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Abstract

Hypomyelination is a rare consequence of *in utero* bovine viral diarrhoea virus (BVDV) infection. We describe a BVDV outbreak in a naïve Holstein dairy herd in northern Italy, with an unusually high prevalence of calves with neurological signs, generalised tremors and ataxia. Histological analysis showed that hypomyelination was the predominant lesion and that the most typical BVDV neuropathological findings (e.g. cerebellar hypoplasia) were absent. Virological and molecular analyses showed that non-cytopathic BVDV genotype 1b was associated with the calves' neurological signs and excluded other viruses responsible for congenital infection or neurological disorders. Whole-genome sequencing of BVDVs from the brain of a calf with neurological signs and the whole blood of a persistently infected herd-mate with no such sign showed >99.7%

sequence identity. Analysis of the quasispecies distribution revealed the greatest variation rates in regions coding for the structural proteins E1 and E2. Variation was slightly greater in the brain- than in the blood-derived sequence and occurred at different sites, suggesting the occurrence of distinct evolutionary processes in the two persistently infected calves. Molecular characterisation of BVDV genomes from five other calves with neurological signs from the same farm confirmed that the E1 and E2 regions were the most variable. Several factors, including genetic variability and host factors, appear to have contributed to the observed unique BVDV disease phenotype, characterised by hypomyelination and neurological signs.

Keywords: Bovine viral diarrhoea virus, Whole-genome sequencing, Congenital tremor, Hypomyelination, *Pestivirus*

1. Introduction

A wide range of bovine congenital central nervous system (CNS) malformations that may be associated with teratogenic virus infection [e.g. Schmallenberg virus (SBV), bluetongue virus (BTV), Akabane virus, Aino virus and bovine viral diarrhoea virus (BVDV; *Pestivirus*)] have been described (Kessell et al., 2011). The consequences of these viral infections depend on the timing of foetal exposure, and not exclusively on the virus type (Kessell et al., 2011). Bovine herpesvirus *5* (BHV-5) is also responsible for bovine neurological disease and encephalitis (Kessell et al., 2011). Other pestiviruses (e.g. border disease virus in small ruminants, classical swine fever virus and atypical porcine pestivirus in pigs) have been linked to congenital tremor and hypomyelination (Anderson et al., 1987; de Groof et al., 2016). Congenital tremor has been reported in cattle with BVDV infection, although this infection is more commonly associated with cerebellar dysplasia (Kessell et al., 2011). BVDV infection at 120–150 gestational days can cause varying degrees of cerebellar hypoplasia. Few genetic reports on associations of BVDV subtypes 1 (species *Pestivirus A*) and 2 (*Pestivirus B*) with congenital tremor and hypomyelination are available, and genetic

investigations have been limited to the 5'UTR virus sequence (Otter et al., 2009; Porter et al., 2010). Here, we describe the high prevalence of congenital tremor and hypomyelination associated with congenital BVDV infection on a dairy farm, and thoroughly characterise the virus responsible for this outbreak.

2. Materials and methods

2.1. Case history and clinical signs

Twelve of 40 neonatal Holstein calves in a BVDV-naïve dairy herd in northern Italy showed generalised congenital tremors, difficulty maintaining the quadrupedal stance and ataxia (Video S1). Ear-notch or blood samples were collected from these 12 calves and 28 apparently healthy calves for virological investigation. Serum was collected from three calves before colostrum intake. Four calves with neurological signs died on the farm and were not necropsied; the remaining eight calves were transferred to the Department of Veterinary Medical Sciences of University of Bologna for clinical observation and sample collection. Two calves (438, 439) died spontaneously, and six calves were euthanized for humane reasons; all were necropsied (Table 1).

2.2. Histological analysis of the encephalon

The brains of the eight hospitalised calves were evaluated grossly and processed for histological investigation. The histological sections were stained with haematoxylin and eosin (HE) and Luxol fast blue and evaluated for the presence of inflammatory lesions and demyelination. The brain of an age-matched calf with no neurological sign was used as a control.

