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Isolation and Characterization of a Novel Orthomyxovirus from a Bothriocroton hydrosauri Tick Removed from a Blotched Blue-Tongued Skink (Tiliqua nigrolutea) in Tasmania, Australia

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Isolation and Characterization of a Novel Orthomyxovirus from a *Bothriocroton hydrosauri* Tick Removed from a Blotched Blue-Tongued Skink (*Tiliqua* nigrolutea) in Tasmania, Australia

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Simple Summary: Human-biting arthropods such as ticks are of interest to public health authorities around the world, owing to their potential to transmit a variety of pathogens, including bacteria and viruses that cause significant morbidity and mortality[1]. In Australia, at least 70 species of soft and hard ticks have been documented[2]. Of these, a variety of tick species have been reported to bite humans, with at least six species (*Amyblomma triguttatum, Bothriocroton hydrosauri, Haemaphysalis novaeguineae, Ixodes cornuatus, Ixodes holocyclus, Ixodes tasmani*) known to transmit pathogens during feeding[3]. Endemic Australian diseases caused by bacterial pathogens transmitted by ticks include (i) Q Fever caused by *Coxiella burnetii*; (ii) Queensland Tick Typhus due to *Rickettsia australis*; and (iii) Flinders Island Spotted Fever, caused by *Rickettsia honei*[4] and (iv) Australian Spotted Fever due to *R. honei* subsp *marmionii* [35].

Abstract: Active and passive surveillance, followed by gene sequencing continues to identify a diverse range of novel bacteria, viruses and other microorganisms in ticks with the potential to cause disease in vertebrate hosts following tick bite. In this study, we describe the isolation and characterization of a novel virus from *Bothriocroton hydrosauri* ticks. In an attempt to isolate rickettsia the inoculation of Vero cell cultures with tick extracts led to the isolation of a virus, identified as a novel tick *Orthomyxovirus* by electron microscopy and gene sequencing. Transmission electron microscopic analysis revealed that *B. hydrosauri* tick virus-1 (BHTV-1) is a spherical orthomyxovirus, 85 nm in size. Multiple developmental stages of the virus were evident *in vitro*. Analysis of putative BHTV-1 amino acid sequences derived from genomic analysis of virus-infected host cell extracts revealed the presence of six putative RNA segments encoding genes, sharing closest sequence similarity to viral sequences belonging to arthropod-borne *Thogotovirus* genus within the *Orthomyxoviridae*. Thogotoviruses are an emerging cause of disease in humans and animals following tick bite. The detection of this new thogotovirus, BHTV-1, in *B. hydrosauri*, a competent vector for human tick-borne infectious diseases, warrants follow-up investigation to determine its prevalence, host range and pathogenic potential.

Keywords: virus; thogotovirus; quaranjavirus; Orthomyxoviridae; genome sequencing and analyses; transmission electron microscopy;

1. Introduction

While bacterial tick-borne diseases in Australia are widely recognized, less is known about the potential for viral pathogens transmitted by Australian ticks. Thus far, at least six viruses have been isolated from human-biting ticks in Australia[3]. These include (i) *I. holocyclus* Iflavirus, a member of the *Iflavirus* genus of positive, sense single stranded RNA (ssRNA) viruses, detected in *I. holocyclus* ticks from Queensland and northern New South Wales[5]; (ii) a diversity of viruses isolated from *Argas robertsi* ticks in the Northern Territory and South-East Queensland[6-8]; (iii) and two viruses, Saumarez Reef virus and Upolu virus (UPOV), isolated from *Ornithodoros capensis* ticks parasitizing sooty terns (*Onychoprion fuscatus*) on the Great Barrier Reef, Queensland[9,10]. UPOV, notably, was initially classified as a Bunyavirus with molecular analyses subsequently reclassifying it as a member of the *Orthomyxoviridae* family[11]. While these viruses have been detected in ticks that bite humans, their pathogenic potential is unclear. It should nevertheless be noted that many of these viruses otherwise belong to families of viruses with well recognized human pathogens, including the *Orthomyxiviridae* and *Flaviviridae[3].*

The preparation of an appropriate public health response to tick-borne diseases relies on the use of state-of-the-art laboratory methods to identify potential pathogenic agents, understand the epidemiology of the putative agents and to determine their actual pathogenic potential[12]. Given that the majority of pathogens associated with tick-borne diseases are obligate intracellular parasites, these methodologies inevitably include a combination of traditional laboratory methods including cell culture and microscopy coupled with molecular methods, including broad-range PCR assays and next-generation sequencing. The use of the latter approaches has revealed an increasing level of microbial diversity harbored by human-biting ticks[12]; while these approaches are useful, studies to isolate the organism are still required if the pathogenic potential of these novel tick-borne microorganisms is to be assessed properly.

A recent molecular study documented a growing number of rickettsiae, obligate intracellular bacterial pathogens from the Order *Rickettsiales*[13], in the Australian reptile tick, *Bothriocroton hydrosauri*. *B. hydrosauri* is the established reservoir of *R. honei*, the aetiological agent of Flinders Island Spotted Fever in Southern Australia[14]. A molecular survey of this tick species on the Australian mainland recently revealed that this tick may carry additional rickettsial agents with novel rickettsiae detected that were genetically distinct from *R. honei*[15]. Similar agents were also documented in *Amblyomma* ticks in Western Australia[16] and the Northern Territory[17].

To explore the diversity of novel rickettsiae hinted at during molecular screening[15], in the current study, we attempted to isolate novel rickettsial agents from *B. hydrosauri*. Instead of culturing a rickettsial agent as expected, we instead successfully isolated and subsequently characterized a novel orthomyxovirus harbored by this human-biting reptile tick.

2. Materials and Methods

2.1. Viral Isolation and Culturing

A live *B. hydrosauri* tick was collected from a blotched blue-tongue skink (*Tiliqua nigrolutea*) at Mount Stuart, Hobart, Tasmania and transported to the Australian Rickettsial Reference Laboratory (ARRL) for further analysis.

The live tick was homogenised in Phosphate Buffered Saline (PBS) using a plastic pestle. The homogenate was filtered through a 0.22 µm filter, with a further 1 mL PBS passed through to wash. The culture medium (RPMI supplemented with 3% fetal calf serum, 4 mM L-glutamine and 25 mM HEPES) was removed from a 25 cm^2 non-vented tissue culture flask containing a fresh, confluent monolayer of Vero cells, and 500 µL tick homogenate was added. The flask was centrifuged at room temperature (500 × g for 30 mins) to promote attachment of any organisms present to the cells. Following centrifugation, 9 mL medium was added and the flask was incubated at 35 °C.

