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Upolu virus and Aransas Bay virus, two presumptive bunyaviruses, are novel members of the family *Orthomyxoviridae*

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Abstract. Emerging and zoonotic pathogens pose continuing threats to human health and ongoing challenges to diagnostics. As nucleic acid tests are playing increasingly prominent roles in diagnostics, the genetic characterization of molecularly uncharacterized agents is expected to significantly enhance detection and surveillance capabilities. We report the identification of two previously unrecognized members of the family *Orthomyxoviridae*, which includes the influenza viruses and the tick-transmitted Thogoto and Dhori viruses. We provide morphologic, serologic and genetic evidence that Upolu virus (UPOV) from Australia and Aransas Bay virus (ABV) from North America, both previously considered potential bunyaviruses based on electron microscopy and physicochemical features, are orthomyxoviruses instead. Their genomes show up to 68% nucleotide sequence conservation to Thogoto virus (segment 2; ~74% at amino acid level) and a more distant relationship to Dhori virus, the two prototype viruses of the recognized species in the genus *Thogotovirus*. Despite sequence similarity, the coding potential of UPOV and ABV differed from Thogoto virus, being instead like that of Dhori virus. Our findings suggest that the tick-transmitted UPOV and ABV represent geographically distinct viruses in the genus *Thogotovirus* of the family *Orthomyxoviridae* that do not fit in the two currently recognized species of that genus.

Importance. Upolu virus (UPOV) and Aransas Bay virus (ABV) are shown to be orthomyxoviruses instead of bunyaviruses as previously thought. Genetic characterization and adequate classification of agents is paramount in this molecular age to devise appropriate surveillance and diagnostics. Although closer to Thogoto virus by sequence, UPOV and ABV differ in their coding potential by lacking a proposed pathogenicity factor. In this respect they are similar to Dhori virus, which despite this lack can cause disease. These findings enable further studies into the evolution and pathogenicity of orthomyxoviruses.

52 **Introduction**

53 Upolu virus (UPOV) strain C5581, an enveloped spherical virus with a diameter of approx. 100
54 nm, was isolated in 1966 from adult *Ornithodoros capensis* ticks that infested a sooty tern
55 (*Onychoprion fuscatus/Sterna fuscata*) colony on Upolu Cay, a small atoll of the Great Barrier
56 Reef, Australia (1). No serologic relationship of UPOV to other viruses was demonstrated until
57 1975 when three antigenically related isolates of Aransas Bay virus (ABV) were obtained from
58 ticks of the same species complex collected from sea-bird nests on islands off the southern
59 Texas coast (2). UPOV and ABV were considered to form a distinct antigenic group.

60 UPOV and ABV do not propagate in mosquitoes but replicate in mammalian cell cultures
61 (African green monkey kidney (Vero), baby hamster kidney (BHK), Madin-Darby canine kidney
62 (MDCK), human embryonic kidney 293 (HEK293) cells; (2, 3)). An incompatibility of tick-derived
63 arboviruses with mosquito physiology has also been observed with other related tick-associated
64 viruses such as Quaranfil and Johnston Atoll (4, 5). Based on physicochemical and
65 morphological features reported for UPOV, the viruses of the Upolu serogroup (UPOV and ABV)
66 were tentatively placed in the family *Bunyaviridae* as two species not assigned to one of the
67 genera of this family of enveloped negative sense, single-stranded RNA viruses with tripartite
68 genomes (3, 6). Here, we report data clearly demonstrating that UPOV and ABV are
69 orthomyxoviruses.

70 The family *Orthomyxoviridae* includes the influenza viruses in the genera *Influenzavirus*
71 *A*, *Influenzavirus B*, and *Influenzavirus C*, infectious salmon anemia virus (ISAV) in the genus
72 *Isavirus* and the tick-transmitted Thogoto (THOV) and Dhori viruses (DHOV) in the genus
73 *Thogotovirus* (6). In addition, several not yet formally classified viruses related to known
74 orthomyxoviruses have been recently described (7, 8). The genomes of orthomyxoviruses
75 consist of 6 (thogotoviruses) to 8 segments (influenzaviruses) of negative sense, single-stranded
76 RNA (9). Replication and transcription take place in the cell nucleus, where the viral polymerase
77 complex, consisting of polymerase basic subunit 1 (PB1), polymerase basic subunit 2 (PB2) and

