UNIVERSITY OF PÉCS

Biological and Sportbiological Doctoral School

Advancing canine distemper virus research through Nanopore Technology for surveillance and genome sequencing

PhD Thesis

Zsófia Lanszki

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INTRODUCTION

Infectious diseases are critical limitation factors regarding the population size and dispersal of wildlife species. Therefore, there has been a growing interest, during the last decades towards the understanding of emerging infectious diseases in wildlife. Detection and investigation of viral diseases are important factors for conserving protected and rare species, however, the elusive nature of several carnivores hampers our understanding of their viruses. Post-mortem examination (autopsy) of animals, furthermore excrements collected on field from passive monitoring programs provides a powerful method to obtain diverse samples, analyse and assess the factors in relation to ecosystem health. The results of retrospective surveillance may provide novel and relevant data for rare and hidden species, including the prevalence and genetic characteristics of certain infectious diseases.

Canine Morbillivirus (canine distemper virus, CDV) is a highly contagious viral agent with high epizootic potential and veterinary health impact. It affects a wide range of animals worldwide, with wild carnivores being particularly at risk. This serious viral disease, known as canine distemper, affects many mammalian species. CDV belongs to the *Paramyxoviridae* family in the *Morbillivirus* genus. It poses a risk to both domestic animals and wild carnivores globally. Without vaccination, dogs have a low chance of survival, and the virus can take weeks to months to be completely eradicated if the dog does survive. This virus endangers a broad range of wild animal populations, can cross species barriers, and represents a

significant risk to conservation and animal health worldwide. The elusive nature of these animals makes virological studies challenging, leading to a significant knowledge gap regarding the evolution of their viruses and the potential effects on population dynamics.

AIMS OF THE THESIS

During the thesis work, our primary goal was to identify the knowledge and technology gaps in canine distemper virus research. This was achieved by thoroughly examining clinical cases and epizootic events related to CDV. Our goal was to develop an efficient and versatile genomic surveillance method that could be widely employed. We aimed to showcase the practical application of this method across multiple setups and scenarios to demonstrate its potential effectiveness. Through this research, we sought to contribute valuable insights to the field of CDV research and foster advancements in genomic surveillance techniques for better disease management and control.

1. Identifying technological gaps in CDV research – Clinical case study with dog
2. Development of a novel genomic surveillance method and its application in outbreak investigation – CDV in red foxes
3. Retrospective genomic surveillance of CDV on a sample bank of Eurasian otters
4. Genomic surveillance among rare and elusive carnivore species - Terrestrial mustelids

5. Understanding the genomic patterns of outbreaks in the past - Red foxes and domestic dogs from Italy

6. Mobile genomic surveillance in a snapshot study setup – CDV in stray dogs of Bangladesh

MATERIALS AND METHODS

1. Clinical case study

In the present study, we traced the course of infection of a 1-year-old mixed-breed male dog. The animal had an unusually long course of persistent CDV infection. The dog excreted the CDV for 17 months with PCR positivity in urine samples collected from February 2019 through June 2020.

2. Outbreak investigation in red foxes

During spring to autumn 2021, according to our current estimates a minimum of 50 red foxes (*Vulpes vulpes*) died of CDV in Hungary, with CDV lesions. Oral, nasal and rectal swab samples were RT-PCR screened for Canine Distemper Virus from red fox carcasses (n=24).

3. Retrospective collection of Eurasian otter samples

Also, as part of a retrospective study, lung-tissue samples (n = 339) from Eurasian otters (*Lutra lutra*) were collected between 2000 and 2021 throughout Hungary.

4. Sample collection from multiple species of the terrestrial mustelids

Spleen and lung tissue samples of 170 road-killed mustelids belonging to six species 64 Steppe polecats (*Mustela eversmanii*), 36 European polecats (*Mustela putorius*), 36 stone martens (*Martes foina*), 18 pine

martens (*Martes martes*), 10 least weasels (*Mustela nivalis*) and 6 stoats (*Mustela erminea*) were collected between 1997 and 2022 throughout Hungary.

