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Exploring the virome of diseased horses

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Metagenomics was used to characterize viral genomes in clinical specimens of horses with various organ-specific diseases of unknown aetiology. A novel parvovirus as well as a previously described hepacivirus closely related to human hepatitis C virus and equid herpesvirus 2 were identified in the cerebrospinal fluid of horses with neurological signs. Four co-infecting picobirnaviruses, including an unusual genome with fused RNA segments, and a divergent anellovirus were found in the plasma of two febrile horses. A novel cyclovirus genome was characterized from the nasal secretion of another febrile animal. Lastly, a small circular DNA genome with a Rep gene, from a virus we called kirkovirus, was identified in the liver and spleen of a horse with fatal idiopathic hepatopathy. This study expands the number of viruses found in horses, and characterizes their genomes to assist future epidemiological studies of their transmission and potential association with various equine diseases.

INTRODUCTION

The ~10 million horses in the USA have been estimated to have an annual economic impact of nearly \$40 billion. Possibly because of their long and close association with humans, the study of equine infectious diseases has been an early and important part of veterinary science. Equine microbiology started as early as the close of the nineteenth century when *Burkholderia mallei* was recognized as the pathogen of glanders and farcy of horse, and a filterable agent, later identified as foot-and-mouth virus, was shown to cause African horse sickness (Slater, 2013). In the last century, numerous equine viral diseases and their causative agents have been described by using classic virological methods, including equine influenza virus, West Nile virus (Toplu *et al.*, 2015), equid herpesviruses, equine encephalitis viruses, equine arteritis virus, African horse sickness virus, equine infectious anemia virus, equine coronavirus (Fielding *et al.*, 2015; Giannitti *et al.*, 2015), Hendra virus and vesicular stomatitis virus (Cullinane & Newton, 2013; Johnson, 2011; Maclachlan *et al.*, 2011; Onmaz *et al.*, 2013; Slater, 2013). However, the aetiology of other equine diseases remains uncertain. Furthermore, because horses can be hosts for zoonotic pathogens such as West Nile virus, influenza A virus, alphaviruses, Hendra virus and rabies virus (Cullinane & Newton, 2013; Johnson, 2011; Maclachlan *et al.*, 2011; Onmaz *et al.*, 2013; Slater, 2013), equine infectious diseases are also relevant from a human public health perspective.

Viral metagenomics has recently been used in numerous animal virus discoveries (Cann *et al.*, 2005; Chandriani *et al.*, 2013; Delwart, 2012; Li *et al.*, 2010b; Ng *et al.*, 2014; Scheel *et al.*, 2015; Shan *et al.*, 2011; Zhang *et al.*, 2014). A deep sequencing approach was used here to characterize eukaryotic viral sequences in a variety of biological sample types from horses with diseases of suspected viral aetiology. The complete or near-complete genomes of eight equine-associated viruses were characterized, providing candidates for epidemiological studies of their disease association.

RESULTS

Overview

Twenty-two barcoded equine DNA libraries consisting of 57 samples from 34 horses were generated and sequenced using paired-end 250 base runs on the Illumina MiSeq platform, generating a total of ~ 48 million reads (Table 1). Raw reads were binned by barcodes and quality-filtered, leaving ~ 16 million high-quality reads, which were *de novo* assembled within each barcode. The resulting sequence contigs and singlets were compared with the viral reference database and the GenBank non-redundant protein database using a BLASTX search with an E value cut-off of 10^{-5}.

Translated sequences similar to those of known or suspected animal viral proteins are summarized in Table 1. We detected viral sequences related to anellovirus, parvoviruses, herpesvirus, hepacivirus and circoviruses (Table 1). The majority of viral sequences were highly divergent (relative to those genomes already in GenBank), whilst others shared high identities with known equine viruses. The hepacivirus sequences showed 91–96 % nucleotide identity to the hepacivirus found in equine serum and

Table 1. Summary of sequences matching to animal virus in 22 barcoded libraries of horse samples

Library ID	Sample type	Animal no.	Clinical signs	Read no.	Virus hits (no. of reads, BLASTX, E value $<1 \times 10^{-5}$)
HB1-12	Blood	6	Depression, loss of appetite, fever and nasal or ocular discharge	4 803 968	**Anellovirus (718)**
HB2	Blood	6		1 061 712	None
HB3	Blood	6		511 992	**Picobirnavirus (380)**
HN1-12	Nasal swab	6		4 790 828	Parvovirus (39), circovirus-like virus (8)
HN2	Nasal swab	6		2 960 334	Adenovirus (1), circovirus-like virus (19), dicistrovirus (2095), picornavirus (6)
HN3	Nasal swab	5		2 014 864	**Cyclovirus (22)**, coronavirus (3), dicistrovirus (53), parvovirus (14)
HF1	Faeces	7		644 730	Circovirus-like virus (69), picobirnavirus (108)
Horse307	CSF	1	Neurological signs	3 096 842	Anellovirus (89), equid herpesvirus 2 (31)
Horse786-P	CSF	1		1 941 796	Hepacivirus (46)
Horse786	CSF	1		2 110 396	None
Horse770	CSF	1		6 68 524	None
Horse788	CSF	1		2 262 308	None
Horse616	CSF	1		2 703 212	None
Horse468	CSF	1		2 390 474	None
Horse498	CSF	1		3 114 772	None
Horse735	CSF	1		2 065 866	None
Horse739	CSF	1		3 116 576	**Parvovirus (1614)**
Horse895	CSF	1		2 824 304	None
Horse625	CSF	1		2 649 916	None
Horse-2	CSF	1		746 086	None
Horse-10	CSF	1		880 420	None
Horse necropsy	Liver/spleen	1	Severe hepatopathy, icterus and neurological signs	816 544	**Po-Circo-like virus (59)**

CSF, cerebrospinal fluid.

liver (Burbelo *et al.*, 2012; Kapoor *et al.*, 2011). Herpesvirus sequences with >80 % nucleotide identity to equid herpesvirus 2 genome were also identified (Telford *et al.*, 1995).

The horse picobirnavirus, parvoviruses, anellovirus, cyclovirus and kirkovirus, whose complete or near-complete genomes were characterized, are described in the following sections.