2.3. Virological and serological investigations

The pre-colostrum sera were analysed for the presence of BVDV antibodies using a BVDV NS2-3 enzyme-linked immunosorbent assay (ELISA) (Sozzi et al., 2020). All blood and ear-notch samples, and brain tissue samples from five calves with neurological signs (cases 433, 438, 439, 441 and 504) were tested for the presence of BVDV RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) using 5'UTR-targeting pan-*Pestivirus* primers (Vilcek et al., 1994). The PCR products were sequenced (Bio-Fab Sequencing Service, Rome, Italy). Phylogenetic analyses

were conducted based on these amplicons with MEGA 7 (https://www.megasoftware.net/) using the neighbour-joining method according to the Kimura two-parameter model, with 1000 bootstrap replicates. The presence of BHV-1 and BHV-5 in brain tissue was investigated by nested PCR (Marin et al., 2016). Direct and indirect diagnoses of BTV and SBV from blood and cerebellar tissue, respectively, were conducted at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy) by real-time PCR and competitive ELISA, respectively (World Organisation for Animal Health, 2019).

2.4. BVDV isolation in cell culture

The inoculum for BVDV isolation was prepared from 1 g brain tissue from case 439 and 200 µl whole blood from case 5. Monolayers of Madin–Darby bovine kidney (MDBK) cells were inoculated and incubated at 37°C with 5% CO_2 for 6 days. BVDV replication was monitored on cells harvested separately at 24, 48 and 72 h post-infection (p.i.) by SYBR Green real-time RT-PCR using primers qBVDVfor1 and qBVDVrev1 (Table 2). Viral cDNA was quantified by comparing threshold cycle values with those of a standard curve obtained from 10-fold dilutions of a plasmid containing the 5'UTR target sequences.

2.5. Whole-genome sequencing by next-generation sequencing and bioinformatics

RNA was extracted from brain tissue (case 439) and whole blood (case 5) using TRI Reagent (Merck KGaA, Darmstadt, Germany). High-throughput sequencing (HTS) cDNA libraries were prepared with the TruSeq stranded total RNA kit (Illumina, San Diego, CA, USA) and sequenced in paired-end mode (2 × 150) on an Illumina HiSeq 3000 device, yielding 222,893,850 and 144,066,985 reads, respectively (National Center for Biotechnology Information short-read archive, Bioproject ID PRJNA658580, BioSample accession nos. SAMN15878142, SAMN15878143). The reads were quality selected using Trimmomatic (ver. 0.33; http://www.usadellab.org/cms/?page=trimmomatic) and mapped to the *Bos taurus* reference genome (Bos_taurus.UMD3.1.dna.toplevel) using STAR (ver. 2.5.3a; https://github.com/alexdobin/STAR/releases/tag/2.5.3a). Quality-selected unmapped reads were

assembled using SPAdes (ver. 3.10.1; http://cab.spbu.ru/files/release3.10.1/) and the resulting scaffolds were compared to virus databases (GenBank viral nucleotide sequences, 22 May 2020; UniProt viral amino-acid sequences, 10 May 2020) using BLASTN (ver. 2.6.0+; https://blast.ncbi.nlm.nih.gov/) and DIAMOND (ver. 0.9.19; https://github.com/bbuchfink/diamond/releases/tag/v0.9.19). Phylogenetic analysis of full-length genome sequences was conducted using Mega X (https://www.megasoftware.net/), the maximum-likelihood method and the general time-reversible model with 1000 bootstrap replicates. Viral quasispecies diversity was assessed by aligning HTS reads to viral scaffolds with Bowtie 2 (ver. 2.3.1; https://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.3.1/) and visualised with the Integrative Genomics Viewer (ver. 2.8.7; http://software.broadinstitute.org/software/igv/). Synonymous and non-synonymous polymorphisms (frequency threshold ≥ 0.05) were determined and plotted to the genome nucleotide position.

2.6. Genetic characterisation of BVDV

To investigate distinct BVDV genome regions, in persistently infected (PI) calves, specific primer combinations targeting the structural and non-structural protein coding regions were used (Table 2). RT-PCRs were performed on RNA extracted from brain tissue (cases 433, 438, 439, 441 and 504) and whole blood (case 5). All amplicons obtained were sequenced. The sequences were aligned to the complete genomes of cases 439 and 5. Pairwise distances were calculated using BioEdit (https://bioedit.software.informer.com/7.2/).