After 6 days of incubation, cytopathic effect was observed by microscopy. Remaining adherent cells were detached from the flask into the medium using a cell scraper and 1 mL of this material was passaged into flasks containing fresh, confluent monolayers of Vero cells with 9 mL culture medium. After a further 6 days of incubation at 35 °C, cytopathic effect was observed again. Flask contents were harvested and aliquots frozen at -80°C in cell culture freezing medium (CCFM; Gibco).

2.2. PCR Screening

Several PCR assays were used investigate the source of the cytopathic effect observed in cell culture. The possible presence of bacterial agents was examined using a eubacterial 16S rRNA PCR[18]. The presence of specific potential rickettsial agents was determined by a rickettsiae-specific qPCR, as previously described[19].

2.3. Transmission Electron Microscopy (TEM)

After the development of cytopathic effect, cell cultures inoculated with passaged media as described above in *2.1 Viral isolation and culturing,* were processed for TEM thin section and negative contrast analyses according to the procedures described in [35].

2.4. Genome Sequencing

Viral RNA from 100 uL of frozen tissue culture supernatant from infected Vero cells was extracted with the Zymo Direct-zol Miniprep kit without *DNase*I digestion and total RNA concentrated to a volume of 16 uL with Zymo's RNA clean and concentrator kit. Sample isothermal amplification and Illumina Nextera XT library preparation was done and samples sequenced on the Illumina MiniSeq Sequencing System generating 150 bp paired-end (PE) reads.

2.5. Genome Annotation, Alignment and Phylogenetic Analyses

Bioinformatics pipeline for viral whole genome *de novo* assembly was done. Host-subtraction was omitted and updated version of CLC Genomics Workbench (v20.0.4) used for read trimming. Geneious Prime (v2020.2.3) was used for read error correction and normalization (BB Norm v38.84) and SPAdes "Multi Cell" (v3.13.0) for *de novo* assembly. Additional *de novo* assemblies were also performed with alternative SPAdes data source options including "RNA", "Single Cell" and "Metagenome" (default parameters) and well as MIRA v4.0 (default parameters). Assembled contigs were then queried against the National Center for Biotechnology Information (NCBI) non-redundant database with BlastX to identify virus-encoding contigs. Contigs for each genome segment were then aligned to closely related viruses from the *Thogtovirus* genus at both the nucleotide and amino acid levels to verify assemblies, and join overlapping contigs, if required, to attempt to obtain the complete viral genome. Final genome segments were verified by mapping back-trimmed reads to identify misassembled regions.

Final genome sequences (submitted to Genbank; were subject to BlastX and BlastN to support viral identification. Phylogenetic analyses was performed using a set of orthomyxovirus sequences retrieved from Genbank (www.ncbi.nlm.nih.gov) to clarify the origin of open reading frames (ORFs) within each genome segment and to identify potential recombination events. All sequences were aligned using the Clustal algorithm (as implemented in MEGA3;[20]) at the nucleotide and amino acid level with additional manual editing to ensure the highest possible quality of alignment. Neighbor-joining analysis at the amino acid level was performed due to the observed high variability of the underlying nucleotide sequences of members of the family *Orthomyxoviridae*. The statistical significance of tree topologies were evaluated by bootstrap resampling of the sequences 1000 times.

3. Results

3.1. Isolation and Morphology of Novel Tick Virus

Cytopathic effect was observed by microscopy six days after inoculation of the Vero cell culture with the filtered tick homogenate. To characterize the potential intracellular pathogen supernatant extracts were screened by PCR. Cellular extracts were negative in the initial screening broad range eubacterial and specific rickettsial PCR tests. Further characterization using random PCR primers followed by sequencing showed that the supernatant contained an Orthomyxovirus in the thogotovirus genus.

Further investigation by TEM revealed the presence of icosahedral to spherical shaped virus particles measuring approximately 85 nm in diameter. The particles observed were located 1) within invaginations of the plasma membrane, 2) at the surface of the plasma membrane or 3) amongst extracellular debris (Figure 1). At higher magnification strands of internal ribonucleic protein were visible (Figure 2d). ‘Tether’ or stalk-like structures were visible between some viral particles and host cell membranes (Figure 2A,B &D) The ‘tethers’ are similar in appearance to those present in Upolu and Aransas Bay virus micrographs [11]. The identification and purpose of these tether-like structures are yet to be investigated; it is possible they may be an envelope remnant resulting from the budding process. However, no virus particles were seen classically budding at the cell surface.