Picobirnavirus

Horse plasma pool HB3 from six horses with depression, loss of appetite, rectal temperature >38.6 °C and nasal or ocular discharge yielded 380 reads matching to picobirnaviruses. One plasma sample in the pool of six was identified as picobirnavirus RNA-positive by reverse transcription (RT)-PCR. The sample was then individually deep sequenced in a separate MiSeq run. Nearly 5000 picobirnavirus reads were obtained, which were then assembled with the criteria of 95 % identity in >35 bases overlap. No other eukaryotic viral sequences were detected in that library. Multiple contigs >1 kb in length showed translated sequence similarities with the RNA-dependent RNA polymerase (RdRp) gene of picobirnavirus, but only low amino acid identity with each other (<40 %). This result indicated the presence of multiple divergent picobirnaviruses in this animal. A large contig of 4.2 kb was generated, which unexpectedly included both Segments 1 and 2, and a coverage map was generated using that contig (Fig. 1). A high read coverage was distributed evenly across the 4.2 kb region, including at the expected junction of Segments 1 and 2 (Fig. 1). To further test the presence of unexpectedly fused genome segments, an RT-nested PCR bridging Segment 1 and 2 was used to amplify a ~500 bp amplicon. The expected size PCR product was sequenced and confirmed to be a junction between both segments (Fig. 1). These results corroborated the presence of a fused picobirnavirus genome, which we named picobirnavirus Equ4 (PBV Equ4; GenBank accession number KR902502). In addition to PBV Equ4, three complete copies of Segment 2 and three complete copies of Segment 1 were also assembled from the same sample, reflecting co-infection of that animal with four distinct picobirnaviruses. The segments were paired according to the rank of their read numbers, and named PBV Equ1, 2 and 3 (GenBank accession numbers KR902503–KR902508).

Picobirnaviruses are currently classified into genogroups I, II and III (GI, GII and GIII) based on RdRp sequences (Bányai *et al.*, 2014; Malik *et al.*, 2014; Smits *et al.*, 2014; Verma *et al.*, 2015). We took RdRp proteins of PBV Equ1–4 together with all available picobirnavirus RdRp proteins ≥450 aa from GenBank (sequences with >95 % nucleotide identity were excluded) to reconstruct a phylogenetic tree (Fig. 2). The GI, GII and GIII genotypes were seen as distinct branches, consistent with previous analyses (Chen *et al.*, 2014; Ganesh *et al.*, 2011; Gillman *et al.*, 2013; Masachessi *et al.*, 2015; Smits *et al.*, 2014). Picobirnavirus Equ2 fell into GI while PBV Equ1, 3 and 4 fell into distinct clades. The pairwise

comparison of the 450 aa RdRp protein sequence of the GI picobirnaviruses showed that the intra-genogroup amino acid identities ranged from 44.8 to 97.1 %, whilst the inter-genogroup amino acid identities (between GI, GII and GIII) ranged from 21.6 to 30.8 %. The high degree of sequence divergence to currently characterized RdRp justified the classification of PBV Equ4, and PBV Equ1 and 3 as members of new genogroups GIV and GV, respectively.

Parvovirus

In one cerebrospinal fluid (CSF) sample from a horse with neurological signs and lymphocytic pleocytosis (nucleated cell count >6 cells μl^{-1}), >1500 reads showed significant similarity with parvoviruses. The presence of this parvovirus DNA was confirmed by PCR. A partial parvovirus genome of 4922 bp with partial NS1 (513 aa), complete VP1 (1077 aa) and a 3′ UTR (138 bp) was acquired by filling genomic gaps by PCR and amplifying 5′ and 3′ extremities using RACE. This virus was named horse parvovirus CSF and deposited in GenBank (accession number KR902500).

Phylogenetic analysis on the basis of the partial NS1 protein sequences (~500 aa) showed that horse parvovirus CSF was most closely related to viruses in the genus *Copiparvovirus* (Fig. 3), with amino acid identity of 29.2–30.1 %. The International Committee on Taxonomy of Viruses proposed that different species within the same parvovirus genus should share >30 % and <85 % amino acid NS1 identity (Cotmore *et al.*, 2014). Considering these criteria, horse parvovirus CSF was classified as a tentative new species in the genus *Copiparvovirus*, which currently comprises parvoviruses infecting pigs and cows (Allander *et al.*, 2001; Cheung *et al.*, 2010; Ni *et al.*, 2014; Xiao *et al.*, 2013). In keeping with the recently updated taxonomy (Cotmore *et al.*, 2014), we tentatively named this species *Ungulate copiparvovirus 3*. Conserved sequence motifs were identified, including the two replication initiator motifs and the three NTP-binding and helicase domains (data not shown). Protein sequence alignment of VP1 revealed horse parvovirus CSF possessed the Ca^{2+}-binding loop (YXGXG) and the catalytic centre (HDXXY) of the putative secretory phospholipase A2 motifs (Sukhumsirichart *et al.*, 2006; Xiao *et al.*, 2013).

