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Isolation and molecular identification of Sunshine virus, a novel paramyxovirus found in Australian snakes

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ABSTRACT

This paper describes the isolation and molecular identification of a novel paramyxovirus found during an investigation of an outbreak of neurorespiratory disease in a collection of Australian pythons. Using Illumina® high-throughput sequencing, a 17,187 nucleotide sequence was assembled from RNA extracts from infected viper heart cells (VH2) displaying widespread cytopathic effects in the form of multinucleate giant cells. The sequence appears to contain all the coding regions of the genome, including the following predicted paramyxoviral open reading frames (ORFs): 3′ – Nucleocapsid (N) – putative Phosphoprotein (P) – Matrix (M) – Fusion (F) – putative attachment protein – Polymerase (L) – 5′. There is also a 540 nucleotide ORF between the N and putative P genes that may be an additional coding region. Phylogenetic analyses of the complete N, M, F and L genes support the clustering of this virus within the family *Paramyxoviridae* but outside both of the current subfamilies: *Paramyxovirinae* and *Pneumovirinae*. We propose to name this new virus, Sunshine virus, after the geographic origin of the first isolate – the Sunshine Coast of Queensland, Australia.

1. Introduction

Members of the family *Paramyxoviridae* are enveloped single-stranded negative-sense RNA viruses and are currently divided into two subfamilies, *Pneumovirinae* and *Paramyxovirinae*, which contain seven and two genera, respectively (ICTV, 2012). Previous to this report, all known paramyxoviruses utilising squamate hosts (snakes and lizards) clustered within the genus *Ferlavirus* (Franke et al., 2001; Marschang et al., 2009; Papp et al., 2010).

An outbreak of neurorespiratory disease in a Swiss serpentarium in 1972 formed the basis for the first description of the isolation of a paramyxovirus from a snake (Folsch and Leloup, 1976). Since this time, paramyxoviruses have been isolated from similarly-affected snakes from other regions of Europe (Ahne et al., 1987; Blahak, 1995; Franke et al., 2001; Manvell et al., 2000), USA (Jacobson et al., 1981, 1980; Potgieter et al., 1987; Richter et al., 1996) and Brazil (Kolesnikovas et al., 2006; Nogueira et al., 2002). Koch's postulates have been fulfilled in five Aruba Island rattlesnakes (*Crotalus unicolor*) to imply a causative association between ferlaviral infection and disease (Jacobson et al., 1997). Besides snakes, ferlaviruses have been associated with disease in tortoises (Marschang et al., 2009; Zangger et al., 1991) and lizards (Jacobson et al., 2001; Marschang et al., 2009). This paper describes the isolation and initial studies of a novel paramyxovirus discovered in a private Australian snake collection that was associated with significant morbidity and some mortalities. The novel paramyxovirus described in this paper is distantly related to the *Ferlavirus* genus and we suggest this new virus be named Sunshine virus, after the origin of the first isolate – the Sunshine Coast of Queensland, Australia.

2. Materials and methods

2.1. Sample collection

In 2008, a private breeder of birds and reptiles from Queensland, Australia acquired seven jungle carpet pythons (*Morelia spilota cheynei*) as a breeding loan from another private keeper. The snakes were to be added to a collection of 70 Australian pythons (*Antaresia* sp., *Morelia spilota* ssp. and *Aspidites* sp.). Following an outbreak of neurorespiratory disease, the entire collection was humanely euthanased. Samples were collected from these animals and then submitted to the primary author (THH) for further investigation. In total, samples from 17 livers, kidneys and lungs, 16 brains and 13 serum samples were collected from 17 snakes. Snakes were selected for sample collection based on clinical signs and/or which snakes they had been in direct contact with. 12 of the 17 snakes were

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symptomatic while the remaining five snakes were in-contact but asymptomatic. Half of each organ was submitted frozen and the other half was submitted fixed in formalin for histopathological examination.

2.2. Virus isolation

Liver and kidney samples from each animal were pooled but lung and brain samples were tested individually. In total, 50 samples (17 pooled liver-kidney, 17 lungs and 16 brains) were processed for virus isolation. Virus isolation was not attempted on any serum sample. Approximately one cubic centimetre (1 cm^3) pieces of fresh frozen organ were individually placed into aliquots of 2 mL of virus isolation media which contained minimum essential Eagle's medium with Earle's salts (MEM, Sigma, Cat. No. M5650) supplemented with 5% (v/v) foetal bovine serum (FBS, GIBCO, Cat. No. 10100-147), 2× enrofloxacin (25 μg/mL using Baytril® 2.5% Oral Solution, Bayer), 2× amphotericin B (5 μg/mL, GIBCO, Cat. No. 15290-018), 5× penicillin G/streptomycin (50 IU/mL and 0.5 mg/mL, respectively, Sigma, Cat. No. P4333-20ML) and 1× L-glutamine (2 mM, GIBCO, Cat. No. 25030-149). Samples were then aseptically and finely diced using sterile scissors. Samples were then vigorously vortexed and clarified (4000*g* × 10 min @ 4 °C).