polymerase acidic subunit (PA), synthesizes negative-strand, viral genomic RNA (vRNA), positive-strand RNA (cRNA) complementary to vRNA, and capped polyadenylated messenger RNAs (mRNA) that are shorter than vRNA and cRNA (10, 11). A function in cap-binding and mRNA synthesis has been assigned to PB2 (12-18), RNA chain elongation to PB1 (19-21), and cRNA and vRNA synthesis as well as cap-cleavage to PA, possibly regulated by phosphorylation (22-26). Whereas the three polymerase subunits are encoded by the three largest genome segments in all orthomyxoviruses, coding assignments for the smaller segments differ between genera. In the tick-transmitted thogotoviruses the fourth largest segment codes for a surface glycoprotein (GP) with distant relationship to that of baculoviruses (27, 28), segment 5 encodes the nucleoprotein (NP) and segment 6 the matrix protein (M), and in some species also an elongated accessory M-long (ML) protein that interferes with the host innate immune response (9).

We present data that demonstrate genetic as well as serologic relationships of UPOV and ABV to the thogotoviruses. The morphology of UPOV and ABV is compatible with that of orthomyxoviruses, serologically they cross-react with THOV, and the complete genome sequences determined for both viruses are more closely related to THOV than to DHOV, but both having coding repertoires similar to DHOV, not THOV. Analysis of the increasing sequence diversity of thogotoviral genomes begins to delineate highly conserved protein domains that may point to novel therapeutic targets of orthomyxoviruses.

Materials and Methods

Viruses. Virus stocks of Upolu virus (UPOV) strain C5581 (1) and Aransas Bay virus (ABV) strain RML65660-8 (2) were obtained from the World Reference Center for Emerging Viruses and Arboviruses collection at the University of Texas Medical Branch at Galveston, USA. Total RNA was extracted with Tri-Reagent (MRC, Cincinnati, OH) from 250 µl of virus stock, suspended in 35 µl nuclease-free water and stored at -80 ^{o}C.

Transmission electron microscopy. Vero E6 cells infected with UPOV or ABV were fixed for 1 h in a mixture of 2.5% paraformaldehyde and 0.1% glutaraldehyde in 0.05 M cacodylate pH 7.3, to which 0.03% picric acid and 0.03% $CaCl_2$ were added. Fixed monolayers were washed with 0.1 M cacodylate, cells scraped, and pelleted cells post-fixed with 1% OsO_4 in 0.1 M cacodylate for 1 h. Cells were washed with distilled water and finally stained *en block* with 2% aqueous uranyl acetate for 20 min at 60 ^{o}C. Preparations were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

Serologic tests. Viral antigens used in serologic tests were not inactivated and prepared by sucrose/acetone extraction of BHK cells, hamster liver or newborn mouse liver (29) infected with the respective viruses. Mouse hyperimmune ascites fluids served as antibody preparations. Four intraperitoneal injections of antigen (10% homogenates of infected newborn mouse brain or liver in phosphate-buffered saline (PBS)) mixed with Freund's complete adjuvant were given at weekly intervals; thereafter mice were inoculated with sarcoma cells, and immune ascitic fluid was collected. Complement fixation (CF) tests were performed in a microtiter plate format by incubation at 4 ^{o}C overnight in the presence of 2 U guinea pig complement (30, 31). On a scale from 0 (complete hemolysis) to 4+ (no hemolysis) CF titers were scored as the highest antibody/antigen dilutions that gave a 3+/4+ fixation of complement; titers ≥1:8 were rated positive. Hemagglutination inhibition (HI) tests were also done in microtiter plates (31, 32). Non-specific hemagglutinin inhibitors were removed by acetone extraction, sera rehydrated in 0.05 M borate, 0.12 M NaCl pH 9, and naturally occurring agglutinins adsorbed to male goose erythrocytes (29). HI was assessed with 4 units of antigen extracted (8.5% sucrose pH 5.75/acetone) from ABV or UPOV infected BHK cells, THOV infected hamster liver, or DHOV infected mouse liver and tested against twofold serial dilutions of pretreated serum beginning at

a dilution of 1:10, and male goose erythrocytes. Animal work was performed under an IACUC approved protocol at the University of Texas Medical Branch.

Unbiased high-throughput sequencing (UHTS), Reverse Transcription – Polymerase Chain Reaction (RT-PCR) and Rapid Amplification of cDNA Ends (RACE). Genomic sequences were generated by applying a combination of UHTS, subsequent consensus RT-PCR and RACE assays. Aliquots of total RNA extracts (0.5 µg) were treated with DNase I (DNA-free; Ambion, Austin, TX, USA) prior to reverse transcription by Superscript III (Invitrogen, Carlsbad, CA, USA) with random octamer primers linked to an arbitrary, defined 17-mer primer sequence. The cDNA was RNase H treated and randomly amplified by PCR with AmpliTaq (Applied Biosystems, Foster City, CA, USA) and a primer mix including the octamer-linked 17-mer-sequence primer in combination with the defined 17-mer-sequence primer in a 1:9 ratio (33). Amplification products >70 bp were purified (MinElute, Qiagen, Hilden, Germany) and ligated to linkers for sequencing on a GS-FLX Sequencer (454 Life Sciences, Branford, CT, USA)(34). Sequence reads were stripped of primer sequences and highly repetitive elements, then clustered and assembled into contiguous fragments (contigs) for comparison by the Basic Local Alignment Search Tool (blast; (35)) to the GenBank database at nt (blastn) and deduced aa level (blastx).