Anellovirus

Anellovirus sequences were detected in horse plasma pool HB1-12 and one horse CSF sample (Table 1). The positive plasma in the pool was identified by PCR and the circular genome was acquired by inverse nested PCR and direct sequencing, yielding a genome of 2197 bases (Fig. 3). It was named torque teno equus virus 1 (TTV Equ1; GenBank accession number KR902501). For the anellovirus in horse CSF, a fragment (~800 bp) of ORF1 was obtained by *de novo* assembly, showing 60 % amino acid identity with TTV Equ1.
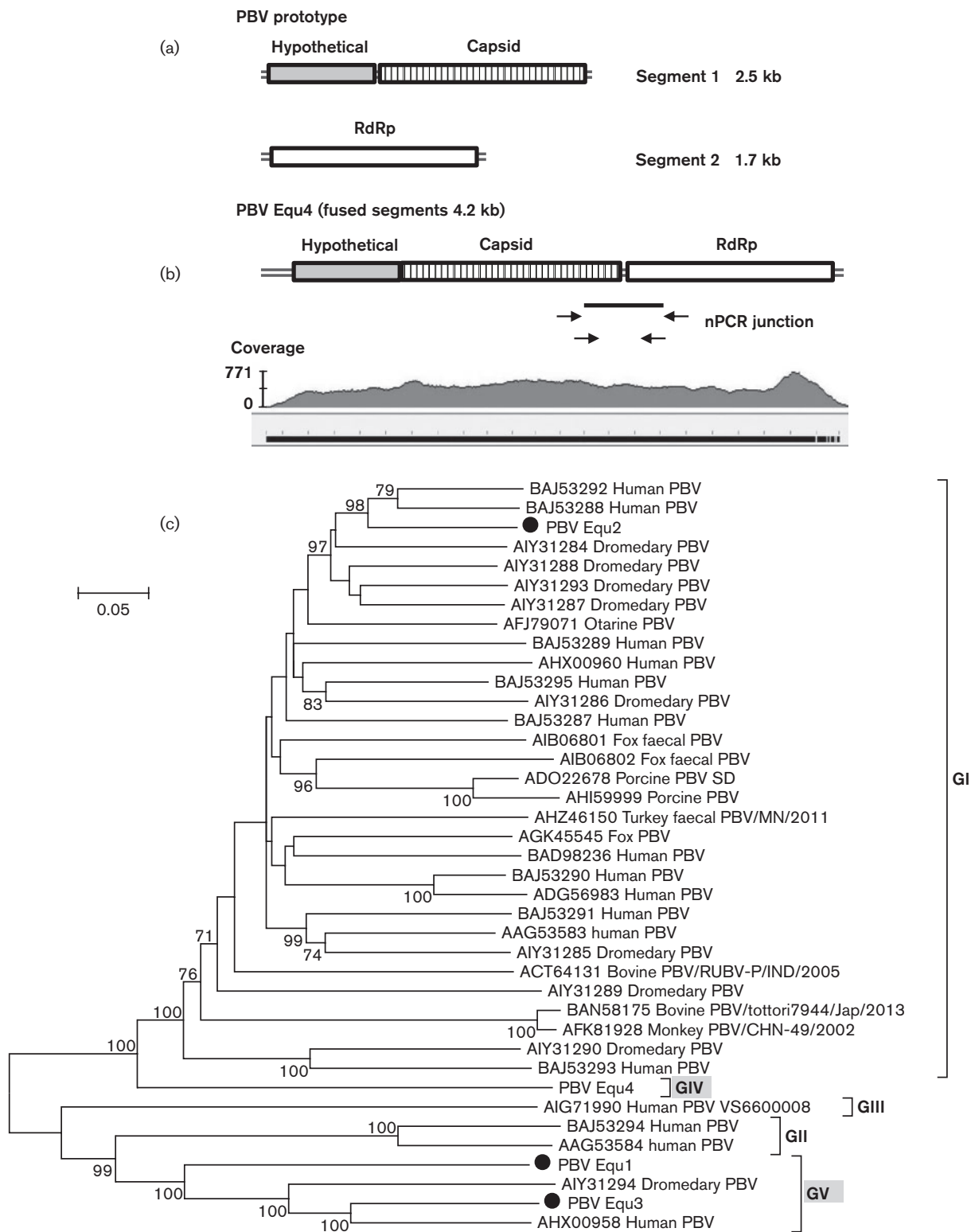
Fig. 1. (a) Diagram of the two RNA segments of picobirnavirus (GenBank accession numbers NC_007026 and NC_007027). (b) Fusion genome and putative ORFs of PBV Equ4. The junction sequence was confirmed by nested PCR (nPCR). The distribution of sequence coverage obtained by next-generation sequencing is shown. RdRp, RNA-dependent RNA polymerase; Cap, capsid. (c) Un-rooted phylogenetic analysis of all picobirnavirus RdRp protein sequences >450 aa available in GenBank using the neighbour-joining method with *p* distance and 1000 bootstrap replications. Genogroups I, II and III are labelled. Proposed genogroups IV, V and VI are highlighted by the grey boxes. Bar, amino acid substitutions per position.
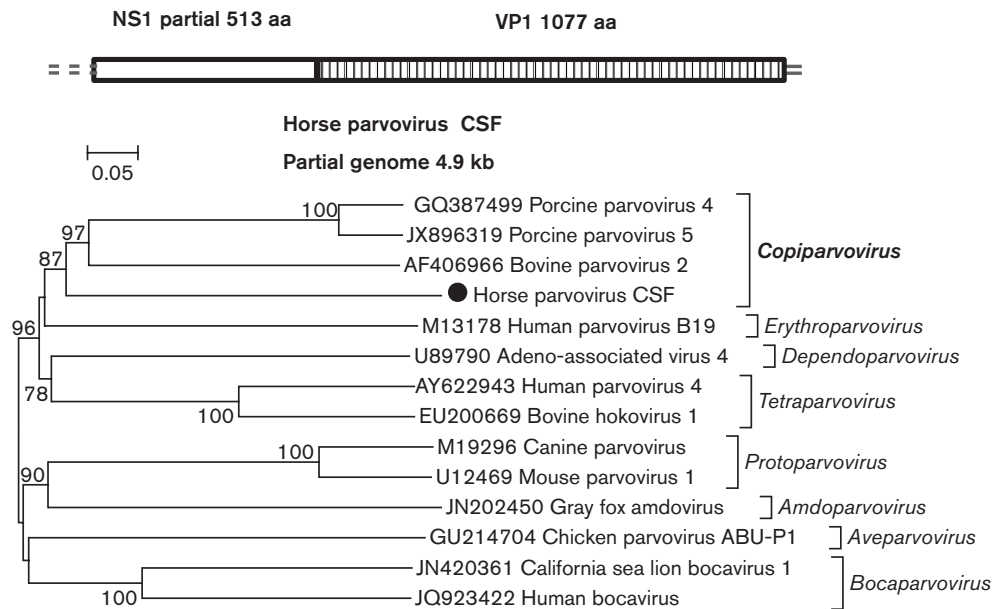
Fig. 2. Un-rooted phylogenetic analysis of horse parvovirus CSF, generated with the near-complete NS1 protein (~500 aa) using the neighbour-joining method with *p* distance and 1000 bootstrap replications. Bar, amino acid substitutions per position. Bootstrap values for each node are shown if >70 %.