3. Results

3.1. Encephalon anatomopathological findings

Macroscopically, no change was observed (Fig. 1A). HE-stained brain sections showed multifocal leukomalacia, multifocal to diffuse white-matter gliosis and neo-angiogenesis in the medulla oblongata and cerebellar peduncles, with no evidence of parasitic, protozoal or mycotic agents [larval parasites, protozoa (e.g. *Neospora* spp.), mycotic hyphae, yeasts]. The morphological diagnosis was mild multifocal encephalitis, consistent with post-inflammatory reaction. In

comparison with the control cerebellum (Fig. 1B), Luxol fast blue–stained brain tissues from affected calves exhibited evident pallor in multiple white-matter cerebellar areas (Fig. 1C), resulting in the diagnosis of white-matter hypomyelination in the vermis and cerebellar hemispheres.

3.2. Virological and serological findings

BVDV antibodies were not detected in pre-colostrum serum (Table 1). Pan-*Pestivirus* RT-PCR showed positivity in all 12 calves with neurological signs and one calf with no such sign (case 5). Nucleotide sequences of RT-PCR amplicons from all animals showed 100% nucleotide identity. These sequences were assigned phylogenetically to *Pestivirus A* (BVDV 1 subgroup b; data not shown). No BTV, SBV, BHV-1, BHV-5 or related serum antibodies were detected.

3.3. Cytopathogenicity of isolated BVDV

The characteristic cytopathic effect (CPE) of cytopathic BVDV strains was not observed in infected MDBK cells. SYBR Green real-time RT-PCR showed an increase in viral RNA between 0 and 72 h p.i. (case 5 blood: 2.8×10^0, 2.35×10^1, 4×10^2 copies/µl at 24, 48, 72 h; case 439 brain: 2.41×10^2, 3.56×10^3; 3.4×10^3 copies/µl at 24, 48, 72 h).

3.4. Complete genome sequences of BVDV1b IT16/439 and BVDV1b IT16/5

HTS and read assembly from case 439 brain RNA extract revealed a 12,244-nt scaffold (IT16/439) that best matched the BVDV type 1 isolate IBSP4ncp (GenBank accession no. KJ620017.1), with ~93% nucleotide identity and 97% amino-acid identity. In total, $>3 \times 10^5$ reads mapped to this scaffold (mean coverage, 2800). Case 5 blood RNA extract yielded a similar scaffold (IT16/5) of the same length ($>1.3 \times 10^7$ mapped reads; mean coverage, 1.43×10^5). The two sequences showed >99.7% identity at the nucleotide and amino-acid levels. No other viral sequence was detected in either sample. Complete genome sequences of the viruses were submitted to GenBank (BVDV1b IT16/439, accession no. MT977118; BVDV1b IT16/5, accession no. MT977117). Phylogenetic analysis showed that both genomes clustered with viruses of the species *Pestivirus A* (Fig. 2). Quasispecies distribution analysis revealed that the greatest variation in both viral sequences was in regions coding for structural proteins E1 and E2 (Fig. 3A). Overall variability was slightly greater

in the brain-derived than in the blood-derived sequence (0.26% vs. 0.19% sites). Sites with variation differed between sequences with no overlap (Fig. 3A).

3.5. Genomic interhost variability

BVDV sequences from calves with neurological signs and hypomyelination (cases 433, 438, 439, 441, 504) and the apparently healthy PI calf (case 5) showed 99.8–100% overall nucleotide and amino-acid identity of the complete sequence encoding Npro (504 nt). The complete E1 sequence (594 nt) showed 99.7–100% nucleotide and 99–100% amino-acid identity. A partial E2 sequence encoding the 314 C-terminal amino acids was obtained and showed 99–100% amino-acid and 98.9–99.8% nucleotide identity. A partial sequence encoding the NS2-3 protein (1168 nt) showed 99.9–100% nucleotide and 100% amino-acid identity. All the sequences obtained were aligned to the IT16/5 and IT16/439 complete genomes; the E1 and E2 coding regions showed the greatest intra- and interhost variability (Fig. 3B).