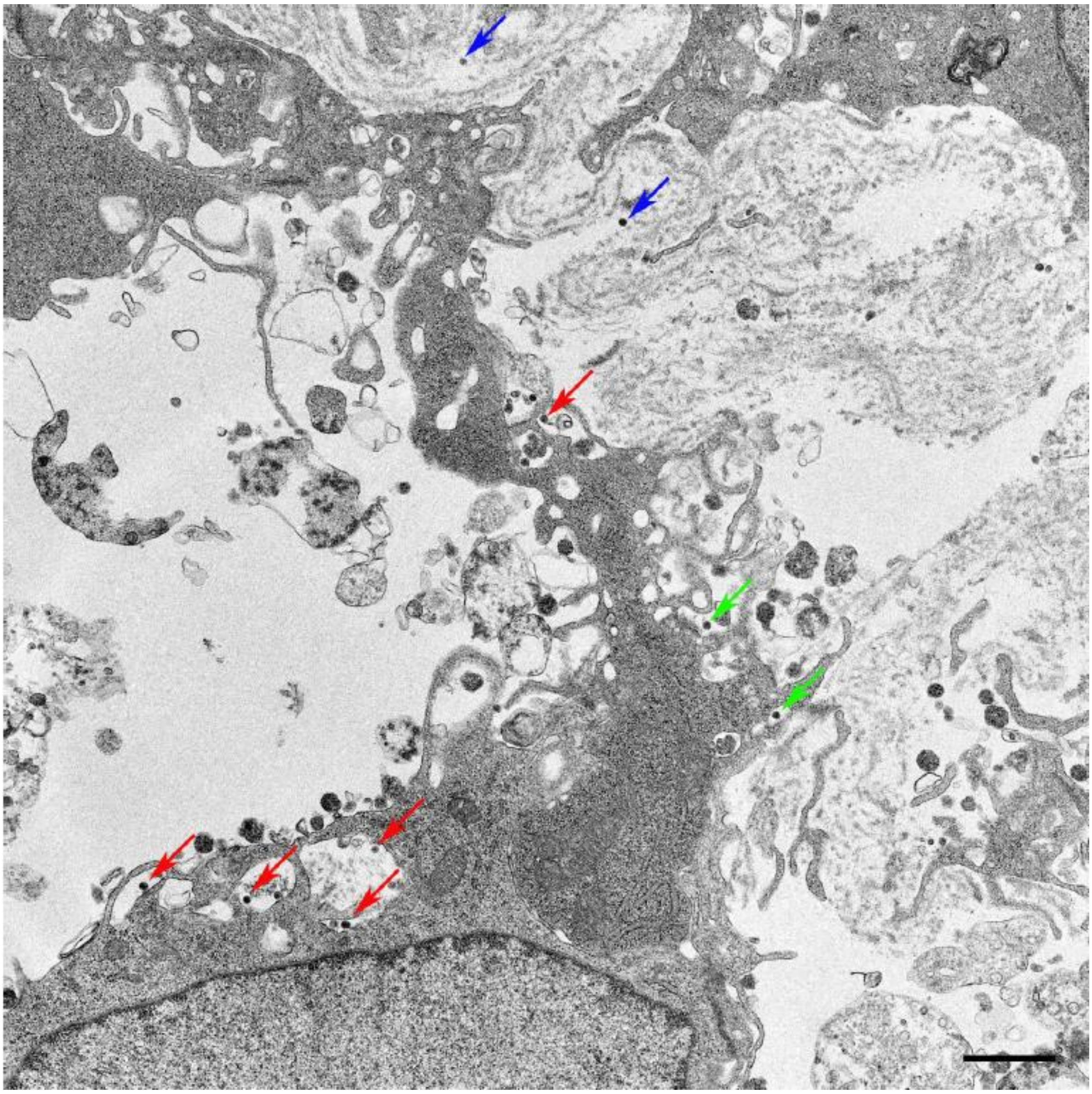

Figure 1. TEM images of putative viral particles detected in infected Vero cells. Red arrows indicate viral particles within smooth membraned vesicles of the cytoplasm of Vero cells. Green arrows indicate viral particles associated with the plasma membrane or lytic vesicles. Blue arrows indicated released viral particles amongst extracellular debris. The scale bar represents 1 μm.

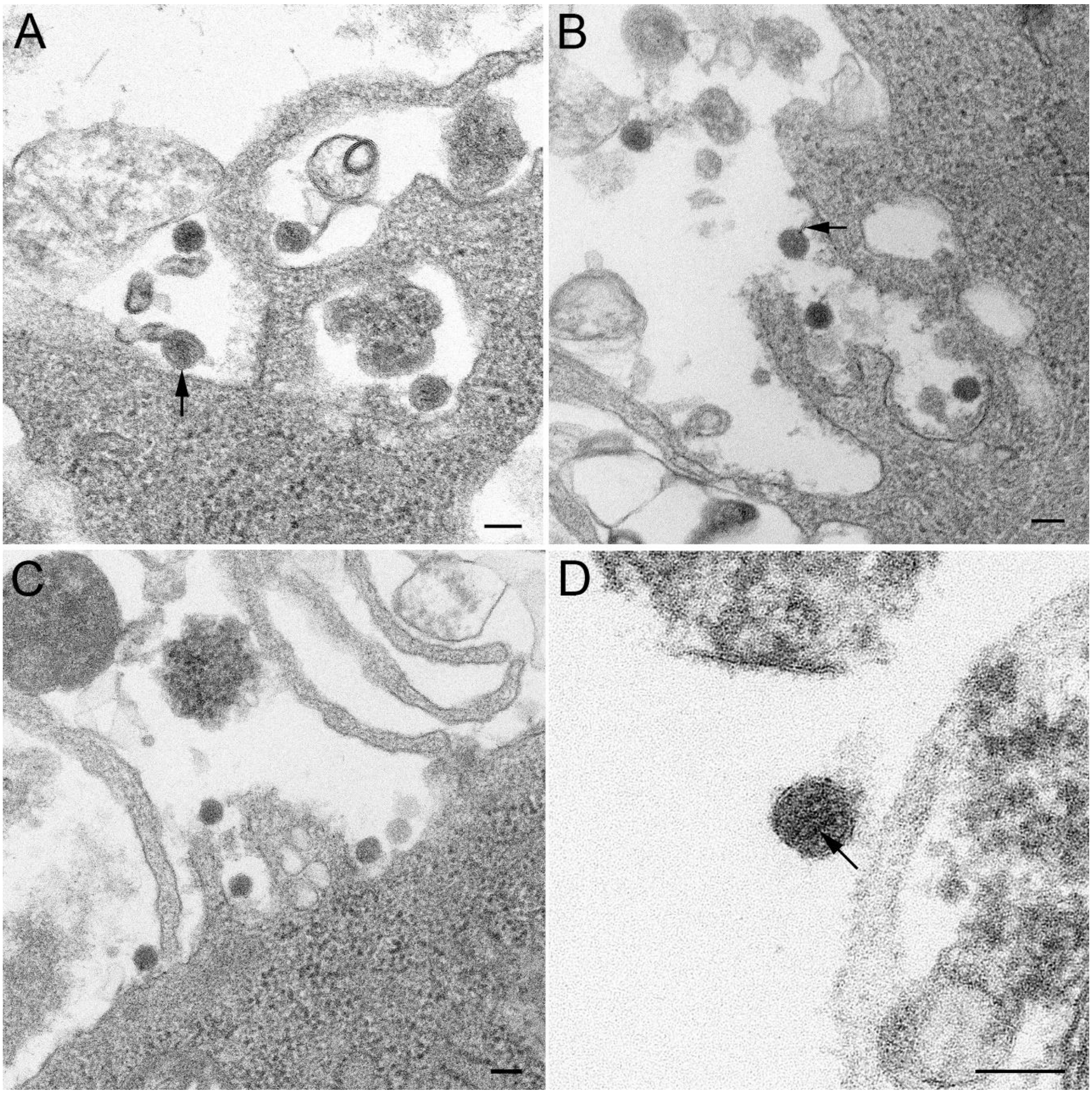

Figure 2. Transmission electron micrographs of different viral developmental stages. *A*, Arrow indicates virus, with putative envelope remnant. *B*, Arrow indicates 'tether' or stalk-like structure. *C*, virus associated with the plasma membrane amongst filopodia. *D*, extracellular virus particle showing internal ribonucleic protein. The scale bar represents 100nm.

