Virus discovery in dogs with non-suppurative encephalitis reveals a high incidence of tick-borne encephalitis virus infections in Switzerland

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Die Virusdiagnostik bei Hunden mit nicht-eitriger Enzephalitis zeigt eine hohe Inzidenz von Frühsommer-Meningoenzephalitis-Virus Infektionen in der Schweiz

Virusinfektionen sind eine häufige Ursache einer disseminierten nicht-eitrigen Enzephalitis bei Hunden. Mit routinemässigen Diagnosemethoden kann es jedoch sein, dass spezifische Viruserkrankungen nicht diagnostiziert werden, weil das Virus weitgehend oder vollständig eliminiert wurde oder weil das Virus unerwartet oder unbekannt ist. Um die virale Ätiologie in Archivfällen von Hunden mit nicht-eitriger Enzephalitis zu untersuchen, wurde ein auf Metatranskriptomik basierender Ansatz, der Hochdurchsatzsequenzierung (HTS) und bioinformatische Analyse kombiniert, verwendet. In formalin-fixiertem, in Paraffin eingebetteten (FFPE) Gehirnmaterial aus den Jahren 1976 bis 2021 wurde eine hohe Inzidenz des durch Zecken übertragenen Frühsommer-Meningoenzephalitis-Virus (FSMEV) nachgewiesen. Darüber hinaus wurde das Canine Staupevirus (CDV) ohne typische demyelinisierende Läsionen identifiziert und das Canine Vesivirus (CaVV) wurde als unerwartetes Virus im Zusammenhang mit nicht-eitriger Enzephalitis entdeckt. Wir haben die Viruspräsenz im Gehirngewebe an den Entzündungsherden durch Immunhistochemie (IHC) und In-situ-Hybridisierung (ISH) nachgewiesen. Diese Ergebnisse unterstreichen den Wert neuer Sequenzierungstechnologien in der Veterinärdiagnostik und erweitern unser Wissen über die Ätiologie der Enzephalitiden bei Hunden.

Schlüsselwörter: Hunde, Enzephalitis, Virus, Metatranskriptomik, Hochdurchsatzsequenzierung

Summary

Viral infections are a frequent cause of disseminated non-suppurative encephalitis in dogs. However, using routine diagnostic methods, the specific virus may remain unknown due to extensive or complete viral clearance or because the virus is unexpected or new. A metatranscriptomics-based approach of combining high-throughput sequencing (HTS) and bioinformatics analysis was used to investigate the viral etiology in archival cases of dogs with non-suppurative encephalitis. In formalin-fixed paraffin embedded (FFPE) brain material from the years 1976 to 2021 a high incidence of tick-borne encephalitis virus (TBEV) was detected. Moreover, canine distemper virus (CDV) was identified without typical demyelinating lesions and canine vesivirus (CaVV) was detected as an unexpected virus associated with non-suppurative encephalitis. We demonstrated the viral presence in brain tissues at the sites of inflammation by immunohistochemistry (IHC) and *in situ* hybridization (ISH). These results highlight the value of emerging sequencing technologies in veterinary diagnostics and expand our knowledge on the etiologies of encephalitis in dogs.

Keywords: dogs, encephalitis, virus, metatranscriptomics, high-throughput sequencing

Introduction

Infectious neurological diseases in dogs present a significant challenge in clinical diagnostics and therapeutics.[42] Viruses are frequently the causative agents of fatal inflammatory central nervous system (CNS) diseases in dogs, resulting in a wide range of neurological symptoms and pathologic lesions of non-suppurative encephalitis.[42] Conventional diagnostics such as polymerase chain reaction (PCR) and immunohistochemistry (IHC) are used for virus detection and selected based on the presumption of the causative virus. However, in a considerable number of cases, the etiological virus remains unidentified, either due to its clearance or due to limited diagnostic capacities.[34,42]

Viral metatranscriptomics is a cutting-edge tool for an unbiased virus detection by combining high-throughput sequencing (HTS) of ribonucleic acid (RNA) and bioinformatics.[14,50] This refined method provides the ability to detect genomic RNA of RNA viruses and transcripts of RNA and deoxyribonucleic acid (DNA) viruses in a given sample and allows the discovery of potentially novel viruses based on sequence similarities and virus genome patterns.[9,14] The aim of this study was to identify the viral etiology in archived brain tissues of dogs with a histological diagnosis of a viral type polioencephalitis by viral metatranscriptomics. We were able to detect a viral etiology in 16/50 cases. We detected canine distemper virus (CDV) in cases without typical demyelinating lesions associated with canine distemper and confirmed tick-borne encephalitis virus (TBEV) as a frequent cause of non-suppurative encephalitis. Furthermore, we were able to discover canine vesivirus (CaVV) as a known but unexpected virus in dogs with encephalitis. Overall, our results further increase the knowledge of the spectrum of neuroinfectious pathogens in dogs and potentially other species.