Various specific primer sets for validation of draft genome sequences were designed based on the UHTS data, as well as sequences of THOV, DHOV and another related orthomyxovirus, Batken virus (BKNV)(primer sequences available on request). Gaps between contigs were filled and the completed draft genomes re-sequenced by overlapping PCR products. Reactions included routinely 1 µl random hexamer-primed cDNA (Superscript II; Invitrogen), primers at 0.2 mM, and Platinum Taq DNA polymerase (Invitrogen). Products were purified (QIAquick PCR purification kit; Qiagen) and directly dideoxy-sequenced on both strands (Genewiz, South Plainfield, NJ, USA). Genomic termini were characterized by Rapid Amplification of cDNA Ends (RACE kits; Invitrogen). For 5'-RACE first strand cDNA was

synthesized from total RNA using a custom gene-specific primer 1 (GSP1) and Superscript III. After purification using S.N.A.P. columns, a homopolymeric tail was added with terminal deoxynucleotidyl transferase (TdT, Invitrogen) and dCTP followed by PCR amplification using Platinum Taq DNA polymerase (Invitrogen) and nested primer GSP2 combined with the 5'-RACE deoxyinosine-containing anchor primer. Depending on the choice of GSP1 and GSP2 the 5' ends of genomic (corresponding to 3' end of antigenomic) or the antigenomic RNA were determined. Products were cloned into pCR-TOPO vector (Invitrogen). Transcriptional termination sites were mapped by 3'-RACE employing the poly-A tail of the (shorter) mRNA transcripts for cDNA priming with Invitrogen oligo-dT-adaptor primer. Thereafter, cDNA was amplified by PCR using a primer complementary to the introduced adaptor sequence and a custom sequence specific primer.

PCRs to assess splicing events were performed with forward primer p1 (5'-GCT AAT CGG GTG GAT GGA TG for UPOV, 5'-GCT GAT CGG GTG GAT GGA C for Jos virus (JOSV, an orthomyxovirus related to THOV (7)) and two reverse primers p2 (5'-GGC CGC TTT TTT TTT TTT TTT TTT ATT AAA AT for UPOV, 5'-ATG CGG CCG CTT TTT TTT TTT TTT TTT TAA CAC C for JOSV) or p3 (5'-ccg ccA GAG ATA TCA AGG CA for UPOV, 5'-gcc gcc AGA GAA ATC AAG GCA for JOSV). Nucleic acid extracts for amplification were generated from crude cell homogenate (cellular RNA; cR) or nuclease-treated (8 ng/µl RNase A (Ambion), 15 min at RT; 0.3 u/µl Benzonase nuclease (Qiagen) and 0.06 u/µl TURBO DNase (Ambion) for 45 min at RT; followed by 8 ng/µl RNase A and 0.08 u/µl RNase H (Invitrogen) for 2 h at 37 ^{o}C) cell culture supernatant (genomic RNA; gR) obtained from virus-infected human embryonic kidney (HEK) 293 cells harvested 72 h post infection. PCR products were analyzed by agarose gel electrophoresis and visualization by GelGreen staining (Biotium, Hayward, CA, USA).

Sequence analyses. Sequence assembly and analysis employed programs of the Wisconsin GCG Package (Version 10.3, Accelrys Inc., San Diego, CA), MEGA 5 (36), Geneious 5.5 (37), and NewblerAssembler 2.4. Identities of nt and aa sequences were calculated with the

Needleman-Wunsch algorithm, applying an EBLOSUM62 substitution matrix (gap open/extension penalties of 12/2 for nt and 6/1 for aa alignments; EMBOSS (38)) and a Perl script to parse the results for all comparisons. Topology and targeting predictions were obtained by using SignalP, NetNGlyc, and TMHMM (www.cbs.dtu.dk/services), Phobius (phobius.sbc.su.se), and Phyre2 (www.sbg.bio.ic.ac.uk/phyre2)(39, 40). Multiple sequence alignments were generated with CLUSTAL (41), and programs implemented in MEGA and Geneious software were applied for phylogenetic analyses.