The largest ORF, ORF1, of TTV Equ1 encodes a putative capsid protein of 635 aa, which as expected is arginine rich in its N terminus. A BLASTP search revealed that the amino acid sequence of ORF1 was closest to that of torque teno sus virus 1a from pig, sharing <30 % amino acid identify. ORF2–4 encoded proteins of 134, 78 and 70 aa, respectively. None of these theoretical proteins showed significant similarity with annotated proteins in GenBank. Based on the complete protein sequence of ORF1 (Biagini, 2009), a neighbour-joining phylogenetic tree was reconstructed to determine the relationship of TTV Equ1 to other anelloviruses (Fig. 3). The result showed that TTV Equ1 fell on a deep-rooted branch on the tree. According to the criterion of <30 % amino acid identity amongst different genera, TTV Equ1 is proposed as a prototype of a new genus of the family *Anelloviridae* that we named *Mutorquevirus*.

Cyclovirus

In a pool of horse nasal secretions (HN3) from animals with respiratory problems, 22 sequence reads were related to the Rep proteins of cycloviruses (Table 1). The entire circular genome was then obtained by inverse PCR and Sanger sequencing, and named equine-associated cyclovirus 1 (CyCV Equ1, GenBank accession number KR902499). The circular genome of CyCV Equ1 was 1843 nt (Fig. 4). It showed typical characteristics of cycloviruses with back-to-back ORFs encoding the putative replication-associated (Rep, 271 aa) and capsid (Cap, 268 aa) proteins (Fig. 4). The characteristic stem–loop structure was found in the 5′ intergenic region (214 nt, between the start codons of the two major ORFs). The 3′ intergenic region between the stop codons of the two major ORFs was only 6 nt long, similar in length to those of other cycloviruses (Li *et al.*, 2010a, 2011). The CyCV Equ1 Rep showed 40–49 % amino acid identity to Rep proteins of circoviruses and cycloviruses, with the closest identity to that of cyclovirus VN-hcf1, identified in CSF from patients with acute central nervous system infections (Tan *et al.*, 2013). The cyclovirus capsid showed no significant sequence identity with those of known circoviruses and cycloviruses. A specific PCR screen showed two more positive samples amongst the 42 blood, nasal or faecal samples from 19 diseased horses (Table 1). One positive sample was the faeces from the same horse with the positive nasal swab; the other one was a nasal swab from a different horse. Blood samples from both horses were PCR-negative. Both horses were from Maryland and tested negative for equid herpesvirus 5. The partial genome (1.5 kb) of this second cyclovirus shared 99 % nucleotide identity with CyCV Equ1 with a total of 15 mutations.

Kirkovirus

The liver and spleen tissues from a horse with fatal idiopathic hepatopathy jaundice (see pathological findings in Methods) were pooled and analysed. Fifty-nine reads distantly related to the Rep proteins of genomes previously described as porcine circovirus-like viruses (Po-Circo-like viruses) from the faeces of both healthy and diarrhoeic pigs (Shan *et al.*, 2011) were detected. The pathological findings of these tissues are shown in Fig. 5. The presence
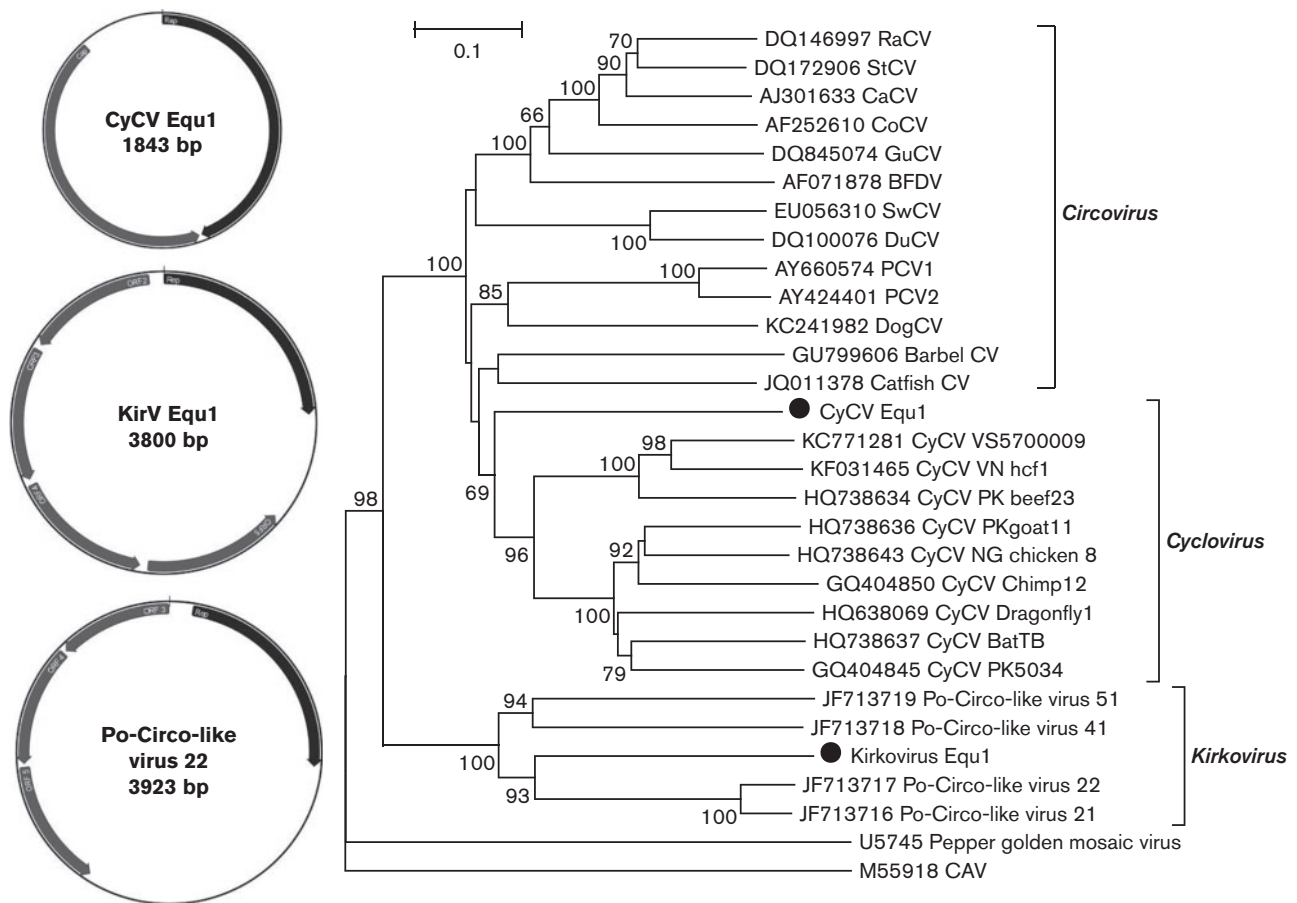
Fig. 3. (a) Genome organization of TTV Equ1. (b) Un-rooted phylogenetic analysis of the complete ORF1 protein using the neighbour-joining method with p distance and 1000 bootstrap replications. Bar, amino acid substitutions per position. Bootstrap values for each node are shown if >60 %. All 11 known anellovirus genera are shown and the new genus proposed by this study is highlighted.