Materials and Methods

Case review and selection

Cases of dogs with a histological diagnosis of non-suppurative encephalitis suggesting a viral etiology were selected from the archives of the Division of Neurological Sciences, Vetsuisse Faculty, University of Bern, based on documented patient history, documented neurological signs and postmortem histopathological findings. A total number of 43 canine cases diagnosed with non-suppurative encephalitis of unknown viral origin between the years 1976 and 2021 were selected for HTS and metatranscriptomics. Based on typical histological lesions of viral encephalitis, which includes perivascular cuffs of mononuclear cells, reactive gliosis, and neuronal necrosis, formalin-fixed and paraffin-embedded (FFPE) brain sections with the most severe lesions were selected for total RNA extraction.[45] In addition, seven cases of dogs with non-suppurative viral encephalitis of unknown etiology were provided by the Institute of Veterinary Pathology of the Vetsuisse Faculty of Zurich. One of these seven samples comprised, additionally to FFPE blocks, fresh frozen (FF) brain tissue. Cases of meningoencephalomyelitis of unknown origin (MUO), which is an umbrella term established for non-infectious and presumably immune-mediated inflammations of the brain of dogs with necrotizing, non-suppurative and/or granulomatous characteristics, were not included in this study.[8]

RNA extraction, Library preparation and High-Throughput Sequencing

Four 10 µm thick sections per case were cut from FFPE blocks. After deparaffinization with xylol, total RNA was extracted using the RNeasy FFPE kit (Qiagen, 4010 Basel, Switzerland) Total RNA from the FF brain sample was extracted using Trizol reagent (Sigma Aldrich, Merck & Cie, 9470 Buchs, Switzerland). Individual RNA extracts were pooled (8 to 12 cases per pool) in equimolar amounts and each pool was spiked with rabies virus RNA, which served as a positive control. To assure that none of the dogs included in the study were affected by rabies virus, all samples were tested by a rabies virus specific RT-PCR[13] beforehand and were negative. All libraries were prepared with the CORALL Total RNA-Seq Library kit (Lexogen, 1030 Vienna, Austria) and sequenced in single-end mode for 100 cycles, resulting in ~100 nucleotide (nt) reads, in an Illumina NovaSeq 6000 machine. Initial sequencing of pooled samples was performed at a target read depth of ~20 Mio. reads per sample.

Bioinformatics

HTS reads were analyzed with an in-house established pipeline for the detection of RNA and DNA viruses[50]: 1) Raw reads were subjected to a quality control process using fastqc (Ver. 0.11.7).[2] 2) Reads were trimmed with fastp (Ver. 0.12.5)[7] at the 5' and 3' end in order to remove molecular identifiers, which were added during the library preparation. 3) Trimmed reads were mapped to the dog reference genome (Canis lupus familiaris 3.1) using STAR (Ver. 2.7.3a).[10] 4) The remaining reads were mapped to the RefSeq viral genome database for the detection of known viruses using bowtie2 (Ver. 2.3.4.1).[20] 5) *De novo* assembly of reads was done with SPAdes (Ver. 3.12.0),[25] resulting in contiguous sequences (contigs) > 500 nt. Finally, viral contigs were mapped to viral reference strains using the Geneious Prime 2023.1 software package (https://www.geneious.com). In addition to the *de novo* assembly, a reference guided assembly of reads and contigs was performed to increase the viral genome coverage. Consensus sequences were generated and subjected to BLASTn (Ver. 2.10.1+)[1] searches, to reveal best viral hits.

RT-qPCR

Viral sequences from initial HTS of pooled samples were assigned to individual samples by RT-qPCR. Taqman pro-

be-based RT-qPCR assays were used for the detection of CDV and TBEV with the AgPath-ID One-Step RT-qPCR System (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA USA 02451) and established primer-probe combinations.[11,32] A BRYT green assay was used for the detection of CaVV with the GoTaq One-Step RT-qPCR (Promega) kit. CaVV primers (Forward 5'-ACCACACTTTCGTTCGACCA-3', Reverse 5'-AACGCTAAGTGCTCTGCCAT-3') were designed using Geneious Prime. For the detection of CDV by RT-qPCR, a cq value below 30 was considered as positive to avoid false positive results due to lab contamination with CDV cDNA, which has been intensively used over decades in our division in research projects. The cut-off for the detection of TBEV and CaVV was set at a cq value of 40.

Immunohistochemistry for the detection of CDV

Brain tissue sections were subjected to immunohistochemistry using the mouse monoclonal antibody D110 directed against the nucleoprotein (N) antigen of CDV.[40] D110 binding was detected using the Dako Real™ Detection System (Agilent Technologies, Santa Clara, CA USA, 95051).

In situ hybridization for the detection of TBEV and CaVV

FFPE brain tissue sections (3 µm) were cut for *in situ* hybridization (ISH). RNA ISH probes targeting the positive strand of the ORF1 of the CaVV genome (nt position 81–987 of NC_004542.1: cat no. 1228351-C1) and the NS3 of the TBEV genome (nt position 4923–5818 of U27495.1: cat no. 575601), respectively were ordered from Advanced Cell Diagnostics. ISH was performed with the RNAscope® 2.5 HD Reagent Kit-RED (Advanced Cell Diagnostics, Newark, CA USA, 94560).

Phylogenetic comparisons

Phylogenetic analysis was conducted for CVD, TBEV and CaVV consensus sequences by comparison to various representative viral reference strains obtained from NCBI GenBank. Sequences for the phylogenetic analysis of CDV and TBEV were imported into MEGA 11 (Ver. 11.0.13)[41] and

Table 1: Details of canine cases with non-suppurative encephalitis with viral hits detected by high-throughput sequencing (HTS).