Results

Recognition of UPOV and ABV as orthomyxoviruses. The failure to obtain amplification products from nucleic acids of UPOV or ABV by reverse transcription-polymerase chain reaction (RT-PCR) using a panel of degenerate bunyaviral consensus primers led us to pursue UHTS. Sequence libraries were prepared from total RNA extracted from ABV virus stock. Sequencing on the Roche GS-FLX platform yielded 94,835 reads with a mean length of 222 bases (range 29-382) that generated contiguous sequence assemblies (contigs) with homology to THOV in regions corresponding to approximately 30% to 80% of the six THOV genome segments (segment 1 ~70%, segment 2 ~60%, segment 3 ~30%, segment 4 ~40%, segment 5 ~70%, and segment 6 ~80%). Continuous coding sequences for UPOV and ABV were subsequently generated through consensus RT-PCR using primers representing the ABV contigs, as well as sequences of THOV, DHOV, and the related orthomyxovirus BKNV (42, 43). Rapid amplification of cDNA ends (RACE) was applied to determine 5' and 3' genomic and 3' mRNA termini (**Table 1, 2**; GenBank Accesssion numbers for UPOV and ABV are KC506156-61 and KC506162-67, respectively).

Morphology of UPOV and ABV virons. Transmission electron microscopy of ultrathin sections showed UPOV and ABV virions in clusters at the cell surface of infected Vero E6 cells (**Fig. 1**). Virions of UPOV were either round with diameters of 75-95 nm, or slightly oval with sizes

ranging from 75 x 85 nm to 105 x 120 nm (**Fig. 1A**). Virions of ABV were more polymorphic and partly larger ranging from 75 x 85 nm up to 105 x 130 and 120 x 140 nm (**Fig. 1B**).

Genetic and serologic characterization of UPOV and ABV. UPOV and ABV display terminal sequences that are semi-complementary and conserved among the six segments and the two viruses (**Table 2**). Overall, the termini of each segment adhere to consensus sequences determined for THOV (3'-UCG UUU UUG UU/CC GU/CC/G/U, and 5'-AGA GAA/U AUC AAG/A GCA/G G/C UUU UUU), although specific differences are evident at the 3'-terminus in positions 6 ('A', similar to DHOV segments 5 and 6), 8 ('C', similar to influenza viruses) and 16-19 (conserved AAA/CA/G, similar to Jos virus (JOSV) (7)), as well as in position 6 of the 5'-terminus of UPOV segment 5 and ABV segment 3 ('C'). In THOV and JOSV the 3' terminal sequence of segment 6 differs from all other segments. No specific difference of the 3' terminal sequence of segment 6 is found in UPOV and ABV, similar to DHOV. Analogous to influenza virus, formation of a forked terminal panhandle has been shown to be essential for promoter function in THOV, although with potential differences in the intra-strand base pairing of vRNA and cRNA 'hook' structures (44-47). Compared to that do the changes in the terminal sequences of UPOV and ABV either locate to the unpaired fork region (3'/5' position 6; **Figure 2**) with no compensating base change at the opposite terminus, or to the paired panhandle region with compensating mutations at the opposite terminus (3' position 11/5'position 12; genomic orientation). In addition, 3' C8 (genomic orientation) allows for a second paired base of a potential 3'-'hook' in several of the segments, and potential wobbling between intra-stand pairing of 3' C2/G9 – 5' G2/C9 and inter-strand pairing of 3' C2/5' G2 – 3'G9/5' C9 may provide options for 'breathing' of the structure (**Figure 2**). Termination of mRNA transcripts occurred at a conserved oligo$(U)_{5\text{-}6}$ signal located 17 nucleotides (nt) from the 5'-end of vRNA templates as indicated by RACE with oligo d(T)-priming. The level of coding sequence similarity between individual segments of UPOV and ABV, and to corresponding segments of other orthomyxoviruses is variable (**Table 3**). Phylogenetic analysis indicates that the evolutionary relationship for all segments is

consistently closest between UPOV and ABV, and that both are closer to the recently characterized JOSV and THOV than to DHOV or the influenza viruses (**Fig. 3**).

