Veterinary Microbiology 156 (2012) 418–424

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Identification of a novel nidovirus associated with a neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*)

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ARTICLE INFO

Article history:
Received 12 October 2011
Received in revised form 1 November 2011
Accepted 15 November 2011

Keywords:
Wobbly possum disease
Neurological disease
Nidovirales
Nidovirus
Arteriviridae
Arterivirus
Next generation sequencing
Emerging infections
Australian brushtail possum

ABSTRACT

A novel, fatal neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*) was first identified in 1995 in a research facility and subsequently in free-living possums in New Zealand and termed wobbly possum disease (WPD). The results of previous transmission studies suggested that the aetiological agent of WPD is most likely a virus. However, the identity of the presumed viral agent had not been elucidated. In the current report, we describe identification of a novel virus from tissues of WPD-affected possums using a combination of next generation sequencing and traditional molecular methods. The proportion of possums positive for the novel virus by PCR was significantly higher ($p < 0.0001$) among animals with WPD than clinically healthy possums, strongly suggesting an aetiological involvement of the virus in WPD. Analysis of the partial genomic sequence of the putative WPD virus indicated that it is a novel nidovirus, most closely related to the current members of the family *Arteriviridae*.

1. Introduction

The Australian brushtail possum (*Trichosurus vulpecula*) is a marsupial native to Australia. It was introduced into New Zealand in the 19th century and has since become a significant pest to the country's ecosystem (Cowan, 2005). Very few viruses have been found among possums in New Zealand, and even fewer have been associated with clinical disease (Perrott et al., 2000b; Rice and Wilks, 1996; Thomson et al., 2002; Zheng and Chiang, 2007). A fatal neurological disease, termed wobbly possum disease (WPD), was first recognised in a research facility in 1995 (Mackintosh et al., 1995). The disease was also observed in free-living possums, and was reproduced under experimental conditions in healthy possums in contact with diseased animals or by intra-peritoneal inoculations of filtered material prepared from spleen or liver homogenates of WPD-affected possums (O'Keefe et al., 1997; Perrott et al., 2000c). The early stages of disease are characterised by behavioural changes (loss of appetite, decreased interest in the environment, temperament changes ranging from timidity to extreme overt aggression), followed by a fine head tremor, progressive ataxia, apparent blindness and reluctance to move (Perrott et al., 2000a, 2000c). Frank blood may be detected in faeces, often in association with mucus. Almost all experimentally infected animals develop severe disease after an incubation of about two weeks. Histologically, the disease is characterised by non-suppurative meningo-encephalitis and infiltrations of mononuclear inflammatory cells, often associated with blood vessels, in several other tissues including liver or spleen (Mackintosh et al., 1995; O'Keefe et al., 1997; Perrott et al., 2000a). Currently, the presence of these histological lesions is the basis for laboratory confirmation of WPD in possums with typical clinical signs. Altogether, the results of the previous investigations

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doi:10.1016/j.vetmic.2011.11.013

suggested that WPD is caused by a transmissible and filterable agent, most likely a virus. However, the identity of the presumed viral agent had not been elucidated. In the current paper, we have re-addressed the search for the aetiological agent of WPD using next generation sequencing (NGS) technology.

2. Materials and methods

2.1. Next generation sequencing

A standard inoculum (SI) that was used during earlier transmission studies (Perrott et al., 2000c) constituted the starting material for sequencing. The SI was enriched for viral nucleic acids by nuclease treatments according to principles described previously (Victoria et al., 2008). Briefly, aliquots of SI were treated with either DNase1 alone or with both DNase1 and RNase, followed by extraction of nucleic acids and cDNA synthesis. The cDNA/DNA was further amplified in a multiple displacement amplification (MDA) reaction using Ilustra GenomiPhi V2 DNA amplification kit (GE Healthcare), phenol–chloroform extracted, ethanol precipitated and submitted to the Massey Genome Service for sequencing on an Illumina $GAII_X$ Genome Analyzer. Following pipe-line processing, the Illumina data were depleted of host sequences by mapping to a repeat masked version of *Monodelphis domestica* genome (the closest available to that of *T. vulpecula*) using BWA (Li and Durbin, 2009), Bowtie (Langmead et al., 2009) and SSAHA2 (Ning et al., 2001) aligners. *De novo* contigs assembled with ABySS (Simpson et al., 2009) and Velvet (Zerbino and Birney, 2008) were compared to viral sequences available in GenBank using BLAST algorithms.