All sequences were submitted to GenBank (accession no. MW605050-MW605068).

4. Discussion

In the outbreak described here, congenital BVDV infection caused neurological disease and histopathological CNS alterations in PI calves. No other virus causing congenital infection or co-infection with a neurovirulent virus (e.g. BHV-5) was detected. The observed congenital tremors were not associated with cerebellar hypoplasia, the most common manifestation of congenital BVDV infection, but with severe hypomyelination. Hypomyelination with congenital BVDV infection has been described, but rarely as the predominant BVDV CNS pathology, and most reports describe few calves with tremors (Binkhorst et al., 1983; Riond et al., 1990; Otter et al., 2009; Porter et al., 2010). Only two other reported BVDV outbreaks involved similar prevalences of congenital tremor and hypomyelination (Otter et al., 2009). BVDV *Pestivirus A* (including subtypes 1a and 1b) and *B* species have been described in animals with these clinical signs and histopathological lesions (Otter et al., 2009; Porter et al., 2010), but no or limited genomic

sequencing was conducted. BVDV strains from the present study belong to the species *Pestivirus A* (subtype 1b), which has been detected on all continents (Yeşilbağ et al., 2017) and in apparently healthy and diseased animals, and is highly prevalent, with wide spatio-temporal distribution, in Italy (Ciulli et al., 2008a; Giammarioli et al., 2008; Luzzago et al., 2014). It was also associated with severe extensive haemorrhage (Fulton et al., 2017). *Pestivirus A* (subtype 1b) and *Pestivirus B* (subtype 2b) showed different tissue distributions during dual natural infection (Spetter et al., 2018), but no specific neurotropism has been reported for the former. In this study, BVDV strains from a clinically healthy PI calf and a calf with congenital tremors and hypomyelination were almost identical; the average frequency of nucleotide variation relative to the consensus sequence was lower in IT16/5 than in IT16/439, suggesting that the viral quasispecies ranges differed. The sites of variation differed between tissue types, which may reflect variability in individual virus swarms among animals (Ridpath et al., 2015). Whole-genome sequencing revealed no genome re-arrangement or insertion, corroborating the absence of a cytopathic phenotype. The cytopathic biotype may not have emerged because all animals examined (except case 5) died or were euthanised a few days after birth. These results confirm the previous detection of non-cytopathic BVDV strains in calves with generalised tremors and hypomyelination (Straver et al., 1983; Porter et al., 2010).

The high sequence identity in several genomic regions suggests high viral strain stability; the virus may tend to remain genetically and antigenically stable in nervous-system tissues (Colitti et al., 2019). The greatest intra- and interhost variability was observed in the BVDV E1 and E2 membrane glycoproteins, confirming previous findings (Dow et al., 2015; Colitti et al., 2019). No genomic marker of neurological pathogenicity was identified in the six cases or on comparison of the entire genomes with other BVDV isolates considered to be neurotropic or causative of neurological symptoms (Oem et al., 2013; Colitti et al., 2019). Factors other than the viral strain, such as the infection timing and BVDV-specific immunity level (unknown in the present case), should be considered in examining the pathogenesis of BVDV-associated CNS disorders.

Hypomyelination is a rare manifestation of *in utero* bovine BVDV infection and its pathogenesis is poorly understood, although it can occur following transplacental pestivirus infection. A new pestivirus was associated with congenital tremors and hypomyelination in new-born piglets (de Groof et al., 2016). This report, of the complete genome sequence of a *Pestivirus A* subtype 1b isolate associated with hypomyelination and neurological disease, confirms the neurotropism of this species. Further studies are needed to establish the molecular basis for this disease phenotype and the potential contributions of host factors.

Declaration of competing interest

The authors declare that they have no competing interest.

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Table 1

Cases, samples and analysis outcomes of the investigated outbreak.