Case ID	Viral hit	Year of autopsy	Canton of origin	Breed	Age	Clinical signs
12268	CaVV	1976	NE	Bernese Mountain Dog	3,5 years	vomiting, diarrhea, neck stiffness, spasticity of all limbs, opisthotonos
18318	TBEV	1986	BE	Greyhound	4 years	high fever, apathy, bilateral miosis, reduced menace reflex, sluggish pupil reactions, tetraparesis, passive manipulations of the neck resulted in severe pain
18772	TBEV	1987	n/a	Pyrenean Sheepdog	5 years	vomiting, diarrhea, apathy, tremors, hypermetria, increased spinal reflexes
19995	CDV	1989	n/a	Dobermann	4 months	CNS disorders, aggressive behavior, trembling, fever, salivation, balance disorder
20565	CDV	1990	TI	Mixed Breed	1 year	behavioral changes, fever, salivation and purulent nasal discharge, balance disorder, ataxia, progressive non-ambulatory tetraparesis
20684	CDV	1990	GE	Chow Chow Mixed Breed	4,5 years	purulent rhinitis, vomiting, ataxia, tendency to fall on right side, abnormal menace reflex
21482	TBEV	1991	BE	Rottweiler	4 years	fever, tetraparesis, ataxia, hypermetria front limbs, hyporeflexia hind limbs, cervical hyperesthesia
27061	TBEV	1998	(D)	n/a	2 years	fever, reduced consciousness, ataxia, difficulties swallowing
31022	TBEV	1999	NE	n/a	n/a	tetraparesis, fever, apathy, anorexia, diarrhea, delayed postural and righting reactions, ventral strabismus left, head tilt to left side, tendency to fall on left side
31140	TBEV	1999	LU	Rottweiler	6 years	fever, generalized spasticity, hyperkinesia, generalized seizures
45100	TBEV	2010	BE	Bobtail	12 years	non-ambulatory tetraparesis, reduced reflexes, reduced muscle tonus, fever, pain cervical vertebrae, megaesophagus
51277	TBEV	2019	BE	Sheltie	5,5 years	tetraparesis, reduced spinal reflexes hind limbs, reduced postural reactions hind limbs, dystonia, tendency to drift to left side
51309	TBEV	2021	BE	Mixed Breed	n/a	ataxia, apathy, behavioral changes, fever, back pain, intention tremor, pace / amble gait, generalized seizures
60481	TBEV	2015	BE	Labrador Retriever	1 year	progressive tetraparesis, ataxia, drifting, apathy
S19–1723	TBEV	2019	TG	Dalmatian	3 weeks	epileptic seizures
51874	CaVV	2022	n/a	Mixed Breed	5 years	paraparesis / paraplegia, proprioceptive deficits, Schiff-Sherrington Syndrome, fever, vomiting, pain cervical vertebrae

[CaVV] canine vesivirus, [TBEV] Tick-borne encephalitis virus, [CDV] canine distemper virus. [TI] Ticino, [GE] Geneva, [D] Germany, [NE] Neuchâtel, [BE], Bern, [TG] Thurgau. [n/a] not available.

aligned using the built-in ClustalW alignment tool. Maximum-likelihood trees were generated based on the Tamura-Nei model[40] with 1'000 bootstrap replicates. Phylogenetic analysis for CDV was based on a partial sequence of the hemagglutinin (H) and for TBEV based on the complete sequence of the envelope (E) gene. Phylogenetic analysis for CaVV was based on the full genome sequences and aligned using the MAFFT (Ver. 7.475)[17] software. A maximum-likelihood tree was constructed with IQ-Tree (Ver. 2.0.3)[23] with 1'000 bootstrap replicates.

Results

A high proportion of dogs with neurological disorders show inflammatory histopathological lesions

In a first step, canine cases (over 6'500) diagnosed with a neurological disease between the years 1937 and 2021 were identified in the archives of the Division of Neurological Sciences of the Vetsuisse Faculty Bern. A high proportion of the cases, 25 % (1633/6553) showed inflammatory lesions with involvement of the brain. Based on neurological and histopathological assessment, 622/1633 cases of encephalitis were associated with a viral etiology or showed lesions suspicious of a viral infection. In 84,2 % (524/622) of these viral encephalitis cases, the viral etiology, e.g., rabies virus or CDV, could be identified by conventional/routine diagnostic assays. However, in 15,8 % (98/622) cases, the etiologic agent could not be determined.

Virus discovery by high-throughput sequencing and conventional/routine diagnostic methods

Viral metatranscriptomics was performed on pooled viral RNA extracted from archived brain tissues from 50 unresolved cases of putative viral encephalitis. We found sequence reads and contigs that mapped to Canine Distemper Virus

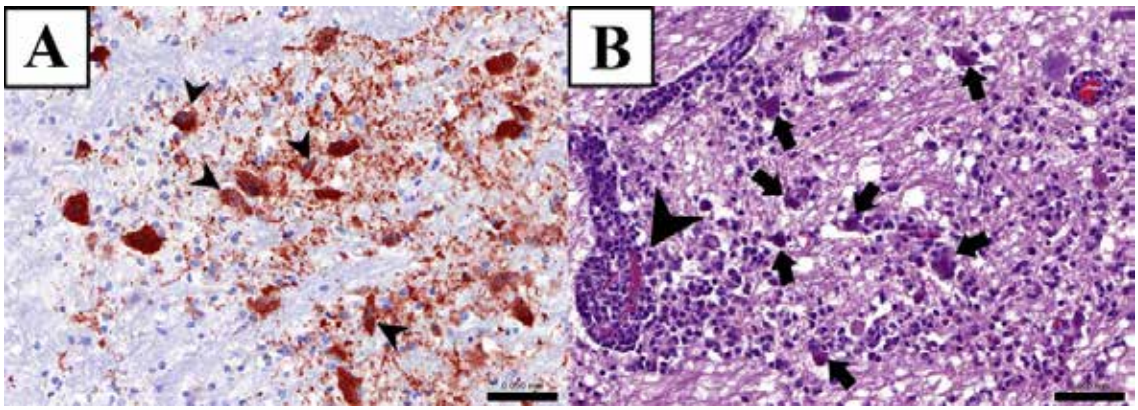

Figure1: Immunohistochemistry for canine distemper virus (CDV) nucleoprotein. **A** Positive immunolabeling shown as red granular staining (arrow heads) in the neuronal somata located in the brainstem of case '19995'. **B** Corresponding hematoxylin & eosin (HE) stained histological slide of case '19995' with inflammatory lesions in the gray matter, perivascular cuff consisting of mononuclear cells (arrowhead) and neuronal necrosis (arrows). Scale bars: 50 µm.