3.2. Genomic Characterization and Phylogenetic Analysis of the Novel Tick Virus

Total RNA extracted from the cell culture supernatant of Vero-infected cells was sequenced to characterize the putative virus cultured. SPAdes Multi-Cell *de novo* assembly of 6,493,760 trimmed, normalized Illumina PE reads led to assembly of 39 contigs with BLASTn and BLASTx analysis used to identify 8 X contigs that contained viral ORFs_. Additional *de novo* assemblies with MIRA and SPAdes (total of 456 contigs; 166 viral encoding genes) were performed to join and or extend ORFs within the original virus containing contigs. Genome analysis resulted in a final total of 6 viral genome segments of 2140 bp (segment 1), 2193 bp (segment 2), 1955 bp (segment 3), 1612 bp (segment 4), 1427 bp (segment 5) and 966bp (segment6). Putative identities of the viral ORFs within each of the 6 genome segments, as determined by BLASTx analysis, are presented in Table 1. Each ORF was found to share closest similarity to viruses in the genus *Thogotovirus* within the family *Orthomyxoviridae*, including (i) ORF1, a complete coding sequence (781 amino acids (aa)) sharing highest similarity to a polymerase basic protein-2 (PB2) from a Jos virus; (ii) ORF2, a complete coding sequence (712 aa) sharing 70.1% similarity to Jos virus polymerase basic protein-1 (PB1); (iii) ORF3, a complete coding sequence (629 aa) sharing 48.9% similarity to Aransas Bay virus polymerase acidic subunit (PA); (iv) ORF4, a partial coding sequence for an Upolu virus glycoprotein-encoding (GP); (v) ORF5, a complete coding sequence (454 aa) with highest similarity to a Thogotovirus nucleoprotein (NP); (vi) ORF6, a complete coding sequence (309 aa) sharing 45.7% sequence similarity to a Jos virus matrix protein long (ML). In addition, assembled data revealed a contig encoding a 269 amino acid truncated spliced variant of the matrix protein (M) as described for the closely related Jos virus M protein in Bussetti et al. 2012. On the basis of this sequence analysis, for the remainder of this manuscript, this novel virus was referred to as *Bothriocroton hydrosauri* Thogotivirus-1 (BHTV-1).

Table 1. Highest percentage BLASTx hits to novel virus ORFs detected after genome assembly.

ORF (size bp)	% identity	Genbank ID	Accession description
ORF1 (2346 bp)	54.4%	AED98375.1 (95% coverage)	Jos virus PB2
ORF2 (2139 bp)	70.1%	AED98371.1 (96% coverage)	Jos virus PB1
ORF3 (1890 bp)	48.9%	AHB34062.1 (96% coverage)	Aransas Bay virus PA
ORF4 (1582 bp)	41.1%	AHB34057.1 (89% coverage)	Upolu virus GP
ORF5 (1365 bp)	59.6%	YP_145809.1 (95% coverage)	Thogotovirus NP
ORF6 (921 bp)	45.7%	AED98373.1 (78% coverage)	Jos virus ML

To gain further insight into the genetic relationship between this BHTV-1 and other closely related viruses, phylogenetic trees were constructed for the individual protein sequences (ORF-1, ORF-2, ORF-3, ORF-4, ORF-5, ORF-6; Figure 3 to Figure 8). As predicted by BLASTx searching, phylogenetic analyses of each of these ORFs revealed that the novel virus, BHTV-1, is most closely related to other tick-borne viruses (Dhorivirus, Bourbonvirus, Josvirus) in the family *Orthomyxoviridae*, genus *Thogtovirus*. Phylogenetic analysis of the putative BHTV-1 ORF-6, however, revealed that this ORF amino acid sequence is most closely related to viruses in the genus *Quaranjavirus* within the family *Orthomyxoviridae* (Figure 4), suggesting a possible reassortment event between BHTV-1 and viruses within the *Quaranjavirus* genus. The most conserved gene within the Orthomyxovirus family was ORF2, the PA gene segment.