of this genome in both tissues was confirmed by PCR. The complete 3800 nt circular genome was acquired by inverse PCR, and encoded a Rep protein of 305 aa plus four other major ORFs (>400 nt) encoding putative proteins of 197, 194, 156 and 188 aa (Fig. 4). The two large intergenic regions were 456 and 131 nt. We named the virus kirkovirus Equ1 (KirV Equ1; GenBank accession number KR902498). The Rep protein of KirV Equ1 virus was most closely related to the Rep proteins of genomes found in pig faeces, with 42–50 % amino acid identity, and only 21–30 % amino acid identity to the Rep proteins of circoviruses and cycloviruses, respectively. Including the pig faeces-associated genomes, the kirkovirus group currently consists of five genomes ranging from 2833 to 3923 bases (Shan et al., 2011). The 3800 nt sized genome of KirV Equ1 was most closely related to those of Po-Circo-like viruses 21 and 22, with a slightly longer genome size of 3912 and 3923 bases and similar genomic organization with their Rep gene in opposite orientation to the other main ORFs.

Sequence alignment of the putative Rep protein of CyCV Equ1 and KirV Equ1 with those of circoviruses, cycloviruses and Po-Circo-like viruses was performed. Conserved Rep motifs were identified in both genomes, including motifs associated with rolling circle replication (FTxNN, HxQG and YCxK) and ATP-dependent helicase motifs (GxGKS, VxxDD and ITSN). A phylogenetic analysis based on the full Rep protein alignment of CyCV Equ1 and KirV Equ1, and representative species of circovirus, cyclovirus and other Rep-containing genomes, showed that CyCV Equ1 fell consistently with other cycloviruses, whilst KirV Equ1 fell into the Po-Circo-like virus cluster, forming a clade distinct from Circoviridae for which we propose the family name Kirkoviridae (Fig. 4).

This horse was subjected to a complete post-mortem examination and demonstrated severe tissue damage. Necropsy findings included diffuse icterus, a flabby liver with dark brown to red capsular and cut surfaces, and diffuse enhancement of the reticular pattern that was more evident on cut sections of the parenchyma (Fig. 5a).

Fig. 4. (a) Genome organization of CyCV Equ1, KirV Equ1 and Po-Circo-like virus genomes. (b) Phylogenetic analysis of Rep proteins using the neighbour-joining method with p distance and 1000 bootstrap replications. Bar, amino acid substitutions per position. Bootstrap values for each node are shown if >70 %.

Microscopically, the most relevant histological lesions were present in the liver, which consisted of severe diffuse parenchymal/lobular collapse with degeneration, necrosis/apoptosis and loss of >80 % of the hepatocytes in the centrilobular, midzonal and periportal areas (panlobular distribution), and diffuse sinusoidal congestion and haemorrhage. Diffuse marked infiltration of histiocytes, lymphocytes, fewer plasma cells and rare neutrophils particularly in the centrilobular and periportal areas, scattered erythrophagocytosis, multifocal moderate perivenous fibrosis in the centrilobular veins, with occasional hepatocellular regeneration (mitosis) and bi-/tri-nucleation were also observed. Portal tracts were variably expanded by histiocytes, lymphocytes, plasma cells, and rare neutrophils and increased amounts of collagen (fibrosis) (Fig. 5b–d). In the cerebral cortex there was diffuse Alzheimer type II astrocytosis consistent with hepatic encephalopathy, which explained the neurological clinical signs. The pathological findings resembled those in the condition known as equine serum hepatitis (or Theiler's disease). All other ancillary tests were negative.

DISCUSSION

The first viral metagenomics study from a mammal other than humans was published in 2005 and analysed dsDNA viruses in faeces from healthy horses (Cann *et al.*, 2005). Following filtration and nuclease treatment, linker ligation, plasmid subcloning, and Sanger sequencing, that study generated 268 sequences, including 169 viral sequences, all but one from bacteriophages (Cann *et al.*, 2005). The second equine metagenomics study was published in 2013 and described a novel member of the genus *Pegivirus* (family *Flaviviridae*) in the serum of horses with non-fatal clinical hepatitis, as well as in the equine blood-derived botulinum antitoxin administered to these animals prior to the onset of their hepatitis (Chandriani *et al.*, 2013).