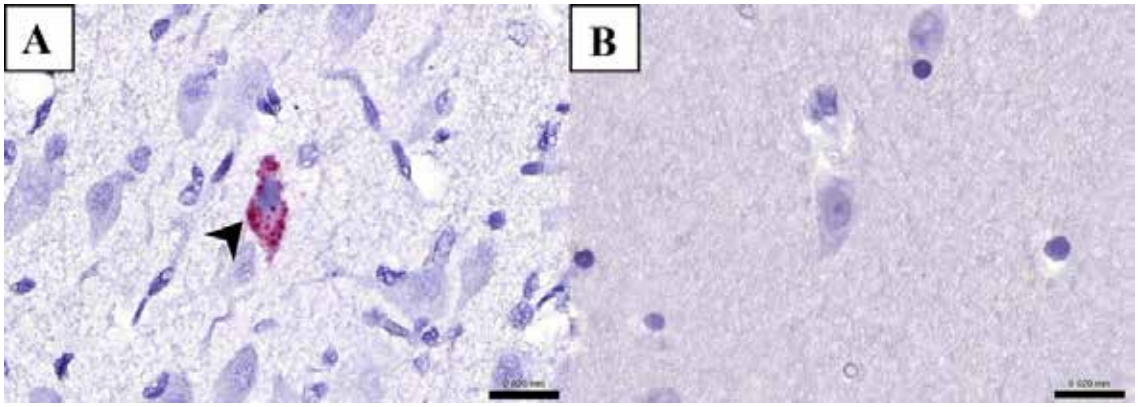

Figure2: *In situ* detection of tick-borne encephalitis virus (TBEV) RNA. **A** Positive hybridization signal shown as red granular staining (arrowhead) can be seen in an intracortical neuron of 'S19–1723'. **B** TBEV negative control brain tissue without hybridization signal. Scale bars: 20 µm.

(CDV, GenBank accession NC_001921.1), Tick-Borne Encephalitis Virus (TBEV, NC_001672.1) and Canine Vesivirus (CaVV, NC_004542.1). The presence of the three viruses in individual samples was confirmed by conventional RT-qPCR: 3/50 samples were positive for CDV, 11/50 samples were positive for TBEV, and 2/50 samples were positive for CaVV. Details on these cases are presented in Table 1.

IHC for CDV showed a strong neuronal labeling in all three RT-qPCR positive cases (exemplified for case '19995' in Figure 1). In the TBEV positive cases, ISH for viral RNA showed mostly a faint signal, located along structures associated with axons. A neuronal signal positive for TBEV RNA was observed in case 'S19–1723' (Figure 2). CaVV RNA was detected by ISH multifocally distributed along the cerebrum and cerebellum displaying a reminiscent pattern of positive perivascular leucocytes (Figure 3).

To generate more comprehensive viral sequences, we re-sequenced samples of all PCR positive cases individually and at higher read depths, ~80 Mio. reads per sample. All samples revealed reads mapping to the respective viruses

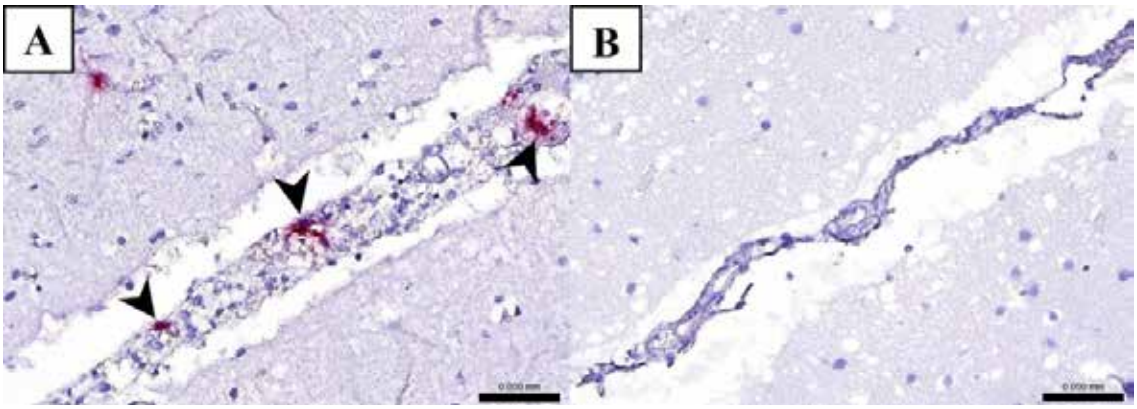

Figure 3: *In situ* detection of canine vesivirus (CaVV) RNA. **A** Positive hybridization signals shown as red granular staining (arrow heads) are associated with cells morphologically compatible with mononuclear cells in a perivascular cuff in the brain stem of case '12268'. **B** Negative control brain tissue without hybridization signal. Scale bars: 50 µm.

