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Virus discovery in dogs with neurological disease and viral encephalitis of unresolved etiology by high-throughput sequencing based metagenomics

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Neurological diseases in dogs are frequently of inflammatory and infectious nature and can pose as a challenge in clinical diagnostics and therapeutics.[1] Viral pathogens are often the causative agent responsible for inflammatory diseases of the central nervous system (CNS) in dogs, causing non-suppurative encephalitis.[1,2] Conventional diagnostics such as PCR and immunohistochemistry (IHC) are used for virus detection and are based on the presumption of the causative virus pathogen. However, the selected diagnostic tool is based on this and identification of the virus may remain unsuccessful, either due to clearance of the virus or due to the lack of available diagnostic capacities.[1,3]
Viral metagenomics is a cutting-edge tool for virus detection that does not rely on a priori knowledge of the causative virus.[4] By combining high-throughput sequencing (HTS) and bioinformatics, it is possible to detect viral sequences in any given sample and to detect potentially novel viruses based on sequence similarities and genome patterns.[4] This project has taken advantage of this technique to be applied on archival formalin-fixed paraffin embedded (FFPE) brain material of dogs suffering of unknown viral encephalitis

Archival FFPE brain samples of dogs diagnosed with a viral encephalitis of unknown etiology were investigated. During mining of the archives of the Division of Neurological Sciences of the Vetsuisse Faculty Bern, a general overview was gained of stored cases of dogs that died with neurological diseases. For this project, 50 cases of dogs between the years 1976 and 2021 and were diagnosed with non-suppurative encephalitis of unknown viral origin were selected for metagenomics analysis. An additional case of a dog with unknown viral encephalitis from the year 2022 was added during the course of this project, for which fresh frozen (FF) brain material was available. RNA from selected FFPE brain sections with the most severe lesions based on corresponding histological slides was extracted. Individual RNA extracts were pooled for library preparation and HTS. Subsequent bioinformatics analysis was performed with an in-house established bioinfo pipeline for the detection of viruses and discovery of potentially new viruses. Viral candidates deriving from this pipeline were confirmed by conventional diagnostic methods, including PCR, immunohistochemistry (IHC) and *in situ* hybridization (ISH) on individual animal (sample) level. Identified samples positive for viruses were re-sequenced at higher read depths to obtain more comprehensive viral genomic sequences.

A general overview was gained during mining of the archives with over 6500 cases of dogs with neurological diseases diagnosed and archived between the years 1937 and 2021. Further investigation showed a high proportion of 25% (1633/6533) of the cases displaying an inflammatory pattern with involvement of the brain. Based on neurological assessment, 622/1633 cases of encephalitis could be clearly traced back to a viral cause. In 84.2% (524/622) of the encephalitis cases the viral cause could be identified, whereas in 15.8% (98/622) of the cases the viral agent could not be identified. Based on the severity of lesions and the availability of tissues, we selected 51 cases of non-suppurative encephalitis of unknown viral origin for metagenomics analysis. We were able to solve the viral etiology in 17/51 cases of dogs (Table 1).

ID	Year	Canton	Age	Breed, Age	Viral Hit	Mapped reads	Coverage [%]	Cq value
19995	1989	(CH)	4 months	Dobermann	CDV	2'498	89.02	29.85
20565	1990	TI	1 year	Mixed Breed		177'310	97.80	22.55
20684	1990	GE	4.5 years	Chow Chow Mixed		1'557	70.38	27.78
18318	1986	BE	4 years	Greyhound	TBEV	12	3.62	35.57
18772	1987	(CH)	5 years	Pyrenean Sheepdog		13	3.33	34.68
21482	1991	BE	4 years	Rottweiler		18	3.85	32.73
27061	1998	(D)	2 years	n/a		39	10.30	29.41
31022	1999	NE	n/a	n/a		220	48.47	30.29
31140	1999	LU	6 years	Rottweiler		4	0.9	31.39
45100	2010	BE	12 years	Bobtail		110	15.06	30.36
51277	2019	BE	5.5 years	Sheltie		15	4.62	32.52
51309	2021	BE	n/a	Mixed Breed		29	7.96	30.36
51485	2020	BE	n/a	Pyrenean Sheepdog		0	-	31.25
60481	2015	BE	1 year	Labrador Retriever		75	19.93	30.36
S19-1723	2019	TG	3 weeks	Dalmatian		3'640	99.45	23.93
12268	1976	NE	3.5 years	Bernese Mountain Dog	CVeV	321	9.8	33.02
51874	2022	(CH)	5 years	Mixed Breed, 5 y		234	8.6	30.99

Table 1: Overview of viral hits assigned to individual case samples. [CH] Switzerland, [TI] Ticino, [GE] Geneva, [D] Germany, [NE] Neuchâtel, [BE], Bern, [TG] Thurgau. [n/a] not available. [CDV] Canine Distemper Virus, [TBEV] Tick-Borne Encephalitis Virus, [CVeV] Canine Vesivirus.