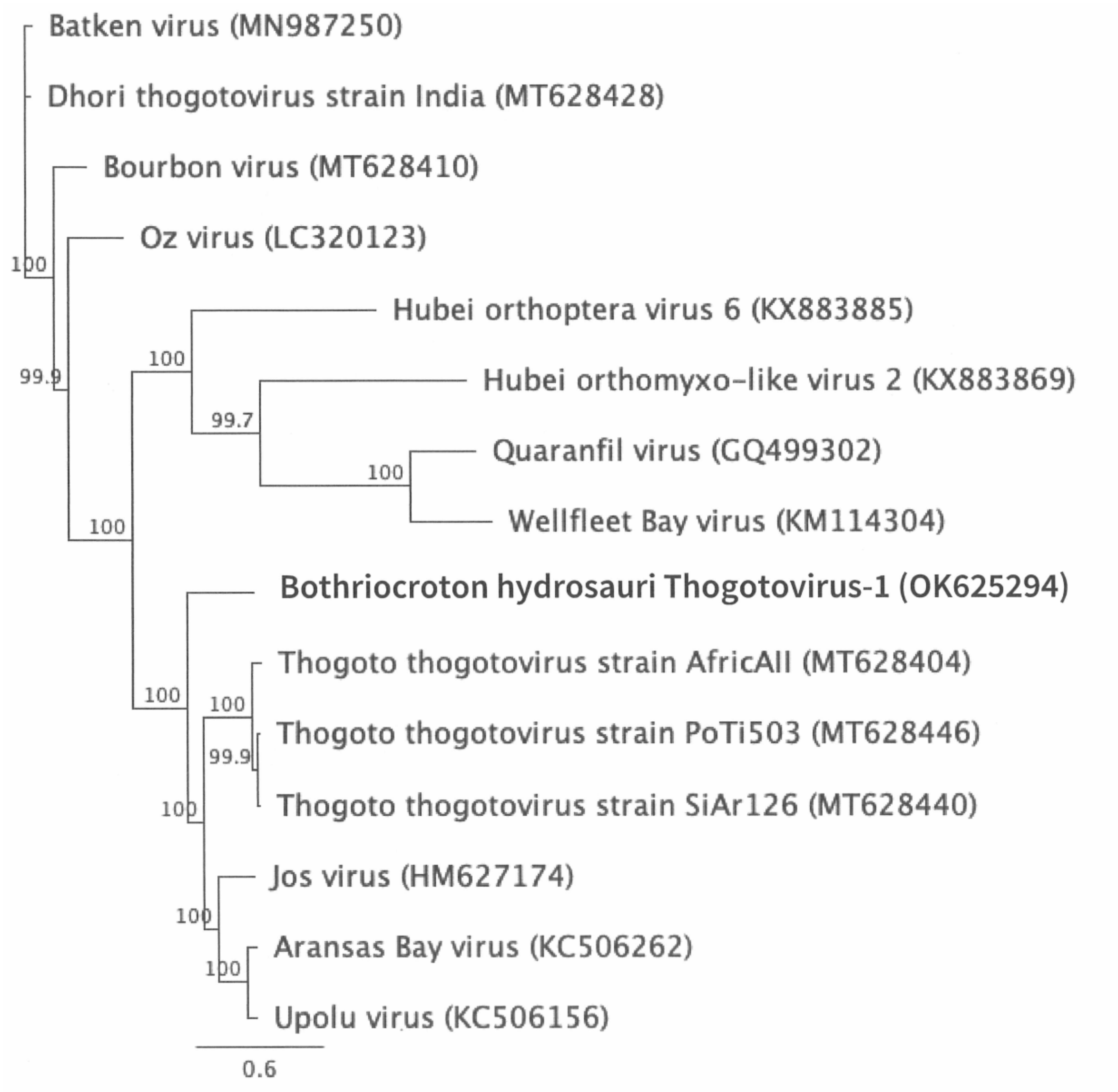

Figure 3. – BHTV-1 Segment 1 Polymerase B2 Gene phylogenetic tree compared to other orthomyxoviruses based on amino acid sequences.

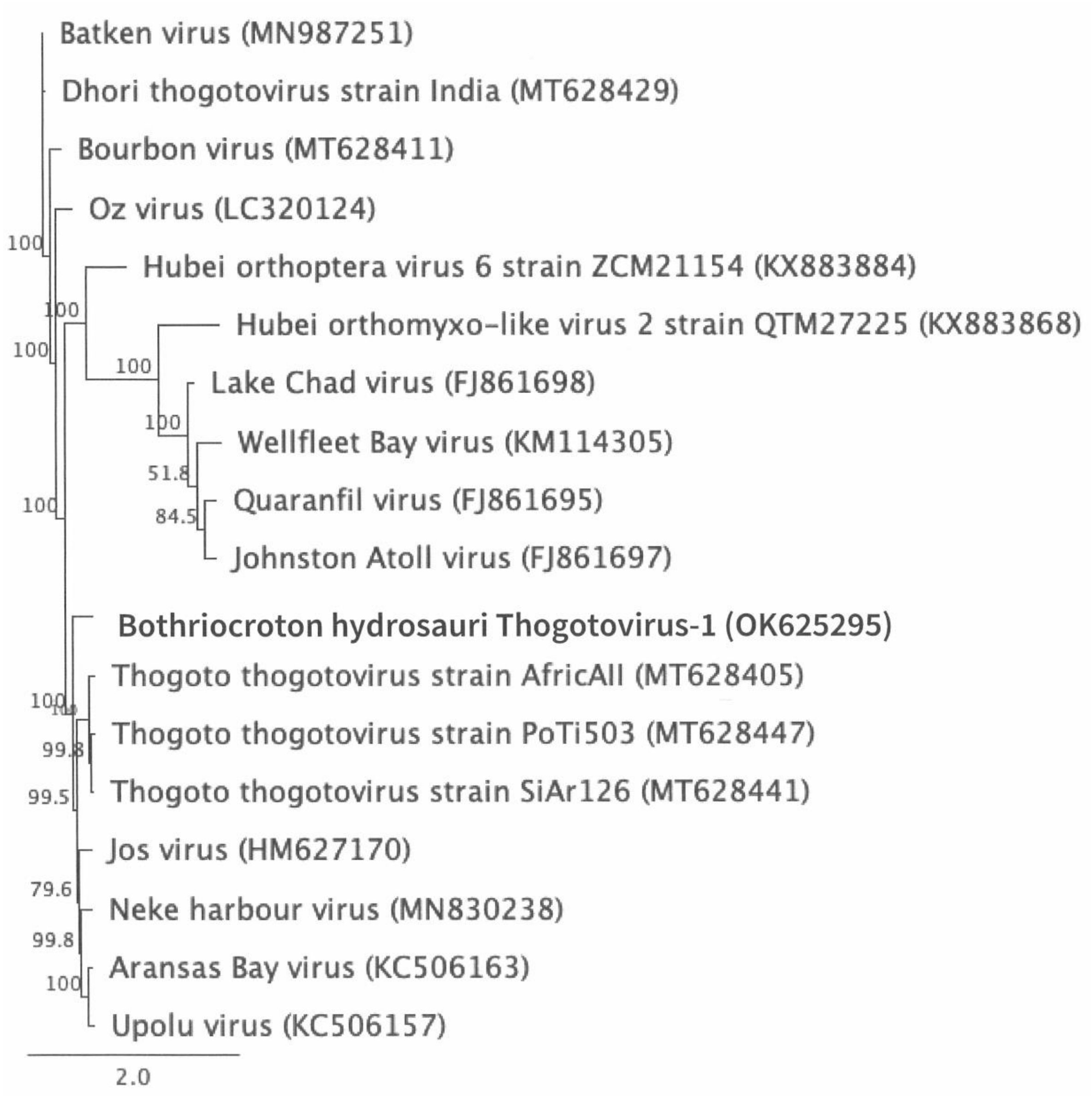

Figure 4. – BHTV-1 Segment 2 Polymerase B1 protein gene phylogenetic tree compared to other orthomyxoviruses based on amino acid sequences.