Calves	Date of birth	Clinical signs	BVDV RT-PCR blood/ear notch	Ab in pre-colostrum sera samples	Sample name	Histopathology	Tissue used for molecular analysis
1	10/7/2016	Tremors	+	/	/	/	/
2	18/7/2016	Tremors	+	/	423	Hypomyelination	/
3	18/7/2016	Tremors	+	/	422	Hypomyelination	/
4-10	23/7-28/7/2016	No	-	/	/	/	/
11	04/08/2016	Tremors	+	/	433	Hypomyelination	Brain
12-13	05/8-08/8/2016	No	-	/	/	/	/
14	08/8/2016	Tremors	+	Negative	434	Hypomyelination	/
15	09/8/2016	Tremors	+	/	/	/	/
16	11/8/2016	No	+	Negative	5	/	Blood
17-27	13/8-27/8/2016	No	-	/	/	/	/
28	27/8/2016	Tremors	+	/	438	Hypomyelination	Brain
29	28/8/2016	Tremors	+	Negative	439	Hypomyelination	Brain
30	3/9/2016	Tremors	+	/	/	/	/
31-33	3/9-6/9/2016	No	-	/	/	/	/
34	8/9/2016	Tremors	+	/	/	/	/
35	09/9/2016	Tremors	+	/	441	Hypomyelination	Brain
36-39	9/8-21-9/2016	No	-	/	/	/	/
40	24/9/2016	Tremors	+	/	504	Hypomyelination	Brain

Primer	Sequence	Amplicon (bp)	Annealing region	Annealing temperature
324-326 (Vilček *et al.*, 1994)	**324:** 5'-ATGCCCTTAGTAGGACTAGCA-3' **326:** 5'-TCAACTCCATGTGCCATGTAC-3'	288	5'UTR	56°C
BD1mod-BD2mod (modified from Vilček *et al.*, 1997)	**BD1mod:** 5'-CTCTGCTGTACATGGCACATG-3' **BD2mod:** 5'-TTGTTRTGGTACARGCCATC-3'	738	Npro	52°C
P1-E2Rev2 (Ciulli *et al.*, 2008b; Sullivan and Akina, 1995)	**P1:** 5'-AACAAACATGGTTGGTGCAACTGGT-3' **E2Rev2:** 5'-GGGCAAACCATCTGGAA-3'	1431	E0-E1_E2	51°C
E2for2-E2rev1 (unpublished)	**E2for2:** 5'-ACTTGAATTTGGTCTTTG-3' **E2rev1:** 5'-AGGTCAAACCAGTATTG-3'	680	E2	52°C
F10-11 (unpublished)	**F10:** 5'-AGGACTTTATGTACTACATGCA-3' **F11:** 5'-CTGTTGTTGCTTTGGCAA-3'	1168	NS2-3	50°C
qBVDVfor1- qBVDVrev1 (unpublished)	**qBVDVfor1**: 5'-AGATGCCACGTGGACGA-3' **qBVDVrev1:** 5'-GCACCCTATCAGGCTGT-3'	121	5UTR	60°C

Figure captions

Fig. 1. Encephalon of a calf with bovine viral diarrhoea virus (case 434), showing normal cerebellar size and topography (A). Bright-field photomicrographs of cerebellar sagittal sections showing myelin staining (Luxol fast blue) in control (B) and affected (C, case 441) calves. Note the severe myelin deficiency in the secondary (WMsl) and tertiary (WMtl) laminae of the white matter in (C). GL, granular layer; PCL, Purkinje cell layer; ML, molecular layer. Scale bar = 200 µm.

Fig. 2. Phylogenetic tree of bovine viral diarrhoea virus full-length genomes. Sequences identified in this study (bold) cluster with *Pestivirus A* (subtype 1b) sequences. Sequence information: GenBank accession number, virus name/subtype/strain/country of origin/year of description. Bootstrap values (1000 replicates) >70% are shown. Substitutions per site are reflected by branch lengths (scale at bottom left).