The largest segments of UPOV and ABV show sequence homology to orthomyxoviral PB2 gene sequences (PF00604 'Flu_PB2'; http://pfam.sanger.ac.uk; **Table 1, 3)**. Although PB2 sequence is least conserved among orthomyxoviral polymerase subunits, UPOV and ABV sequences match closely to those of JOSV and THOV, with DHOV being more distantly related particularly in the C-terminal portion. Only a few amino acid (aa) motifs (D_{89}LG, R_{149}KPV, W_{225}LP, I_{314}CRVALG in UPOV) are conserved with respect to influenzaviruses outside of an N-terminal motif (F_{40}-L_{56} in UPOV) that is recognizable throughout influenza and tick-transmitted viruses and located in a region that is implicated in PB1-binding in influenza A virus (FLUAV)(48). Only limited conservation is noted for the cap-binding domain defined in FLUAV, although the secondary structure of the N-terminal part and aromatic residues corresponding to FLUAV F_{330}, F_{363}, and F_{404} (but not F_{323}/F_{325}) are maintained as previously reported also for THOV (18, 49). Consistent with the nuclear replication of orthomyxoviruses a nuclear localization signal (NLS; K_{745}RRX$_{11}$KRPRR), resembling the bipartite NLS identified in FLUAV (K_{736}RKRX$_{12}$KRIR (50-52)), is present. However, mutational analysis in THOV did not support a functional NLS role for its homologous K_{753}RRR motif (53).

The sequences of UPOV and ABV segments 2 correspond to orthomyxoviral PB1 sequences (PF00602 'Flu_PB1'; http://pfam.sanger.ac.uk; **Table 1, 3),** and shows conservation of the polymerase motifs pre-A, A, B, C, D, and E (20, 54-56). Conservation is also noted for aa maintained between THOV, DHOV, and influenzaviruses in the second half of the N-terminal domain involved in PA binding in FLUAV (Y_{22}-Y_{47} in UPOV)(57-59), and a downstream motif present throughout the orthomyxoviruses (L_{118}-T_{124} in UPOV). PB1s of UPOV and ABV have a rather neutral pI (**Table 1**), more similar to PB1 of THOV than that of influenzaviruses. No conservation is obvious in the region of the FLUAV bipartite NLS (60), as it is also the case in THOV and DHOV.

Segments 3 of UPOV and ABV encode a PA-like protein (PF00603 'Flu_PA'; http://pfam.sanger.ac.uk; **Table 1, 3**). The endonuclease motif $PDX_n(D/E)$ described for FLUAV (24, 25) corresponds in UPOV and ABV to a $P_{96}HX_{16}D$ motif that is not surrounded by additional characteristic primary or secondary sequence conservation reported for FLUAV. Elevated conservation is noted in the C-terminal part of the sequence (around Q_{426}-F_{452} in UPOV) that has been implicated in interaction with PB1 in FLUAV (61).

The putative glycoproteins (GP) of UPOV and ABV are coded by segment 4 (**Table 1, 3**). Instead of showing conservation with respect to influenzavirus-like orthomyxoviral GPs, the overall structure of UPOV and ABV GP is similar to corresponding proteins of THOV and the 'baculovirus gp64 envelope glycoprotein family' (PF03273; http://pfam.sanger.ac.uk;(27, 28), including conservation of glycosylation sites around positions 183 and 415/428 of UPOV (**Fig. 4**). Primary sequence conservation is observed in the N-terminal region containing a potential fusion peptide cleavage site (V_{59}GY-WGS$_{116}$ in UPOV; homologous to A_{61}GY-WGS$_{118}$ proposed for THOV (28)), and for motifs W_{155}RCGV, upstream of the only strictly conserved glycosylation site N_{183}GS, and S_{351}LSKIDERLIG, S_{391}NC, D_{401}GRW, and G_{444}VIEDEEGWNF. Significant differences are noted for the cytoplasmic tail regions of GPs of the various orthomyxovirus species (**Fig. 4**). Serologic analyses by hemagglutination inhibition (HI) test indicate limited cross-reactivity between UPOV, ABV, and THOV (**Table 4**). Interestingly, antigenic relatedness was greater between ABV and THOV than between UPOV and THOV or UPOV and ABV, pointing to sequence areas divergent between UPOV and ABV as potentially involved in HI epitopes (possibly including in UPOV I_{43}-E_{55}, W_{98}-C_{110}, L_{122}-K_{134}, K_{171}-V_{175}, C_{225}-H_{235}, L_{364}-K_{371}, W_{404}-I_{424}, and particularly L_{264}-H_{306} that includes indel regions).

The nucleoprotein (NP) of orthomyxoviruses represents the main type-specific antigen recognized in complement fixation tests (CF; **Table 4**), and has been widely used to assess phylogenetic relationships. The ORF coded by segment 5 of UPOV and ABV is conserved with respect to 'influenza virus nucleoprotein' (PF00506; http://pfam.sanger.ac.uk; **Table 1, 3**).