In order to further define the virome of horses with unexplained signs of disease and/or severe liver damage we also used non-specific amplification (Li *et al.*, 2015) and deep sequencing of enriched viral nucleic acids to characterize viral sequences following sequence similarity searches (BLASTX) to all eukaryotic viral genomes in GenBank.
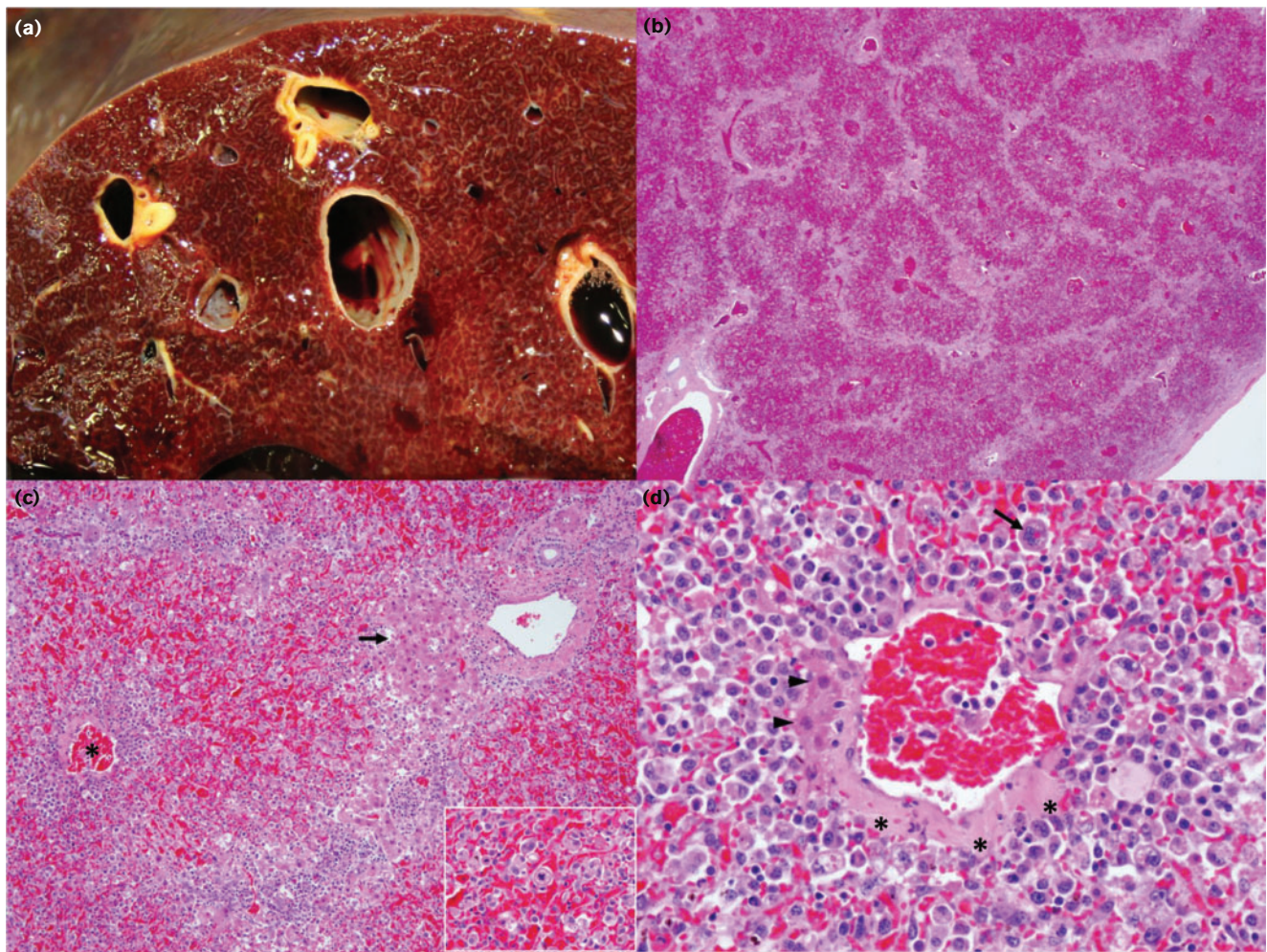
Fig. 5. Pathological findings in the horse with severe hepatopathy. (a) Gross view of the liver, cut section. Diffuse enhancement of the reticular pattern characterized by a delicate tan/pale meshwork that delineates the periphery of the hepatic lobules is shown. In many lobules a similar tan/pale discoloration is evident in the centrilobular areas. The parenchyma between the portal and centrilobular areas is dark brown to red. The walls of the portal veins and hepatic arteries/arterioles are yellowish (icterus). Bar, 2 cm. (b) Liver histopathology, haematoxylin and eosin stain. Subgross microscopic view of the enhanced lobular pattern shown in Fig. 1(a), with marked diffuse congestion and haemorrhage in the midzonal areas. The hepatic lobules have an irregular instead of hexagonal shape, a consequence of parenchymal collapse. Bar, 500 μm. (c) Liver histopathology, haematoxylin and eosin stain. Higher magnification of a hepatic lobule showing a portal tract (upper right corner) and a centrilobular vein (*). There is severe diffuse panlobular hepatocellular loss with few remaining hepatic cords in the periportal region (arrow), and marked congestion and haemorrhage in the midzonal and periportal areas. The centrilobular and periportal areas are hypercellular due to inflammatory cell infiltrates. Portal inflammation and fibrosis are also observed. Inset: closer view of a mitotic figure in a hepatocyte in the midzonal area (regenerative attempt). Bar, 100 μm. (d) Liver histopathology, haematoxylin and eosin stain. Centrilobular area and central vein. There is almost complete hepatocellular loss with very few remaining degenerate (arrowheads) and necrotic hepatocytes, although the centrilobular area is hypercellular due to infiltration of abundant histiocytes, fewer lymphocytes, and rare neutrophils. Numerous histiocytes/Kupffer cells contain intracytoplasmic haemosiderin granules, and occasional bi-/tri-nucleated cells contain intracytoplasmic red blood cells (erythrophagocytosis, arrow). Semicircular perivenous fibrosis is also shown (*). Bar, 20 μm.

Biological samples from horses manifesting different clinical signs were analysed, yielding several novel viruses.

Picobirnaviruses are small, non-enveloped viruses with a bi-segmented dsRNA genome, which belong to the family *Picobirnaviridae*, originally described in 1988 (Pereira *et al.*, 1988). The large Segment 1 (2.2–2.7 kb) contains two ORFs encoding the capsid protein and a protein of unknown function. The smaller Segment 2 (1.2–1.9 kb) contains a single ORF that encodes viral RdRp (Fig. 1a) (Bányai *et al.*, 2014; Malik *et al.*, 2014; Smits *et al.*, 2014;

Verma et al., 2015). Picobirnaviruses have been reported in faecal and respiratory samples from >20 animal species, including humans, and display a high level of genetic diversity (Bányai et al., 2014; Chen et al., 2014; Gillman et al., 2013; Malik et al., 2014; Masachessi et al., 2015; Ng et al., 2014; Smits et al., 2014; Verma et al., 2015; Woo et al., 2014). The pathogenicity of picobirnavirus alone or as co-infections remains unclear.

Two studies reported picobirnaviruses in the respiratory tract of pigs and humans (Smits et al., 2011, 2012). No prior report of picobirnavirus detection in plasma could be identified in the literature. Here, we detected picobirnaviruses in the plasma of a horse with depression, loss of appetite and fever that tested negative for equid herpesvirus 5. Analysis of viral RdRp and capsid sequences showed that at least four highly distinct picobirnaviruses were replicating in this animal. Given that viraemia is often associated with fever, it is conceivable that this horse's clinical signs were related to its co-infection and viraemia with multiple distinct picobirnaviruses.

