studies of the genetic relationships of BoHV and BVDV isolates circulating in wild and domestic ungulates to determine if moose or roe deer can be infection reservoir are required. The

results obtained from identifying adenoviruses in moose and roe deer suggest the circulation of a new virus in cervids. Based on the results of phylogenetic analysis, it can be concluded that the detected adenovirus belongs to the genus Mastadenovirus. The detected virus probably affects the respiratory tract of animals, and most likely does not cause an acute disease.

The final classification of any new virus should be based on the most complete study of its biological properties and features of genome organization. A necessary tool for studying the genome is next-generation sequencing technology, which, if a sufficient amount of virus accumulates, allows one to decipher the structure of the genome in a short time. Unfortunately, in most cases, in vitro cultivation of wild animal adenoviruses and assessment of its biological characteristics is difficult due to the lack of appropriate cell cultures. In such cases, as noted in a review article on animal adenoviruses, one must rely on information obtained from deciphering individual fragments of DNA, which, despite its limitations, provides information about the diversity of adenoviruses (Harrach et al., 2019).

CONCLUSION

In conclusion, among the viral pathogens with zoonotic potential - hepatitis E virus and group A rotavirus, only rotavirus was detected in the studied animals. SARS-COV-2, which was detected in white-tailed deer, was not detected in cervids samples from Moscow region. However, to reduce the risk of transmission of viral pathogens, it is important to observe measures to prevent zoonotic diseases when interacting with animals, cutting carcasses, and consuming wild deer meat products. For the first time, a new mastadenovirus has been detected in different deer species, likely causing a mild respiratory disease. The work showed that viral infections do not pose a danger to the deer population of various species in the Moscow region. Further research is needed to study the biological characteristics of the new viral pathogen. Molecular studies indicate the circulation of BoHV-1, BoHV-5, BVDV in cervids in the Moscow region. Some authors point to the role of wild artiodactyls as a natural reservoir of viral pathogens of livestock diseases. This research work does not suggest a direct link between cervid infections and livestock diseases in the same region. The potential transmission routes of viruses between wild ruminants and livestock are still poorly understood. Additional molecular genetic studies are needed to confirm the circulation of the same strains among hunting animals and livestock.

DECLARATIONS

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Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contribution

S. Yatsentyuk designed the study, M. Krasnikova writing the manuscript. K. Dolinskaya collecting samples and data. A. Pchelnikov analysis data and manuscript writing. All authors drafted and revised the manuscript as read, evaluation and approved the final manuscript.

Acknowledgements

This work was supported by the Russian Science Foundation under grant NO. 22-26-00093, https://rscf.ru/en/project/22-26-00093/.

Competing interests

The authors declare no competing interests in this research and publication.

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