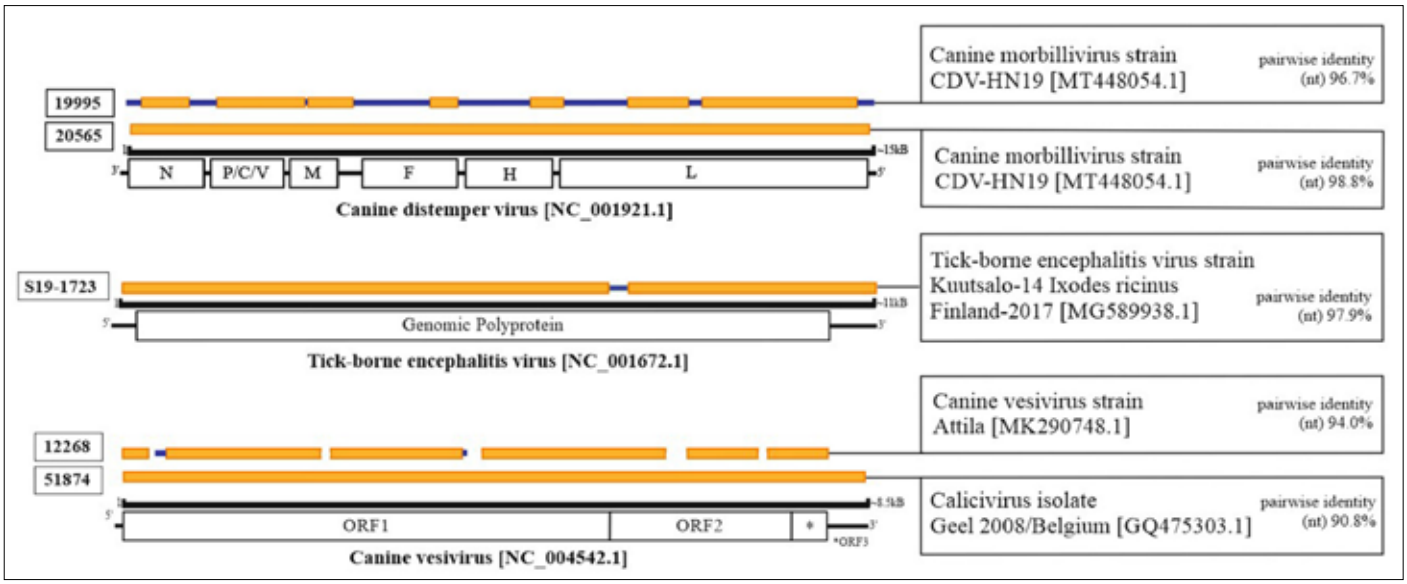

Figure 4: Schematic presentation of the reference guided assembly of contigs and mapped reads covering the viral reference genome of canine distemper virus (CDV), tick-borne encephalitis virus (TBEV) and canine vesivirus (CaVV). Consensus sequences of assembled contigs (orange) and mapped reads (blue) were generated. For two CDV cases ('19995' and '20565') a full CDV genome coverage could be generated. A full TBEV genome coverage could only be generated for the TBEV case 'S19–1723', for which mapped reads bridged the sequence gap between the two assembled contigs. As for the CaVV cases, only the case '51874' generated a full CaVV coverage, whereas in the case '12268' reads mapping to the CaVV lacked to fully bridge the gaps between contigs. BLASTn searches performed on consensus sequences and best hits are shown along with pairwise identity percentages on nucleotide (nt) level. Accession numbers in square brackets were obtained from NCBI GenBank.

detected by RT-qPCR, but the number of reads differed between samples, which reflects differences in viral RNA loads (Table 2). *De novo* assembled larger contigs were obtained for two CDV cases ('19995', '20565'), for one TBEV case ('S19–1723') and for both CaVV cases ('12268', '51874'), but not in the other cases (Figure 4).

Phylogenetic comparison

We compared CDV, TBEV and CaVV sequences to representative members of their virus species and genera by phylogenetic analysis. The phylogenetic tree for CDV showed a clustering of the two CDV cases ('19995' and '20565') with CDV strains Rockborn, which belong to a vaccine lineage, and HN19 (Figure 5A). The TBEV sequence found in case 'S19–1723' showed a close phylogenetic relation to TBEV sequences isolated from ticks in Switzerland in 2010 (Figure 5B). Finally, for CaVV, the case '51874' matched to representative members of the *Caliciviridae* family, genus *Vesivirus* (Figure 5C).