5. Sample collection from red fox and dogs in Italy

In Italy, the collection of samples from dogs (n=19) and red foxes (n=3) was between 2005 and 2019. The storage and processing of the samples were carried out at the University of Bari at the Department of Veterinary Medicine of Bari.

6. Sample collection from stray dogs in Bangladesh

We collected oral swab samples (n=257) from stray dogs in two major cities of Bangladesh, Rajshahi and Chattogram in 2023. Random sampling in Rajshahi was performed between 20-28 February (n=135) and in Chattogram between 4-5 March (n=122) among stray dogs with the aid of local veterinary students and animal rescuers.

The samples were screened for CDV using a Real-Time RT-PCR method. The complete viral genome was sequenced using a novel, pan-genotype CDV-specific amplicon-based sequencing method with Oxford Nanopore sequencing technology. Phylogenetic analysis was performed in all cases. Case of mustelids the potential recombinant CDV genomes were tested through recombination analysis using similarity plot and bootscan analyses in SimPlot software package.

RESULT AND DISCUSSION

1. Clinical case study

The urine samples were PCR-positive in February, March, April, May, July, August, September, October and November 2019 and January, February and June 2020 and finally tested negative in August 2020. The dog excreted the virus RNA for 17 months, which we successfully verified and quantified with qRT-PCR. Based on the phylogenetic analysis involving all known genotypes, the CDV strain explored in the case study belongs to the Arctic-like genetic lineage of the virus. It is positioned in the genetic cluster of previously reported CDV sequences from Italy, Austria, Hungary and Switzerland.

Examining the Hemagglutinin gene gives us the opportunity to identify the specific CDV lineage and it's a widely used method for genetic classification. However, using the complete genome gives valuable additional opportunity to gain insight into more detailed evolutionary patterns (such as unique mutations or recombination patterns). Therefore, the complete genome analysis is a preferable tool than H gene analysis.

2. Red foxes from Hungary

Carcasses of 6/5 cubs and 1/1 adult fox from the spring period, and 5/3 cubs, 10/10 juvenile and 2/2 adult foxes from the summer period were positive for CDV with RT-PCR. A total of 21 of the 24 foxes tested were positive. Finally, 19 complete CDV genomes were sequenced from foxes with a pan-genotype CDV-specific amplicon-

based sequencing method resulting in high sequencing coverage. The development of the sequencing protocol is a main result of the current thesis. Based on the phylogenetic analysis of all currently recognized genotypes, the CDV strains of this study belong to the Europe lineage. They are positioned in the genetic cluster of previously reported CDV sequences in Europe.

In the case of human outbreaks (such as the recent COVID-19 pandemic), the development and use of rapid, sensitive and specific NGS-based genomic epidemiological tools is very common, especially the use of amplicon sequencing-based approaches. Following this trend, using similar methods is getting more common in animal disease surveillance as well. Based on our current knowledge this is the first NGS-based amplicon sequencing method for CDV.

3. Eurasian otters from Hungary

Eurasian otter samples were collected from all nineteen counties throughout Hungary. From these, canine distemper virus RNA was identified by real-time RT-PCR screening in 2/339 samples. Both originated from western Hungary; one was collected in 2006 and the other in 2010. The first infected animal was a young (4–5 months old) male in poor body condition and was found to be deceased due to natural infection, on the edge of a marshland (Kis-Balaton). The second was an adult male in good body condition, found as road-killed near a river (Rába). Both viral sequences from otters were grouped to a European lineage based on the hemagglutinin-gene and complete

genome phylogenetic classification. In the current study we present the first complete CDV genome and H gene sequence from the Eurasian otters, enriching the diversity of available CDV genomes.

Although the Eurasian otter is a well-studied "key species" of aquatic habitats in ecological and zoological terms, there is a considerable lack of knowledge of the microbiological context across its distribution range. The detection of CDV in Eurasian otters in Hungary provides valuable insights for their conservation. Our findings allow us to make practical recommendations to protect the species.