It has been extensively demonstrated that two small RNA segments code for picobirnaviruses, as reflected by the viral family name (Pereira et al., 1988). Unexpectedly we also detected a fused form of Segment 1 and 2 by deep sequencing and confirmed its presence using RT-PCR bridging the fused segments. The fused genome accounted for the majority of the picobirnavirus reads whilst the other picobirnavirus reads could be assembled into at least three other pairs of separate Segments 1 and 2. Pools HN3 and HF1 included the nasal swab and faecal samples from the picobirnavirus plasma positive horse, respectively (Table 1). A low number of horse PBV Equ2 and Equ4 reads were also detected in these pools, indicating that in this animal picobirnavirus RNA may have also been present in nasal secretions and faeces. We therefore found picobirnaviruses co-infections, including a novel fused picobirnavirus genome, in the plasma of a febrile horse. No other eukaryotic viral sequences were detected in that same sample, supporting a possible role for picobirnavirus viraemia in this animal's clinical signs.

Parvoviruses are small, non-enveloped viruses with linear ssDNA genomes of 4–6 kb. The subfamily Parvovirinae infecting vertebrates is currently classified into eight genera, which includes many human and animal pathogens (Cotmore et al., 2014). Prototypic parvovirus genomes encode two major ORFs: a non-structural protein (NS1) and capsid protein (VP1). An additional ORF3 (NP) is found in the genus Bocaparvovirus (Allander et al., 2005). A new member of the genus Copiparvovirus, horse parvovirus CSF, was sequenced here from the CSF of a horse with neurological deficits and lymphocytic pleocytosis. Human parvovirus B19 and PARV4 have previously been reported in the CSF of patients with encephalitis (Barah et al., 2014; Benjamin et al., 2011; Douvoyiannis et al., 2009; Prakash et al., 2015). The detection of a novel parvovirus in the CSF in this animal may therefore be related to its neurological signs or reflect increased blood–brain barrier permeability.

Anelloviruses are small, non-enveloped viruses with a negative-sense, circular ssDNA genome. Currently, the family Anelloviridae contains 11 genera, named from Alphatorquevirus to Lambdatorquevirus (Biagini, 2009). We classified the horse anellovirus genome characterized here in a new genus we named Mutorquevirus. Following the initial discovery of torque teno virus in 1997 (Nishizawa et al., 1997), a large number of diverse anelloviruses have been characterized in humans and other mammals (Biagini, 2009; Ng et al., 2009; Nishiyama et al., 2014; Okamoto, 2009a, b; Young et al., 2015). The circular genome of anelloviruses ranges from 2 to 4 kb and usually consists of three to five ORFs, and they have been reported in the blood, CSF, tissue, respiratory and faecal samples from humans and many other mammals, both healthy and diseased (Mancuso et al., 2013; Mi et al., 2014; Nishiyama et al., 2014; Nishizawa et al., 1997; Okamoto, 2009b; Pollicino et al., 2003). However, no causal link between anellovirus infection and specific disease has been identified, and viral pathogenicity remains unproven with the possible exception of the porcine anellovirus TTSuV2, usually in the context of co-infections (Aramouni et al., 2013; Mei et al., 2011). Anelloviruses are ubiquitous viruses whose viral load increases with immunosuppression (Aramouni et al., 2013; De Vlaminck et al., 2013; Focosi et al., 2014; Li et al., 2013; Young et al., 2015). The detection of highly divergent anellovirus may therefore be unrelated to this horse's clinical signs, and rather a consequence of its poor health status and possible immunosuppression.

Next-generation sequencing (NGS), rolling circle amplification, inverse PCR and degenerate/consensus PCR recently led to the discovery of numerous small circular, Rep-containing ssDNA genomes (Delwart & Li, 2012; Labonté & Suttle, 2013; Rosario et al., 2012). These highly diverse Rep-containing genomes infect hosts as diverse as fungi and mammals (Ellis, 2014; Yu et al., 2010). Amongst the highly variable Rep-containing genomes are the cycloviruses that make up a sister clade to the clearly pathogenic circoviruses infecting birds and mammals. Cyclovirus DNAs have been detected in the faeces, muscle tissue, blood, CSF and nasopharyngeal aspirates of human and other mammals (Garigliany et al., 2014; Li et al., 2010a, 2011; Phan et al., 2014; Smits et al., 2013; Tan et al., 2015). Different cycloviruses (sharing <40 % amino acid identity in their capsid proteins with mammal-associated cycloviruses) have also been identified in the abdomen of dragonflies and cockroaches (Padilla-Rodriguez et al., 2013; Rosario et al., 2011). The origin and significance of the cyclovirus DNA found here in a non-sterile respiratory secretion remain uncertain. The co-detection of numerous sequence reads from insect viruses such as dicistrovirus in pools of equine respiratory secretions (Table 1) indicated that viruses in the environment may also be inhaled onto these respiratory secretions. Whether cycloviruses can replicate in mammalian tissues, including the respiratory

tract, or are environmental contaminants remains to be determined.

A distinct clade of Rep-containing genomes, which contained three or more ORFs and were labelled as porcine circovirus-like (Po-Circo-like) viruses, was recently characterized from pig faeces (Shan *et al.*, 2011). A related genome was detected here in both liver and spleen tissues of a horse with severe fatal idiopathic hepatopathy, icterus and hepatic encephalopathy, with anatomopathological findings compatible with, although not pathognomonic for, equine serum hepatitis (Theiler's disease). Testing these samples for the presence of the recently described equine pegivirus associated with non-fatal clinical hepatitis in horses by RT-PCR was negative (Chandriani *et al.*, 2013). Detection of only the kirkovirus genome in the liver of a horse with fatal hepatopathy indicates that a pathogenic role for this still poorly characterized viral family we named *Kirkoviridae* should be considered. As this genome was detected in liver rather than faeces or respiratory fluids, viral contamination due to ingestion or inhalation is less likely.