Discussion

Canine distemper virus in cases lacking hallmark lesions

We found CDV, a morbillivirus of the family *Paramyxoviridae,* in the three canine encephalitis cases ('19995', '20565' and '20684') from the years 1989/90 by HTS, IHC and RT-qPCR. On histopathological examination, the brain of the three cases did not reveal a demyelination of the white matter, which is a typical finding in chronic phases of CDV.[44] Instead, the lesions were compatible with a neurotropic viral infection, characterized by a non-suppurative polioencephalitis. The lack of demyelinating lesions has been described in cases of post-vaccination encephalitis. This is a rare complication following CDV vaccination with live attenuated viruses, especially observed in the Rockborn vaccine lineage.[3,5,12,21] Our phylogenetic comparison showed a close relation to the Rockborn strain as well as the Rockborn-like strain CDV-HN19.[30,35] The Rockborn vaccine strain was developed in the 1950s but was withdrawn from several markets in the mid 1990s , since it was considered to be less attenuated and less safe compared to other CDV vaccines, e.g. Onderstepoort strain.[21,30] In Switzerland the license for Candur SHL® (Hoechst GmbH, Hoechst GmbH,, 65926 Frankfurt am Main, Germany) which was based on the Rockborn strain, expired in the year 2000. The vaccination history of dog '19995' was not known and the other dogs '20565' and '20684' only received a first vaccination course against CDV. The patient records of dog '20565' documented the use of Vetamun®, which contained the live attenuated Onderstepoort CDV strain but was withdrawn from the Swiss market in 2007.[15] Our phylogenetic analysis based on our HTS results from the dog '20565' showed a close relation to the 'HN19' strain and Rockborn-like vaccine lineage, rather than the Onderstepoort vaccine strain. In this case, we presume that the dog '20565' did not suffer from post-vaccination encephalitis but instead was infected with a Rockborn-like CDV strain without typical demyelinating properties before the complete vaccination could grant an effective immunity.

Table 2: Mapping results of confirmed canine viral encephalitis cases. Shown are the number of mapped reads, read depth and reference genome coverage (Accession numbers obtained from NCBI GenBank) and the read depth per confirmed viral case.

Viral Hit	Cq in RT-qPCR	Case ID	Year of autopsy	Number of mapped reads (100 nt)	Read depth	Coverage [%] of the reference genome sequence
CDV [NC001921.1]	29,85	19995	1989	2'498	15,6	89,0
	22,55	20565	1990	177'310	807,3	97,8
	27,78	20684	1990	1'557	6,8	70,4
TBEV [NC001672.1]	35,57	18318	1986	12	0,1	3,6
	34,68	18772	1987	13	0,1	3,3
	32,73	21482	1991	18	0,1	3,9
	29,41	27061	1998	39	0,2	10,3
	30,29	31022	1999	220	8,2	48,5
	31,39	31140	1999	4	0,1	0,9
	30,36	45100	2010	110	0,6	15,1
	32,52	51277	2019	15	0,1	4,6
	29,61	51309	2021	29	0,2	8,0
	28,89	60481	2015	75	0,4	19,9
	23,93	S19–1723	2019	3'640	23,4	99,5
CaVV [NC004542.1]	33,02	12268	1976	321	2,7	9,8
	30,99	51874	2022	234	8,6	8,6

[CDV] Canine Distemper Virus, [TBEV] Tick-Borne Encephalitis Virus, [CaVV] Canine Vesivirus.

Currently, CDV cases in domestic animals in Switzerland are rare as a result of vaccination schemes and the use of life attenuated vaccine strains with increased biosafety (strains Onderstepoort, Lederle, BA5, Bio 11/A).[15] Our findings highlight the importance of maintaining vaccination protocols and surveillance of circulating CDV strains.[26,49]

Tick-borne encephalitis virus

We found 11/50 cases positive for TBEV. The number of TBEV HTS reads varied between the cases and covered between 0,9 and 99,5 % of the viral genome. However, our analysis could detect two longer *de novo* assembled sequences in one case ('S19–1723'). Both contigs were most similar