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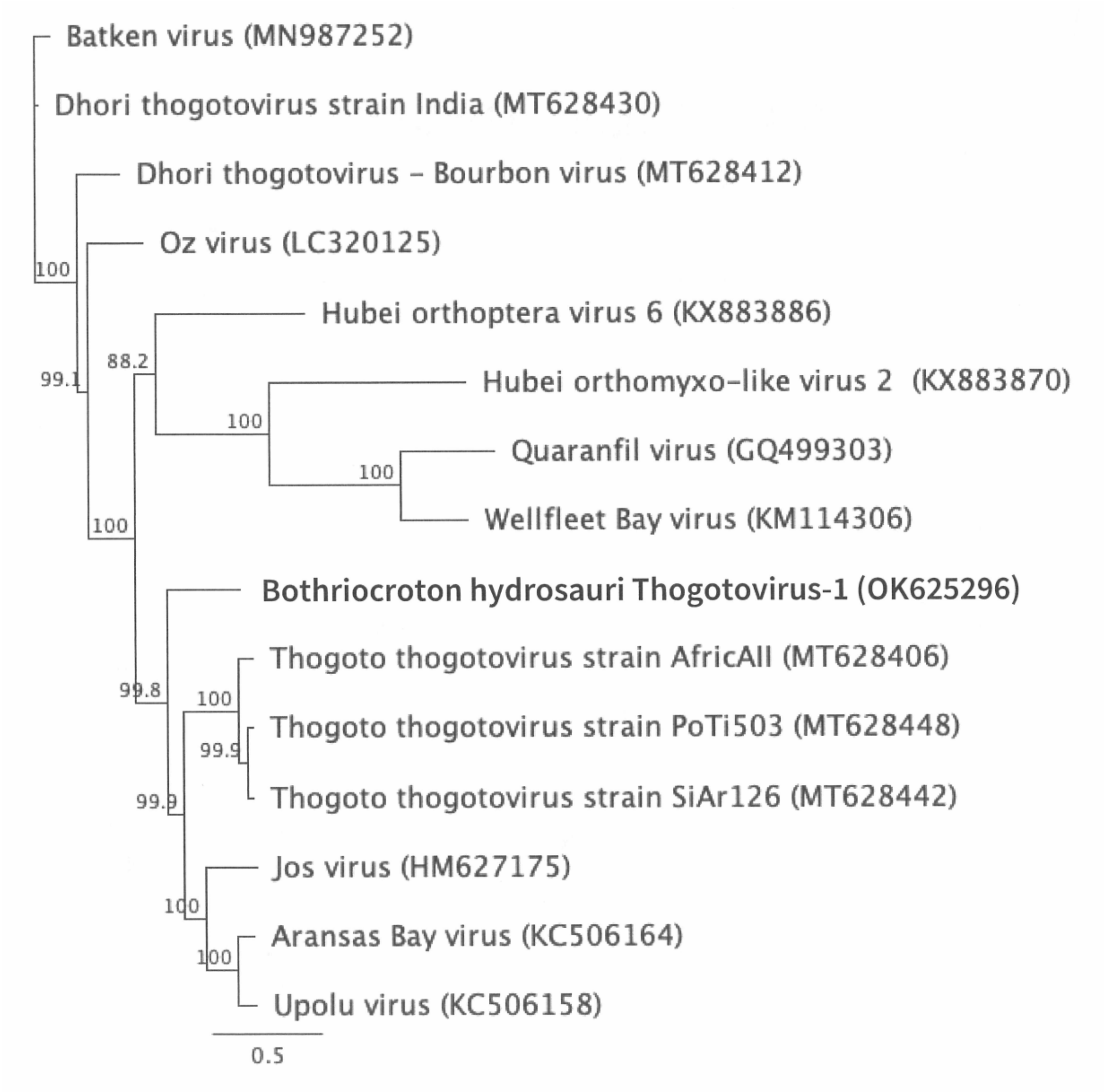

Figure 5. – BHTV-1 Segment 3 Polymerase A protein gene phylogenetic tree compared to other orthomyxoviruses based on amino acid sequences.

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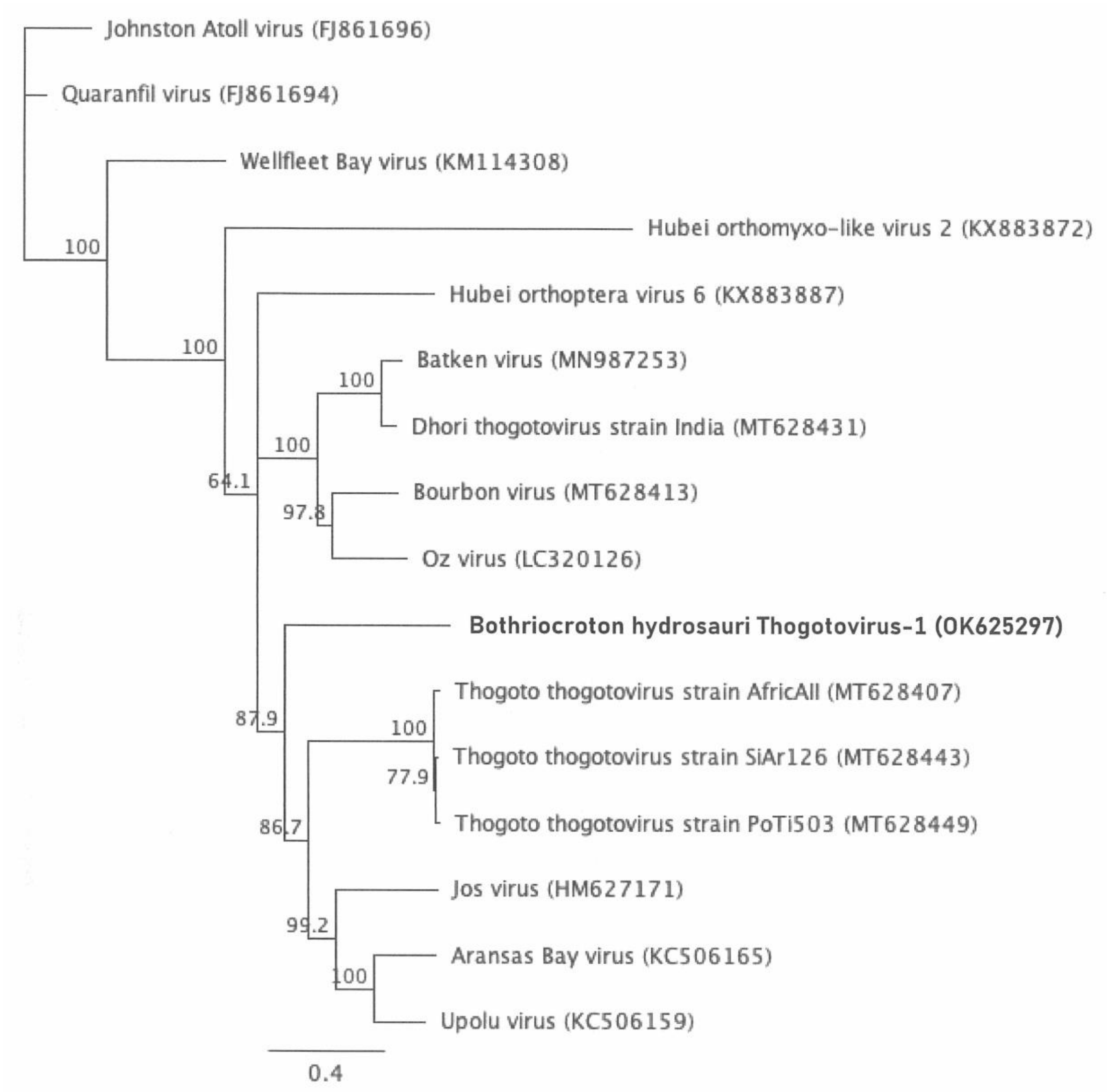

Figure 6. – BHTV-1 Segment 4 Glycoprotein gene phylogenetic tree compared to other orthomyxoviruses based on amino acid sequences.