CONCLUSIONS

We describe here several novel viral genomes in horses suffering from different organ-specific signs. The picobirnaviruses, copiparvovirus and anellovirus are most likely to be genuine equine viruses based on the presumed sterile nature of the anatomical sites in which they were detected and their phylogenetic relationships to related viruses infecting mammals. Less confidence can be assigned to the equine tropism of the cyclovirus as it was detected in a non-sterile site (respiratory fluid) and belongs to a viral genus whose replication in vertebrate cells has not yet been demonstrated conclusively. Whilst the kirkovirus genome was detected in presumably sterile sites (spleen and liver), there are presently no data, such as specific seroconversion or replication in mammalian cells, to more firmly demonstrate its replication in horses. In the absence of further evidence, such as association with clinical signs using case–control studies or of disease causation following animal inoculations, a causative role for any of these viruses in these horse diseases remains tentative.

METHODS

Horse sample collection and veterinary testing. All samples were stored at −80 °C until metagenomics analyses by this study (Table 1). The plasma (*n*=18), nasal swabs (*n*=17) and faecal samples (*n*=7) were collected from horses from California, Maryland and Pennsylvania, whose signs consisted of fever (>38.6 °C), depression, loss of appetite and ocular or nasal discharge. The samples were submitted to the School of Veterinary Medicine at the University of California, Davis for equid herpesvirus 5 testing.

CSF samples were collected from 14 Californian horses with acute neurological signs that were referred to the William R. Pritchard Veterinary Medical Teaching Hospital with cytological evidence of lymphocytic pleocytosis, but negative in routine screening for viral and protozoal pathogens.

The liver and spleen tissues from a horse with severe fatal hepatopathy were collected at necropsy of a 9-year-old intact male paint horse from California, with a 14 h history of anorexia, incoordination, ataxia, partial unilateral facial paralysis, yellow discoloration of the sclera (icterus) and terminal laboured breathing. The animal had not received any injectable biologics of equine origin within 60 days prior to the onset of the clinical signs. The horse was humanely euthanized in terminal stages of the disease due to poor prognosis, and the carcass submitted to the California Animal Health and Food Safety laboratory (University of California, Davis) for necropsy and diagnostic work-up. At necropsy, sections of all major organs were immersion-fixed in 10 % neutral buffered formalin for 24 h, embedded in paraffin, microtome-sectioned at 4 μm, and stained with haematoxylin and eosin for microscopic examination (histology). Fresh tissue samples and serum were collected for routine ancillary testing to investigate infectious diseases. These included testing for West Nile virus, equid herpesvirus 1 and equine pegivirus by PCR, equine infectious anemia virus antibodies on serum by agar gel immunodiffusion test, rabies virus by direct fluorescent antibody test on the brain, and bacterial aerobic cultures from liver, lung and brain.

Sample preparation. Horse samples including plasma, nasal swabs, faeces, CSF and tissues were processed as described previously (Li *et al.*, 2015). Briefly, liver and spleen samples (∼25 mg) were immersed in 800 μl ice-cold Hank's balanced saline solution and disrupted with a tissue homogenizer for 30 s on ice. The resulting homogenates were placed on dry ice for 5 min and thawed at room temperature. Freezing and thawing were repeated twice in order to improve tissue disruption and viral particle release. Nasal swabs and faecal samples (∼50 mg) were immersed in 800 μl ice-cold Hank's balanced saline solution and vortexed for 10 min. Then, the processed tissues, nasal swabs and faeces, as well as plasma and CSF, were clarified by centrifugation at 13 000 *g* for 5 min and the supernatants were filtered through a 0.45 μm filter (Millipore) to further remove cellular debris. The viral particle-containing filtrates were digested with a mixture of DNases and RNases to remove unprotected nucleic acids (Victoria *et al.*, 2009). Viral nucleic acids were then extracted using a QIAamp Viral RNA Mini kit (Qiagen) or MagMAX Viral RNA Isolation kit (Ambion) and resuspended in 60 μl water plus 40 U RNase inhibitor (Fermentas) and stored at −80 °C. The CSF samples were pre-amplified to obtain enough DNA, as reported previously (Li *et al.*, 2015).

NGS and data analysis. Multiplexed viral DNA/RNA libraries were constructed using a Nextera XT DNA sample preparation kit (Illumina). The quality of the libraries was assessed using a 2100 Bioanalyzer (Agilent Technologies) and the quantification was estimated by a KAPA Library Quant kit (Kapa Biosystems) following the manufacturer's instructions. The resulting library of ssDNA fragments was sequenced using a MiSeq Illumina platform with a 2 × 250 cycle MiSeq Reagent kit version 2 (Illumina). The different sample pools or individual samples were analysed by libraries with unique barcodes (Table 1).

Paired-end reads of 250 bp generated by MiSeq were debarcoded using vendor software from Illumina. A virus discovery pipeline running on a 32-node Linux cluster was used to process the data. Bacterial reads were subtracted by mapping the reads to bacterial RefSeq genomes release 66 using bowtie2. Clonal reads were removed and low sequencing quality tails were trimmed using Phred quality score 10 as the threshold. Adaptors were trimmed using the default parameters of VecScreen. The cleaned reads were *de novo* assembled using multiple sequence assembly programs (Deng *et al.*, 2015). The assembled contigs and singlets were translated and aligned to a viral proteome database (consisting of all annotated full or near-full eukaryotic viral genomes) using BLASTX.

The significant hits to virus were then aligned to a non-virus-non-redundant (NVNR) universal proteome database using BLASTX. Hits with a more significant E value to NVNR than to virus were removed.

Genome sequencing and analyses. For the virus of interest, the presence of virus genome sequences assembled from NGS reads was confirmed by PCR and Sanger sequencing. Using primers based on the sequences obtained by NGS, multiple methods including nested PCR, inverse PCR, and 5′ and 3′ RACE, were then used to obtain the genome of target viruses. Putative ORFs with a coding capacity > 100 aa were predicted by ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein sequence alignments were made by using CLUSTAL W with the default settings. Phylogenetic analyses based on aligned amino acid sequences were generated by the neighbour-joining method in MEGA using amino acid p distances with 1000 bootstrap replicates. Maximum-parsimony and maximum-likelihood methods confirmed the topology of the neighbour-joining tree (data not shown). The genome coverage of PBV Equ4 was determined by Geneious 7 (Biomatters).

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