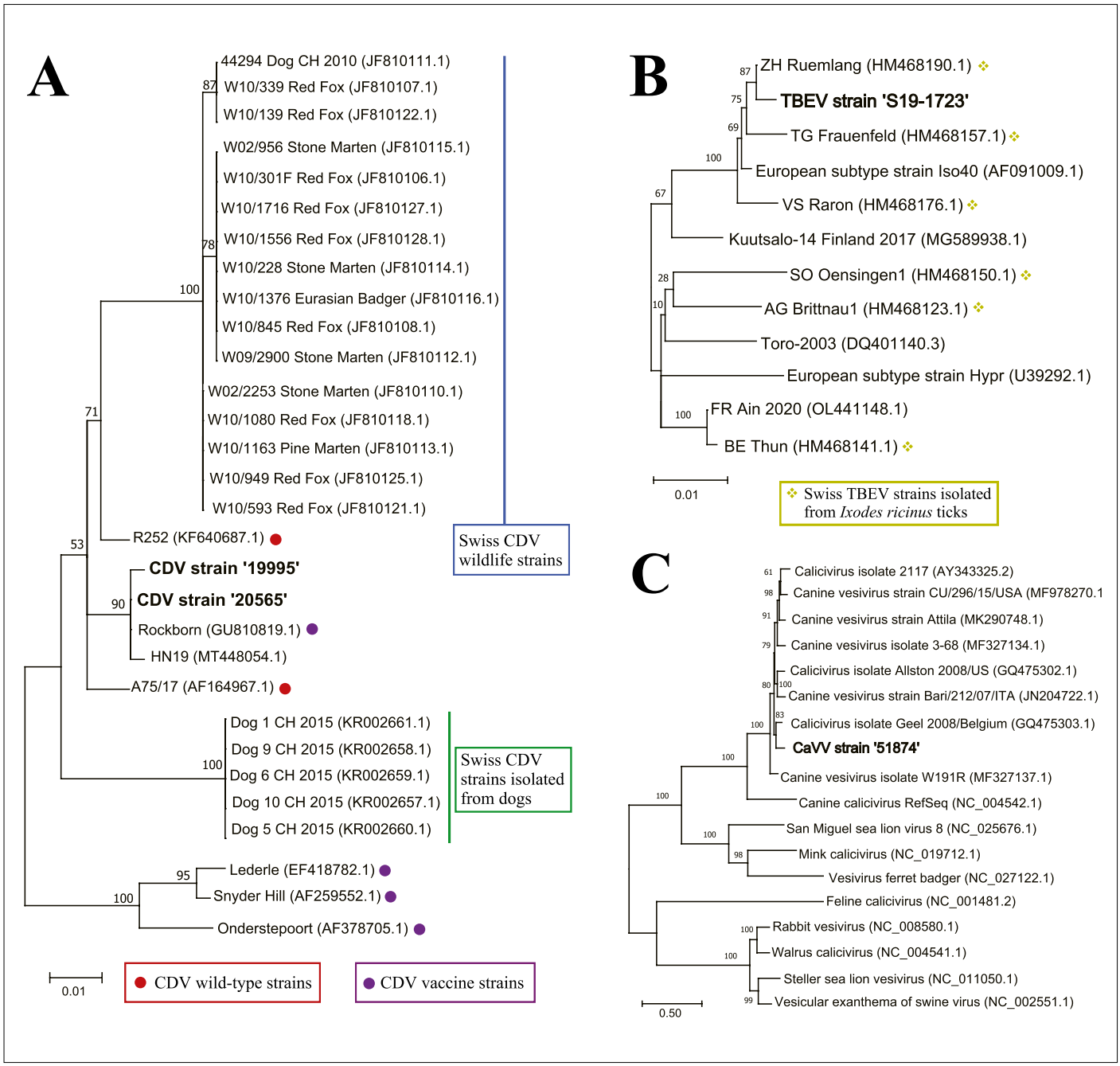

Figure 5: Phylogenetic comparisons for canine distemper virus (CDV), tick-borne encephalitis virus (TBEV) and canine vesivirus (CaVV). The consensus sequences of mapped reads and assembled contigs were compared to representative reference strains obtained from GenBank (Accession numbers are shown in brackets). Maximum-likelihood trees of aligned sequences were generated with 1'000 bootstrap replicates. **A** The phylogenetic tree for CDV was constructed based on a partial nucleotide (nt) sequence of the hemagglutinin (H) gene. **B** The phylogenetic tree for TBEV was constructed based on a partial nt sequence encoding for the envelope (E) protein. **C** The phylogenetic tree for CaVV was constructed based on the complete genome sequence and included representative members of the family Caliciviridae, genus Vesivirus.

to the TBEV strain 'Kuutsalo-14 Ixodes ricinus Finland-2017', which belongs to the European TBEV subtype.[19] TBEV is an arbovirus of the family *Flaviviridae*, genus Flavivirus, and is mainly transmitted by *Ixodes ricinus* ticks.[36] TBEV is the most dominant arbovirus in Switzerland and the increasing number of TBEV cases in humans observed over the last decade has prompted attention towards this emerging virus and its geographical spread.[33] While dogs are frequently infected with TBEV without developing clinical symptoms, an increase of clinical cases has also been observed in dogs in the last decade.[27] Diagnostically, the detection of TBEV can be challenging due to the rapid clearance of the virus from the CNS.[47] By RT-qPCR and HTS we detected very low loads of TBEV RNA in some of the FFPE brain tissue samples. Interestingly, the sample with the strongest positivity was obtained from a three-week-old puppy, which is a peculiar finding, given the main transmission of TBEV by ticks, and is currently being further investigated.

Our findings suggest a high incidence of TBEV infections in dogs with non-suppurative encephalitis and highlight the advantages of an unbiased HTS approach for virus detection. To diagnose suspected TBEV cases, history of tick-exposure, neurological assessment, positive serology, and neuropathological findings should be taken into consideration.[28] TBEV vaccines are available for humans, and the development of veterinary vaccines for domestic animals has been proposed, none have been licensed yet.[31] Dog owners are advised to be cautious towards tick-exposure during high season and consider the use of tick protection for their dogs. Our study emphasizes the importance of awareness towards TBEV as an emerging arthropod-borne and zoonotic virus, particularly in Switzerland where it is endemic throughout the country.[6]

Canine vesivirus

CaVV was as an unexpected virus that we detected in the canine encephalitis cases, both in FFPE brain material after decades of storage as well as in FF brain material from a recent case. CaVV is not considered a typical neurotropic virus associated with neurological diseases in dogs and other species. Therefore, the detection of CaVV is not included in routine post mortem diagnostic work-up cases of dogs with non-suppurative encephalitis of viral origin.[24,34,42] Vesiviruses are members of the family Caliciviridae and are small, non-enveloped single-stranded (+) RNA viruses.[46] Unlike other caliciviruses, it appears that vesiviruses are able to cross species barriers and may cause a wide range of clinical signs and lesions, harboring a zoonotic risk for animals and humans.[38] Recent studies have investigated the association between gastrointestinal disease and canine vesivirus infections.[29,43] In one case report, a dog with enteric disease and meningoencephalitis was described and CaVV was confirmed in the brain by *in situ* hybridization.[29] Similarly, the two CaVV dogs in the present study also displayed gastrointestinal symptoms. *In situ* detection of CaVV RNA in the brain was restricted to inflammatory infiltrates, indicating that the virus was transported to the brain by inflammatory cells as part of a systemic CaVV infection. Additional research is necessary to better investigate the pathogenesis and tissue tropism of CaVV. The detection of CaVV in two different cases provides further insight into CaVV as a potentially pathogenic virus.