4. Terrestrial mustelids from Hungary

Canine distemper virus RNA was detected in three out of the six investigated species: 2 positives out of 64 steppe polecats, 1 positive out of 36 European polecats and 2 positives out of 36 stone martens. Samples screened from 18 pine martens, 10 least weasels and 6 stoats were negative. Complete genomes were successfully retrieved from all positive samples. Based on the phylogenetic analysis of complete genomes, all these sequences belong to the Europe lineage. The SimPlot analysis confirmed the recombination of the Hemagglutinin gene with a closely related, Europe lineage strain. Also, it confirmed multiple additional recombination points in the genome.

We present the circulation of CDV throughout the country over several years, supporting the endemic nature of this virus among mustelids. An important finding of the current study is the detection

of CDV in wild-living steppe polecats. In the current study we present the first two complete CDV genomes from the steppe polecat, enriching the diversity of available CDV genomes. By revealing the presence of a recombinant CDV strain in these animals we demonstrated the importance of generating complete genomic data. This approach may ultimately lead to better understanding CDV evolution, since partial genome fragments are not suitable to understand the impact of recombination events in CDV evolution or the role of coding regions other than H. Our study demonstrated that road-killed carcasses are a valuable source of CDV surveillance in wildlife species.

5. Red foxes and domestic dogs from Italy

During our work, we were able to sequence the coding region of the genome from samples from the past, and based on the phylogenetic analysis, in the case of the dogs 4 sequences belong to Europe lineage, and 14 sequences belong to Arctic-like lineage. The sequences from 3 red foxes were all grouped into the Europe lineage. Our study aimed to fill the gap in knowledge regarding the emergence and patterns of the Arctic-lineage in Italy, particularly in the middle region. Through our research, we successfully identified the underlying patterns of CDV dispersal in mid-Italy. Nevertheless, our research emphasized the importance of genome-scale monitoring of CDV evolution, which may serve as a first step measure to comprehend genomic evolution. Retrospective examination methods offer a valuable opportunity for the rapid evaluation of current

epidemics and the ability to infer future spread patterns and potential host species. These methods aid in understanding the virus and play a crucial role in preparing for future epidemics.

6. Stray dogs from Bangladesh

Overall, 3.9% (10/257) of sampled animals were positive for CDV by RT-PCR. We detected CDV RNA only in the samples collected in Rajshahi city where 7.4% (10/135) of the swab samples were positive. During the sampling activity all positive dogs were observed as asymptomatic. During complete genome sequencing, we managed to obtain 4 complete sequences. Based on the results, the detected virus belonged to the India-1/Asia-5 lineage.

In this study, we utilized RT-PCR and Oxford nanopore sequencing techniques to confirm the presence of canine distemper virus in Bangladesh. To the best of our knowledge, these are the first CDV sequence data obtained from the country. The research was conducted in collaboration with local experts, with a focus on knowledge transfer and improving the diagnosis of CDV in stray dogs. In this work we successfully detected and sequenced the complete genome of CDV in Bangladesh using a mobile laboratory approach with the Nanopore Sequencing method. The findings underscore the importance of monitoring and preventing the spread of CDV in regions with low vaccination coverage, ultimately contributing to the well-being of both animals and humans.

CONCLUSION

In this thesis we describe the significant progress that has been made from the recognition of the knowledge and technological gaps in CDV diagnostics to the development and widespread application of novel genomic epidemiological methods. Such methods are already applied in human outbreak investigation but less common in animal health research. Canine distemper is a serious viral disease that affects many species around the world and has notable conservational effects on certain species. Rapid and effective understanding of the long-term presence of CDV in free-living mammals is of great importance. As we provided novel genomic information of CDV from multiple continents and geographic areas, we also highlighted the importance of analyzing genome-scale data by identifying recombination patterns and epidemiological scenarios.

PUBLICATIONS

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Publication related to the thesis topic

Lanszki, Z., Lanszki, J., Tóth, G. E., Cserkész, T., Csorba, G., Görföl, T., Csathó, A.I., Jakab, F., Kemenesi, G. (2022) Detection and sequence analysis of Canine morbillivirus in multiple species of the Mustelidae family. *BMC Veterinary Research*, 18, 450. DOI:10.1186/s12917-022-03551-7
IF: 2.600

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