Although only low conservation is observed for the N-terminal NLS characterized in the NP of FLUAV (62, 63), which is also he case in THOV and DHOV, higher conservation is noted in the second half of a region that is proposed for RNA interaction in FLUAV (L_{134}, V_{137}, L_{139}, T_{143}, I_{147}, $Q_{150}K$, V_{160}, A_{168}, G_{170}, I_{173}, R_{176}, and G_{186} in UPOV)(64, 65). Conservation is also evident in the previously characterized internal NP regions 2 to 5 (66). This includes in region 4 sequence corresponding to a proposed nuclear accumulation motif of FLUAV (S_{329}AGEDLGLLS in UPOV)(67, 68), and in region 5 a motif similar to a C-terminal bipartite NLS motif found in THOV and JOSV ($K_{388}RX_9KGKR$ in UPOV)(7), but not in DHOV. The internal bipartite NLS characterized in THOV and FLUAV is conserved ($K_{195}RX_9KTKR$ in UPOV)(69).

Segments 6 of UPOV and ABV show no homology to entries in the protein families database. The nt sequences align only with segment 6 sequence of JOSV and the C-terminal quarter of that of THOV, but not to those of DHOV or the influenza viruses (**Table 1, 3**). Limited conservation with respect to DHOV is discernable at the deduced aa level for a short motif (A_{249}KGVSYQVL in UPOV) and strictly conserved aa E_{175}, $N_{181}T$, E_{212}, $Y_{224}D$, G_{232}, E_{236}, I_{240} located in the C-terminal region that has been proposed for the matrix protein (M) of THOV to inhibit viral polymerase activity (70). Segments 6 of UPOV, ABV and DHOV have longer UTRs than those of THOV and JOSV (DHOV, 121 nt; UPOV and ABV, 128 and 138 nt, respectively). PCR analyses of genomic and mRNA preparations indicated that only a single size segment 6 mRNA transcript was generated by UPOV, whereas two differently sized mRNA transcripts were generated by JOSV (**Fig. 5**; (7)). This correlates with different coding strategies used by the viruses. Whereas segment 6 of DHOV codes only for an M protein (71) that terminates in an analogous position as the putative M ORF of UPOV and ABV, THOV and JOSV are known to generate two products through splicing (72); ML is generated from non-spliced transcripts resulting in a UTR of 20 nt (73), while M is generated from a spliced transcript by creation of a stop codon at the splice junction, which is located in a position corresponding to the stop codons for M in UPOV, ABV and DHOV (**Fig. 5C**). Of note, sequence conservation between UPOV and

ABV is highest for segment 6, and this segment's sequence is also one of the closest to JOSV and THOV (**Table 3**), despite the observed differences in coding potential.

Discussion

The analyses of the genome sequences of UPOV from Australia and ABV from North America show that they are up to ~75% identical at the aa level (nt ~68%; **Table 3**) to viruses in the family *Orthomyxoviridae*. The genetic distances of these are smallest with respect to JOSV and THOV, ranging from approx. 76% aa/68% nt (PB1) to 52% aa/60% nt (GP) identity with JOSV, and approx. 74% aa/68% nt (PB1) to 43% aa/56% nt (GP) identity with THOV. However, the coding strategy of segment 6 of UPOV and ABV differs from that of JOSV and THOV and is similar to that of DHOV. Differences in the commonly conserved segment termini are also compatible with a significant evolutionary distance of UPOV and ABV from the species *Thogoto virus*. The species *Dhori virus* includes two viruses, DHOV and BKNV, which share approx. 97% and 90% aa (87%/80% nt) identity among their available partial NP and GP sequences, respectively. In comparison, DHOV and THOV share only between 42% and 35% aa (55%/54% nt) identity for their NP and GP sequences, respectively. This is also reflected by the serological reaction between the viruses. Whereas DHOV and BKNV cross-react, DHOV and THOV are antigenically distinct. This provided the basis to include BKNV together with DHOV in a single species *Dhori virus*, separate from the species *Thogoto virus* (6, 43). Both, UPOV and ABV are antigenically closer to THOV than to DHOV in HI tests, whereas differences exist by CF test in their cross-reactivity to THOV and between each other when ABV antigen/UPOV antibody is tested. These serologic results combined with a <60% aa (<60% nt) sequence identity of their NP or GP sequences to THOV or DHOV and the observed differences in coding capacity suggest that UPOV and ABV should be considered as separate species within the genus *Thogotovirus*, distinct from the species *Dhori virus* and *Thogoto virus*. In addition, aa sequence identities between UPOV and ABV of as little as 86% (nt 78%), and even less for the