HTS based applications and their limitations

A significant advantage of viral metatranscriptomics by unbiased sequencing is the ability to detect unexpected viruses and identify novel or highly divergent viruses.[9,14] This approach solved the viral etiology in 16/50 archival cases of dogs with viral encephalitis of unknown origin. However, 34/50 cases remain without identification of an etiological agent. A possible explanation for the unresolved cases could reside in the discrepancy of the severity of inflammation that does not correspond to the viral load. Furthermore, histological descriptions of a virus-type encephalitis are not always specific for a virus but may also occur in other types of inflammation. It is also worth noting that HTS has its limitations, particularly when applied to FFPE material, which is the most common tissue type stored in pathology archives. FFPE material undergoes chemical modification during the formalin fixation process, leading to crosslinking and fragmentation of nucleic acids.[16,22] This presents a challenge when sequencing FFPE material. Nevertheless, we addressed these issues and optimized preparation conditions for virus identification in FFPE material, as demonstrated by the retrieval and identification of viral sequences in FFPE brain material even after decades of storage.[48] However, the difficulty of obtaining full-length viral genomes remains a challenge. In cases of low viral nucleic acid abundance, *de novo* assembly of viral genome sequences can remain unsuccessful.[4] This was evident in the TBEV cases, for which *de novo* assembly could not detect TBEV contigs in 10 out of the 11 TBEV samples with a relative low number of viral reads. In such cases, hits for viral sequences should be interpreted cautiously and in relation to the disease to make an accurate causality assessment.[37] We used a refined metatranscriptomics approach to identify RNA and DNA viruses, however, the identification of DNA viruses can be challenging in cases of persistent infections with low occurrence of transcription and translation.[50] This limitation could also be a possible explanation for the unresolved cases. Nevertheless, we were successful in identifying the viral causes in archival canine cases of non-suppurative encephalitis and demonstrate the benefits of HTS as an unbiased virus discovery tool. The integration of HTS-based protocols in veterinary virus diagnostics could significantly contribute to virus surveillance and provide considerable insight into the genetic diversity of viruses.[18]

Conclusion

This study has demonstrated the efficacy of HTS in detecting viral agents responsible for non-suppurative encephalitis in brain tissues of unresolved cases of dogs, even after decades of storage. We found a high number of TBEV cases in Switzerland. Furthermore, CDV cases lacking typical demyelinating lesions were resolved. As an unexpected virus, CaVV was identified as the plausible causative agent in a decade old and a recent case, expanding our knowledge of viral encephalitis in canine patients. Overall, our findings contribute to improving diagnostic techniques, therapeutic interventions, and prophylactic measures in veterinary and human medicine.

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La découverte de virus chez des chiens atteints d'encéphalite non suppurée révèle une incidence élevée d'infections par le virus de l'encéphalite à tiques en Suisse.

Les infections virales sont une cause fréquente d'encéphalite non suppurée disséminée chez le chien. Cependant, en utilisant les méthodes de diagnostic de routine, le virus spécifique peut rester inconnu en raison d'une clairance virale importante ou complète ou parce que le virus est inattendu ou nouveau. Une approche métatranscriptomique combinant le séquençage à haut débit et l'analyse bioinformatique a été utilisée pour étudier l'étiologie virale dans des cas archivés de chiens atteints d'encéphalite non suppurée. Une incidence élevée du virus de l'encéphalite à tiques (TBEV) a été détectée dans le matériel cérébral fixé au formol et inclus dans la paraffine (FFPE) des années 1976 à 2021. En outre, le virus de la maladie de Carré (CDV) a été identifié sans lésions démyélinisantes typiques et le vésivirus canin (CaVV) a été détecté comme un virus inattendu associé à une encéphalite non suppurative. Nous avons démontré la présence virale dans les tissus cérébraux au niveau des sites d'inflammation par immunohistochimie (IHC) et hybridation in situ (ISH). Ces résultats soulignent la valeur des technologies de séquençage émergentes dans le diagnostic vétérinaire et élargissent nos connaissances sur les étiologies de l'encéphalite chez les chiens.

Mots clés: Chiens, encéphalite, virus, métatranscriptomique, séquençage à haut débit

La scoperta di virus nei cani con encefalite non suppurativa rivela l'alta incidenza di infezioni da virus della meningoencefalite trasmessa dalle zecche in Svizzera

Le infezioni virali sono una causa frequente di encefalite non suppurativa disseminata nei cani. Tuttavia, utilizzando metodi diagnostici di routine, il virus specifico può rimanere sconosciuto a causa della diffusione virale estesa o completa o perché il virus è inatteso o nuovo. È stato utilizzato un approccio basato su metatranscriptomica che combina sequenziamento ad alta capacità (NGS) e analisi bioinformatica per indagare l'eziologia virale in casi, già archiviati, di cani affetti da encefalite non suppurativa. Nel materiale cerebrale in formalina-fissato e incluso in paraffina (FFPE) degli anni 1976–2021 è stata rilevata un'alta incidenza di virus della meningoencefalite trasmessa dalle zecche (TBEV). Inoltre, è stato identificato il virus del cimurro del cane (CDV) senza lesioni demielinizzanti tipiche e il vesivirus canino (CaVV) è stato rilevato come un virus inatteso associato all'encefalite non suppurativa. Abbiamo dimostrato la presenza virale nei tessuti cerebrali nei siti di infiammazione mediante immunistochimica (IHC) e ibridazione *in situ* (ISH). Questi risultati evidenziano il valore delle tecnologie emergenti di sequenziamento nella diagnostica veterinaria e ampliano le nostre conoscenze sulle eziologie dell'encefalite nei cani.

Parole chiave: Cani, encefalite, virus, metatranscriptomica, sequenziamento ad alta capacità

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