Three case samples were confirmed positive for Canine Distemper Virus (CDV) by PCR and IHC. Notably, these three CDV cases did not display typical demyelinating lesions and inclusion bodies associated with CDV and were therefore left undiagnosed. Furthermore, we were able to detect Tick-Borne Encephalitis Virus (TBEV) as a frequent causative virus and 12/51 cases were confirmed as positive by PCR and ISH. Interestingly, the strongest positivity for TBEV originated from a three-week-old puppy, which is an unusual finding given the transmission cycle of TBEV via tick bites. Furthermore, our unbiased approach was able to detect Canine Vesivirus (CVeV) as known but unexpected virus by PCR and ISH both in an archived FFPE brain sample from 1976 as well as in an FF brain sample from a recent case from 2022 (Figure 1).

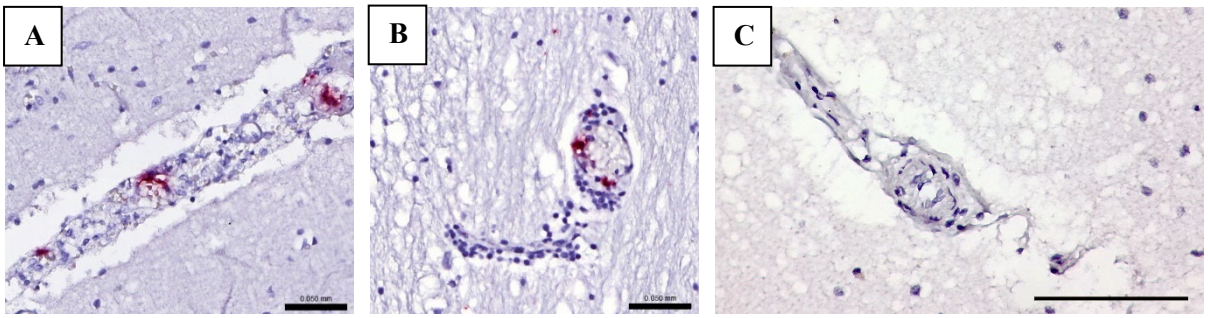

Fig. 1 *In situ* detection of CVeV RNA. **A** Positive signals (red granular staining) can be seen in the cells morphologically compatible with mononuclear cells located in the meninges of the cerebellum from case sample '12268'. **B** Positive signal detected in a vascular structure in cells morphologically compatible with endothelial cells and mononuclear cells located in the thalamus of case sample '51874'. **C** Negative control sample without detection of CVeV RNA. Scale bars: 50 µm.

Overall, our unbiased metagenomics approach was able solve the viral etiology in 17/51 archived cases of dogs suffering from encephalitis. Our results provide further insight into pathogenicity diversity of CDV and allow for a more precise identification of genomic variants. Furthermore, TBEV was identified as a frequent viral agent in canine encephalitis cases where conventional diagnostic methods failed to confirm the strong suspicion of TBEV involvement. The unusual finding of the TBEV positive puppy was followed up by further work-up and found that two of its siblings, which were not included in this study, were also positive for TBEV. An unanswered question remains in regards of the transmission route of TBEV given the very young age of the animals. These findings are currently being further investigated. And finally, we were able to detect CVeV as an unexpected virus in encephalitis cases and contribute to increasing the knowledge of the pathogenesis of viral encephalitis in dogs. We are currently in the final stages of writing a manuscript that will be published as a scientific paper in the journal of the Swiss Veterinarians Association (SAT Schweizer Archiv für Tierheilkunde).

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[3] Schwab S, Herden C, Seeliger F, Papaioannou N, Psalla D, Polizopulou Z, Baumgärtner W. Non-suppurative meningoencephalitis of unknown origin in cats and dogs: an immunohistochemical study. J Comp Pathol. 2007 Feb-Apr;136(2-3):96-110. doi: 10.1016/j.jcpa.2006.11.006. Epub 2007 Feb 1. PMID: 17275833; PMCID: PMC7126569.

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