338 immunoreactive GP, combined with the serologic differences observed between them, may
339 justify their classification as two separate species.
340 Due to their distinct structure, the GPs of the tick-infecting orthomyxoviruses have been
341 classified as Class 3 penetrenes, distinct from the Class 2 penetrenes in alphaviruses and
342 flaviviruses, and the Class 1 penetrenes in the influenza viruses, (28). Furthermore, it has been
343 hypothesized, based on sequence homologies that GPs of viruses in the genus *Thogotovirus*
344 may have been derived from a common ancestor with insect baculoviruses (27, 28). Thus, the
345 tick-infecting orthomyxoviruses represent an evolutionary lineage distinct from the
346 influenzaviruses, and an ancestral relationship of either orthomyxoviral line to the other is not
347 apparent from available data (**Fig. 3**). Nonetheless, the tick-adapted orthomyxoviral GPs are
348 compatible with mammalian receptors as exemplified by reports of human THOV and DHOV
349 infections. In central Africa and regions of southern Europe THOV has also been isolated from
350 various ruminant species (61, 74). The geographic distribution of DHOV includes primarily India
351 and eastern Russia, but also East Africa, Egypt and other Mediterranean countries where
352 serologic data indicate circulation in ruminants as well as waterfowl (61, 75-78). Migratory
353 waterfowl are also reservoirs of influenza A viruses (78, 79). Cases of natural human infection
354 have been reported for THOV from Africa (77); and accidental laboratory infections with DHOV
355 indicate that this virus can also act as a human pathogen (76), despite the lack of an ML protein
356 (71, 73). UPOV and ABV productively infect BHK, Vero or HEK 293 cells and are lethal to
357 newborn mice after intracerebral inoculation (1, 2, 61), suggesting that mammalian pathogenicity
358 is also conceivable for UPOV and ABV.
359 In FLUAV, reassortment of genome segments is a well-known phenomenon that leads to
360 sudden genetic shifts that can result in dramatic chances in pathogenicity. Reassortment in
361 arthropod and vertebrate hosts has also been demonstrated for THOV in experimental settings
362 (80, 81). The dissemination of genetically related tick-transmitted orthomyxoviruses over large
363 distances by migratory birds (74) may support genome segment reassortment culminating in the

364 emergence of novel genotypes with altered pathogenicity and host range. Indeed, the recent
365 implication of other tick-borne orthomyxoviruses in a proposed genus *Quaraavirus* in human
366 febrile illness (82) and the discovery of variants with high bird pathogenicity (83, 84) reinforce the
367 need for comprehensive surveillance and characterization of this growing group of viruses to
368 closely monitor their potential as emerging pathogens.
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636 **Table 1.** Properties of UPOV and ABV genome segments.

Virus	Segment	Segment length [nt]	5'-UTR [nt]	ORF length [aa]	3'-UTR [nt]	predicted MW [kDa]	pI	FLUAV/THOV Homolog
UPOV	1	2,385	27	774	36	89.4	9.0	PB2
ABV		2,384	27	774	35	89.1	9.0	
UPOV	2	2,245	45	716	52	81.3	7.5	PB1
ABV		2,246	45	716	53	81.4	8.0	
UPOV	3	1,984	35	629	62	72.5	5.7	PA
ABV		1,984	35	629	62	72.5	5.7	
UPOV	4	1,635	23	524	40	59.1	8.7	GP
ABV		1,630	23	521	44	59.1	6.4	
UPOV	5	1,542	30	470	102	53.2	9.0	NP
ABV		1,544	32	470	102	53.1	9.1	
UPOV	6	973	32	271	128	30.2	6.6	M
ABV		983	32	271	138	30.2	6.6	

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642 **Table 2.** Sequence conservation at the termini of genome segments.