Fig. 3. Molecular characterisation of bovine viral diarrhoea virus genomes from congenitally persistently infected calves with and without neurological disease. (A) Assembly of high-throughput sequencing reads of two calves (case 439, neurological disease, brain tissue sample, isolate IT16/439; case 5, no neurological disease, blood sample, isolate IT16/5) yielded two full-genome sequences of similar size (12,224 nt). The quasispecies distribution [based on sequence variation frequency (threshold > 0.05)] shows synonymous (grey) and non-synonymous (black) amino-acid changes. Variation sites are indicated by numbers referring to polyprotein sequences. A schematic representation of the BVDV genome is shown at the top. Structural proteins: Npro, C, Erns, E1, E2, p7; non-structural proteins: NS2, NS3, NS4A, NS4B, NS5A, NS5B. The scale at the bottom indicates the nucleotide position in the genome. (B) Variability of the Npro, E1, E2 and NS2-3 sequences obtained by Sanger sequencing from five calves with neurological signs relative to the complete genome sequences of the IT16/439 and IT16/5 isolates. Black lines show nucleotide differences from the IT16/5 genome of the calf without neurological signs.

Supplementary Video S1. Calves from the outbreak investigated with generalised tremors, difficulty maintaining the quadrupedal stance and ataxia.

Figure 1

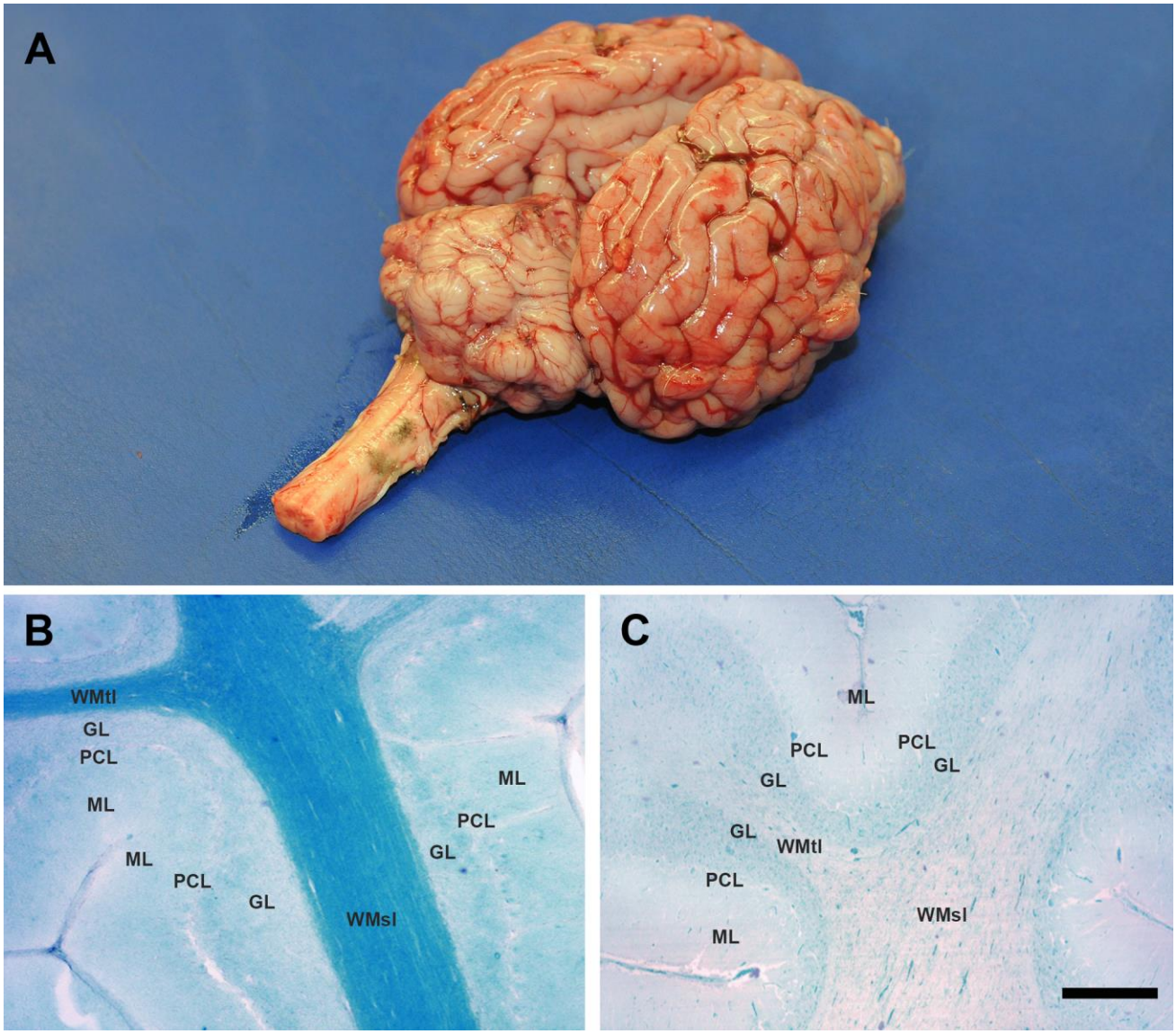
A
B
WMtl
GL
PCL
ML
ML
PCL
GL
ML
PCL
GL
WMsl
C
ML
PCL
PCL
GL
GL
WMtl
PCL
WMsl
ML