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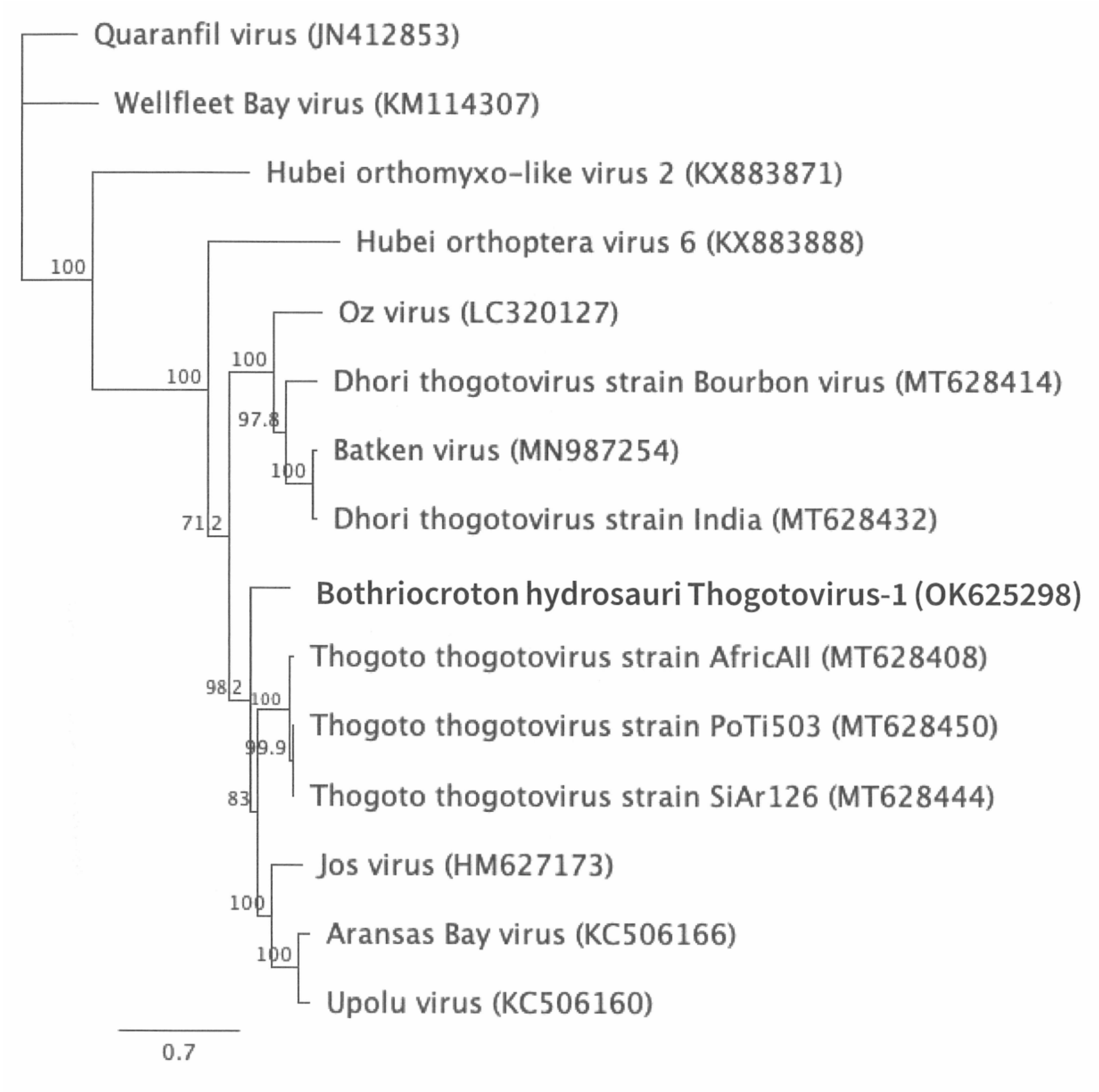

Figure 7. – BHTV-1 Segment 5 Nucleoprotein gene phylogenetic tree compared to other orthomyxoviruses based on amino acid sequences.

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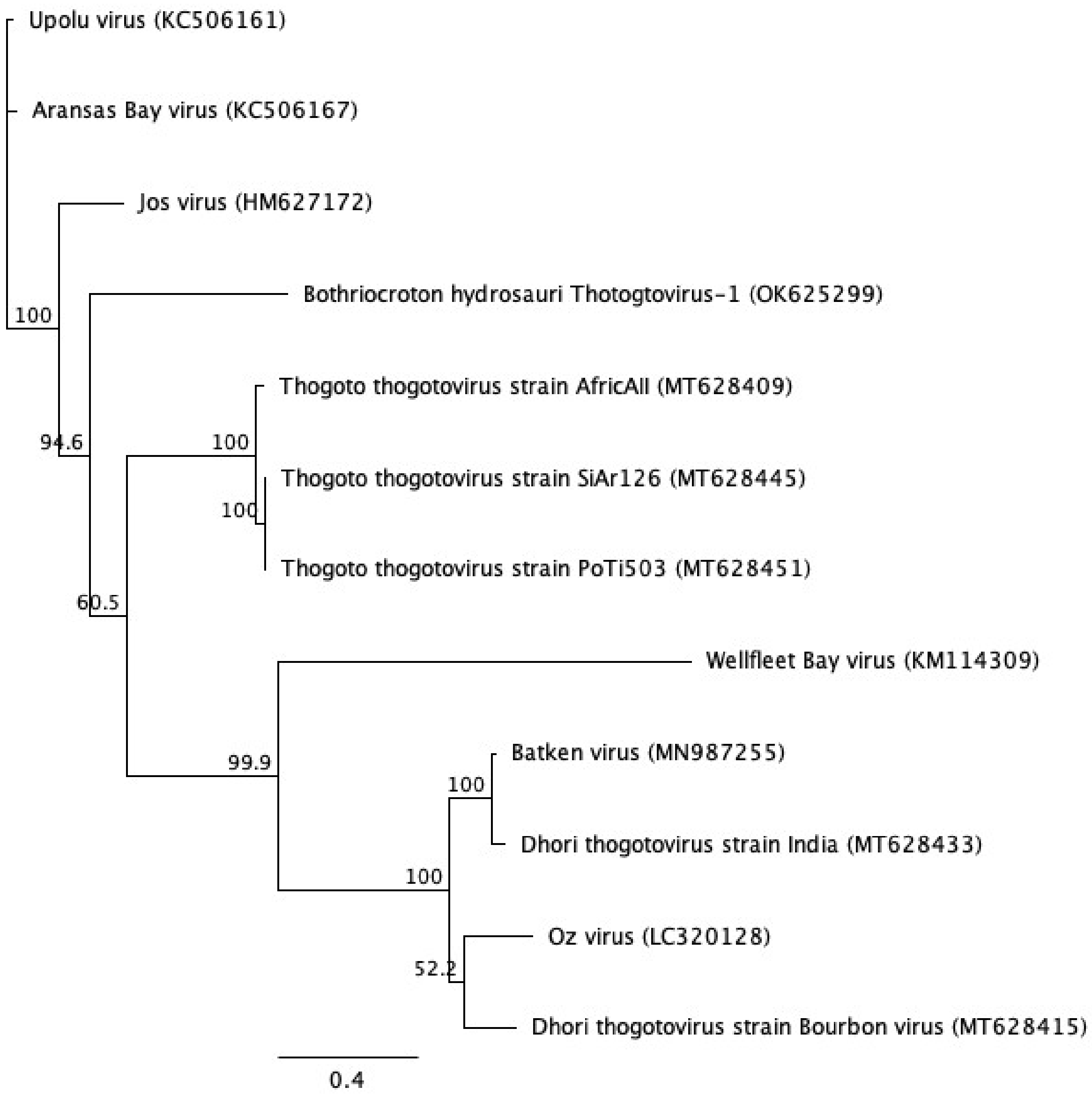