2.2. Screening of possum tissues for arterivirus-like sequences

To further investigate the association between the novel virus and WPD, the initial arterivirus-like contig was used to design a pair of primers to amplify a 321 bp product. Each PCR reaction consisted of 0.3 μM of each primer (WPD.A4.F: ACGTGTGTCGCGAGCTGTGG and WPD.A4.R: ACGTGGCTGGGGGTGACGAT) in 1× PCR buffer (Fast Start master mix, Roche). Cycling conditions consisted of the initial denaturation (95 °C for 10 min), followed by 35 cycles of denaturation (95 °C for 15 s), annealing (65 °C for 15 s) and elongation (72 °C for 1 min), and the final extension (72 °C for 7 min). The PCR results were confirmed by dot blot hybridisations with the virus-specific digoxigenin labelled PCR probe (DIG probe). The PCR assay was used to screen archival tissues (spleens or filtered spleen homogenates) from WPD-affected and healthy possums for the presence of novel viral sequences. Tissues from the following possums were tested: (1) healthy possums ($n = 18$) that were kept in captivity as part of an unrelated trial (Zheng and Chiang, 2007) for at least two months without showing any signs suggestive of WPD; (2) possums with histologically confirmed WPD following natural exposure ($n = 8$); (3) possums with WPD following challenge ($n = 6$) and control possums ($n = 2$) from the 1996/97 transmission studies (Perrott et al., 2000c).

2.3. In situ hybridisation

Nine tissue samples from two WPD-affected and seven tissue samples from two healthy possums were tested for the presence of novel sequences by *in situ* hybridisation (ISH). The ISH was performed essentially as described by others (Liu et al., 2000), with the exception that the sections were digested with proteinase K (Roche) [20 μg/mL] for 10–15 min at 37 °C, and hybridisations with the DIG probe diluted 1:100 in hybridisation buffer were carried overnight at 42 °C. The hybridised probe was detected using alkaline phosphate conjugated anti DIG Fab fragments (Roche) and NBT/BCIP substrate solution (Roche), and the slides were counterstained with FastRed.

2.4. Assembly and analysis of the partial genomic sequence of the novel virus

The partial genome of the putative WPD virus was assembled and analysed using Geneious software, including third party plugins (Drummond et al., 2010). The protein topology was assessed using TMHHM algorithms (Krogh et al., 2001). The presence of possible N-glycosylation sites was assessed using NetNGlyc 1 server (available at http://www.cbs.dtu.dk/services/NetNGlyc/) and the presence of N-terminal signal peptides using the SignalP 4.0 (Petersen et al., 2011). Secondary structure of RNA was predicted using pknotsRG and KnotInFrame tools (available at http://bibiserv.techfak.uni-bielefeld.de/bibi/Tools_RNA_Studio.html). The NGS assembly was extended by rapid amplification of cDNA ends (RACE). Templates for RACE consisted of MDA-amplified SI prepared for NGS, unprocessed SI cDNA, or cDNA from tissues of another histologically confirmed case of WPD. The assembly was confirmed by amplification and sequencing of an overlapping set of PCR products using cDNA from the SI as a template (Fig. 1). The final assembly was then used as reference for mapping NGS data to increase the sequence depth. To further define the relationships between the virus and other nidoviruses, a phylogenetic tree was constructed based on the conserved HEL1 domain. The tree was inferred from predicted amino acid sequence alignments by a maximum likelihood method with the JTT model of amino acid substitutions using a PhyML Geneious plugin with a 1000 bootstrap value (Guindon and Gascuel, 2003).

2.5. Statistical analysis

Association between detection of the novel virus and clinical disease was estimated based on the analysis of a 2×2 contingency table with p values calculated using Fisher's exact test (GraphPad InStat version 3.10 for Windows).

2.6. Nucleotide sequence accession number

The sequence of the novel virus was deposited in GenBank under accession number JN116253.