Figure 2

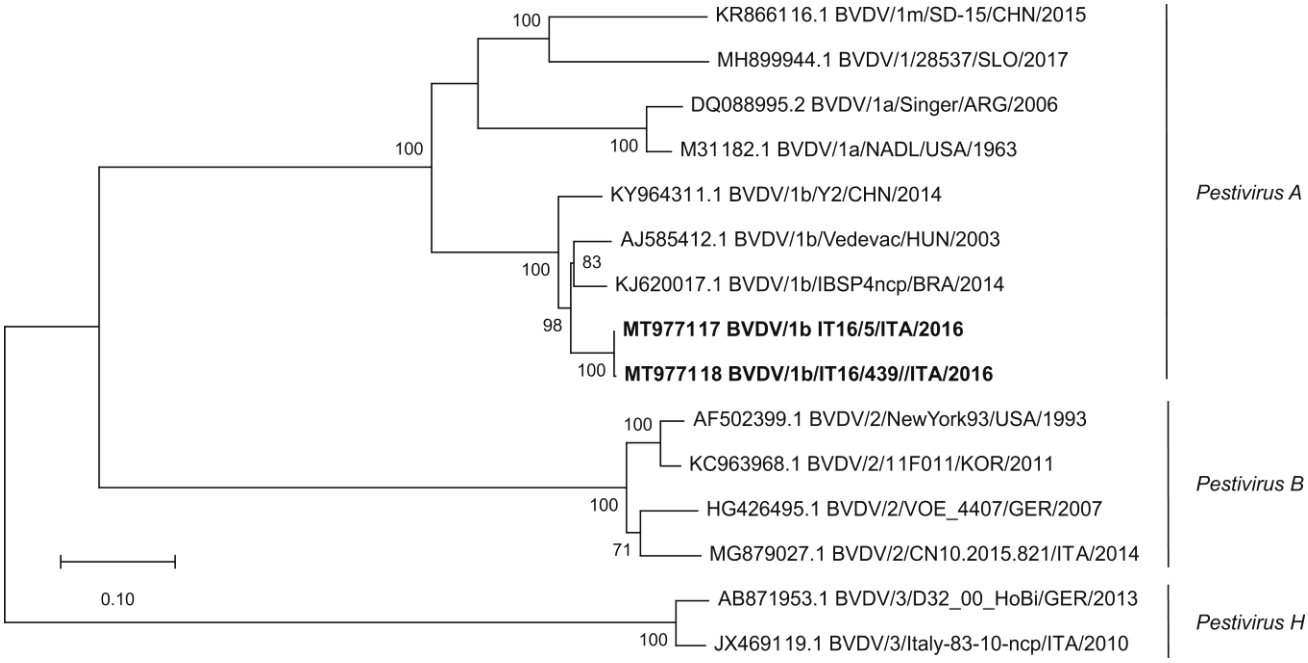

Figure 3