Viper heart cells (VH2, ATCC CCL-140) were grown at 30 °C and 5% CO_2. At 80–100% confluency, the cell culture medium was removed, the cells were rinsed with 1× phosphate-buffered saline (PBS) and supernatant from the clarified tissue suspension was added to the cells. Flasks and plates were left to incubate at room temperature for 1 h. The tissue suspension supernatant was removed, cells were rinsed several times with 1× PBS and then virus isolation media was added. Cells were observed daily for cytopathic effects (CPE). Seven days after inoculation, cells were frozen and then left to thaw at room temperature. Medium was clarified as above and supernatant was then used to replace the maintenance media of 75–80% confluent VH2 cells. Wells were left at room temperature for 1 h and then maintenance media was added. For some virus isolation attempts, this freeze–thawing passage was repeated once more. Viral titre was determined using the Reed–Muench method as previously described (Mahy and Kangro, 1996).

2.3. Polymerase chain reaction and high-throughput sequencing

For the purposes of polymerase chain reaction (PCR), aliquots of unprocessed media and frozen-thawed, clarified cell lysate were taken from infected flasks. Nucleic acid was extracted using the MagMAX™ Viral RNA Isolation Kit (Ambion, Austin, Texas, Cat. No. AM1939) according to the manufacturer's protocols. RNA was reverse transcribed into cDNA using Superscript® III reverse transcriptase (Invitrogen, Mulgrave, Victoria, Cat. No. 18080044) and either random hexamers or gene-specific primers (data not shown). Primers that were tested on the extracted nucleic acid are listed in the Appendix in Table A1. For some of these PCRs, the stringency was lowered by decreasing the annealing temperature (data not shown).

For Illumina® high-throughput sequencing, RNA was extracted from a 25 cm^2 flask of VH2 cells, infected with Sunshine virus that was displaying extensive CPE. Medium was removed and the monolayer was rinsed with PBS. 1 mL of Trizol® LS (Invitrogen, Mulgrave, Victoria, Cat. No. 10296010) was added to the flask, pipetted thoroughly and then transferred to a new tube. 333 μL of chloroform was added, the tube was vortexed and then left at room temperature for 5 min. The contents of the tube were then transferred to a phase lock gel heavy separator tube and centrifuged at 12,000 g for 15 min at 4 °C. The volume of clear supernatant was mixed with 100% ethanol to an ethanol concentration of 33% (v/v). This was added to an RNeasy® spin column (Qiagen, Doncaster, Victoria, Cat. No. 74104) and centrifuged at 8,000 g for 1 min. Flow through was discarded and the remaining washes were performed in accordance with the manufacturer's instructions. Ribosomal RNA (rRNA) was removed from total RNA using the Ribominus™ Eukaryote Kit (Invitrogen, Mulgrave, Victoria, Cat. No. A10837-02) and the rRNA-depleted RNA was sent to Fasteris (Geneva, Switzerland) for further processing. Fasteris then performed the following steps: zinc breakage of RNA, cDNA synthesis, ends repair, adaptor ligation, gel purification, PCR amplification, Illumina® sequencing and *de novo* assembly.

Five thousand eight hundred and eighteen unique contigs were assembled. The Basic Local Alignment Search Tool (BLASTN; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to try to determine the identity of these unique sequences. Batch searches were performed and the results downloaded. Results were then searched for the word "virus". From the 5818 unique contigs, 326 had BLAST hits for the word "virus". The viral family was then determined for each BLAST hit and viruses not known to occur in vertebrates were excluded. This excluded host DNA (e.g. virus receptors) and viruses of algae, fungi, invertebrates, plant and protozoa. This left 212 unique contigs representing 25 classified families. Next, ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to identify open reading frames (ORF) and then search the protein database of GenBank (National Center for Biotechnology Information, Maryland) for similarities. From the 212 unique contigs that were analysed in this way, only one (an 11,709 nucleotide contig) showed strong similarity to a virus. This sequence had one ORF that was similar to the fusion glycoprotein superfamily and a second ORF that was similar to paramyxovirus RNA-dependent RNA-polymerase.

The raw sequencing data was then reassembled using CLC Genomics Workbench® software (CLC Bio©) to look for additional sequence information belonging to the putative paramyxoviral genome and a 17,187 nucleotide sequence was assembled: 5478 nucleotides longer than the 11,709 nucleotide contig assembled by Fasteris®.

Using the putative paramyxoviral sequence information that had been generated, non-degenerate primers were then designed (Table 1) based on the RNA-dependent RNA-polymerase gene. These primers would then be used in PCRs to look for Sunshine virus in other samples (manuscript submitted). To determine that this new virus was not endogenous to the VH2 cell line, all three primer pairs were used on infected and uninfected cells.

Table 1
Primer sequences, and anticipated amplicon size, used for the detection of the polymerase gene of Sunshine virus. PCR = polymerase chain reaction. bp = base pairs.

Primer set (number of nucleotides from 3' end of polymerase gene)	PCR product size (bp)
SunshineS1 (2444): 5'GGAAAGGGAGGTCTATG SunshineAS1 (2596): 5'ATTCAACATCTGGGGTC	153
SunshineS2 (2240): 5'TTCAAGGAGATAACCAGG SunshineAS2 (2469): 5'CGGGATTCCCATAGAC	230
SunshineS2 (2240): 5'TTCAAGGAGATAACCAGG SunshineAS1 (2596): 5'ATTCAACATCTGGGGTC	357