Figure 8. – BHTV-1 Segment 6 Matrix protein gene phylogenetic tree compared to other orthomyxoviruses based on amino acid sequences.

4. Discussion

In the current study, we identified a novel tick-borne orthomyxovirus, BHTV-1, during attempts to isolate novel rickettsial agents from B. hydrosauri ticks. No rickettsia were detected during this study. Genetic characterization of this novel virus shows that it is closely related to viruses within the Thogotovirus genus of the *Orthomyxoviridae* family. Like other members of the *Orthomyxoviridae* family, Thogotoviruses are single-stranded, negative sense segmented RNA viruses (ssRNA)[21]. These viruses have been reported in a range of host species, including rodents[22], domesticated livestock[23,24] and humans[25,26]. In the latter host, viruses from this genus have been linked to serious infection outcomes, including fever and neurological symptoms[27]. Virus isolation and molecular studies suggest that the primary reservoir for thogotoviruses are hard tick species [28,29], with transmission to humans the result of tick bite[27]. The isolation of BHTV-1 represents the second detection of a putative Thogotovirus genus virus from an Australian hard tick with UPOV previously isolated from *O. capnesis* ticks infesting sooty terns[9]. Human-biting *B. hydrosauri* ticks have already proven as competent reservoirs of tick-borne diseases with this tick species the host of *R. honei*, the causative agent of Flinders Island Spotted Fever in Australia[14,30]. Its detection in a tick removed from a blotched, blue-tongued, skink likely suggests that the virus can potentially infect reptiles as well, which is an observation that is yet to be described for thogotoviruses. As present, the human

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health significance of the detection and description of BHTV-1 is unclear but given that (a) *B. hydrosauri* are human-biting ticks responsible for transmission of tick-borne diseases; and (b) there is growing evidence around the world for the pathogenic potential of thogotoviruses, further research into the prevalence and host range of this virus is warranted.

Orthomyxovirus genomes consist of six segments (thogotoviruses) to eight segments (influenza A viruses)[31]. Our genomic and phylogenetic analysis identified thogotovirus homologues for six of the ORFs described in thogotovirus family members, including viral polymerase complex proteins, (polymerase basic subunit 1 (PB1), polymerase basic subunit 2 (PB2), polymerase acidic subunit (PA)), a surface glycoprotein, a nucleoprotein and matrix protein[11]. Phylogenetic analysis of the predicted amino acid sequences for each of these ORFs revealed that four of six BHTV-1 proteins shared closest phylogenetic relationships to thogotovirus and Jos virus. Experimental infection studies have shown that tick-derived Dhori virus can cause fulminant, systemic and fatal disease in infected mice[32] while the latter thogotovirus was linked to a human tick bite-associated death reported in the United States in 2014[27]. In contrast to the results for the other BHTV-1 proteins, phylogenetic analysis of the predicted glycoprotein and revealed closest similarity to viruses within the *Thogotovirus* genus, *Orthomyxoviridae* family, Upulu and Aransus Bay. Given the potential geographic and host overlap between viruses from each of these orthomyxovirus genera, it is conceivable that co-infection (presumably in a tick) and potential reassortment events between species may have occurred within the evolutionary history of BHTV-1.

An obvious limitation of this study was that viral isolation was performed on only a subset of *B. hydrosauri* ticks isolated from blotched blue-tongued skinks. Furthermore, while viral sequences were verified by mapping trimmed reads to reveal misassembled regions, RACE-PCR and traditional PCR and Sanger sequencing are required to confirm the BHTV-1 segments sequenced and to obtain complete genome ends.

5. Conclusions

In this study, a novel thogotovirus, BHTV-1, was isolated from a reptile-infesting *B. hydrosauri* tick in Tasmania. *B. hydrosauri* ticks are competent vectors for tick-borne diseases in Australia and have a growing reputation for causing disease in humans and animals. This may be the first known report of reassortment between different genera of orthomyxoviruses, in this case between one thogotoviruses genus and another. The effect on the pathogenicity of the reassortant virus is unknown and further studies are required to determine the prevalence and potential for these viruses to cause tick-borne diseases in Australia and elsewhere.

Author Contributions: conceptualization, SG, JS and GV; virus isolation, GV, virus growth and sample preparation, PS; gene sequencing and analysis, MT; electron microscopy, SC; support and funding for testing at ACDP, GM; manuscript preparation, PS; review and editing of manuscript, all authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: SG and JS are Directors of the Australian Rickettsial Reference Laboratory, a not-for-profit diagnostic and research laboratory which undertakes diagnostic testing for Rickettsia, Coxiella and Borrelia on human patient specimens. The Australian Government and individual patients pay for these tests. The other authors declare no conflict of interest.

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