UPOV Segment	3' terminus (genomic orientation)	5' terminus (genomic orientation)
1 (PB2)	3'- UCG UU**A** U**C**G UUC GUC AA**A**A GUA	5'- AGA GAU AUC AAA GCA G UUU UUU
2 (PB1)	UCG UU**A** U**C**G UCC GUC AA**A**A GU**U**	AGA GAU AUC AAG GCA G UUU UUU
3 (PA)	UCG UU**A** U**C**G UUC GUC AA**A**A GU**U**	AGA GAA AUC AAA GCA G UUU UUU
4 (HP)	UCG UU**A** UUG UCC GUC AA**A**A GU**U**	AGA GAA AUC AAG GCA G UUU UUU
5 (NP)	UCG UUU U**C**G UCC GUC AA**A**A GU**U**	AGA GA**C** AUC AAG GCA G UUU UU**C**
6 (M)	UCG UU**A** UUG UCC GUC AACA G**AU**	AGA GAU AUC AAG GCA G UUU UUU
ABV Segment		
1 (PB2)	3'- UCG UU**A** U**C**G UUC GUC AA**AG** UGA	5'- AGA GAU AUC AAA GCA G UUU UUU
2 (PB1)	UCG UU**A** U**C**G UCC GUC AA**A**A **A**GU	AGA GAU AUC AAG GCA G UUU UUU
3 (PA)	UCG UU**A** U**C**G UUC GUC AA**A**A GU**U**	AGA GAA AUC AAA GCA G UUU UU**C**
4 (HP)	UCG UUU U**C**G UCC GUC AA**A**A GU**U**	AGA GAA AUC AAG GCA G UUU UUU
5 (NP)	UCG UUU U**C**G UCC GUC AA**A**A GU**U**	AGA GAU AUC AAG GCA G UUU UUU
6 (M)	UCG UU**A** U**C**G UCC GUC AA**AG** **AAU**	AGA GAU AUC AAG GCA G UUU UUU
THOV Segment	cons: UCG UUU UUG UyC Gyb wvCw kkk	cons: AGA GAw AUC AAr GCr S UUU UUU
1 (PB2)	3'- UCG UUU UUG UUC GC**U** ACCU GU**C**	5'- AGA GAA AUC AAG GCG **A** UUU UU**C**
2 (PB1)	UCG UUU UUG UCC GCG AG**G**U UUG	AGA GAA AUC AAG GCG C UUU UUU
3 (PA)	UCG UUU UUG UUC GUG AACU GUA	AGA GAA AUG AAA GCA C UUU UUU
4 (HP)	UCG UUU UUG UUC GUC UACA **A**GG	**N**GA GAU AUC AAA GCA G UUU UUU
5 (NP)	UCG UUU UUG UCC GUC AG**U**U UUA	AGA GAA AUC AAG GCA G UUU UUU
6 (M)	UC**A** **C**CU UUG UCC GUC ACCU **C**UA	AGA GAA AUC AAG GCA G UUU UUU
DHOV Segment		
1 (PB2)	3'- UCG UUU UUG UUC GUC AA**A**U **C**UG	5'- AGA GAA AUC AAA GCA G UUU UU**C**
2 (PB1)	UCG UUU UUG UUC GUC AACU GU**C**	AGA GAU AUC AAA GCA G UUU UUU
3 (PA)	UCG UUU UUG UUC GUC AA**UG** GUG	AGA GAA AUC AAA GCA G UUU UUU
4 (HP)	UCG UUU UUG UUC GUC AA**UG** **C**UA	AGA GAA AUC AAA GCA G UUU UU**C**
5 (NP)	UCG UU**A** UUG UUC GUC AA**AG** **C**U**U**	AGA GAU AUC AAA GCA G UUU UUU
6 (M)	UCG UU**A** UUG UUC GUC A**UG**A U**CU**	AGA GAA AUC AAA GCA G UUU UUU
JOSV Segment		
1 (PB2)	3'- UCG UUU UUG UUC **N**UC AA**A**A GU**U**	5'- AGA GAA AUC AAA GCA G UUU UUU
2 (PB1)	UCG UUU UUG UCC GUC AA**AG** GG**U**	n.a.
3 (PA)	n.a.	guuucccaguaggucuc AGA GAU AUC AAG GCA G UUU UUU
4 (HP)	UCG UUU UU**C** UCC **U**C**A** AA**A**A **CCU**	AGA GAA AUC AAG GCA G UUU UUU
5 (NP)	UCG UUU UU**C** UCC **UG**U ACCU **C**GA	n.a.
6 (M)	g UC**A** **C**CU UUG UCC GUC AA**A**A G**CU**	AGA GAA AUC AAG GCA G UUU UUU

643 THOV: GenBank Acc. No's. NC_006504, 06-08, 006495-96; DHOV: GU969308-13; JOSV: HM627170-75; n. a.: not available.

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648 **Table 3.** Percent sequence identities.

S1 [nt] / PB2 [aa]	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
UPOV		78.3	67.9	61.7	54.2	46.6
ABV	92.1		68.1	62.1	54.4	47.8
JOSV	71.1	71.3		62.4	53.5	49.7
THOV	61.2	60.9	61.0		54.2	48.0
DHOV	36.8	36.6	36.3	36.6		46.5
FLUAV	22.5	21.9	20.7	22.3	22.7	

S2 [nt] / PB1 [aa]	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
UPOV		78.8	67.8	68.1	63.5	51.5
ABV	92.7		68.1	68.2	62.9	51.2
JOSV	75.1	76.1		66.2	61.5	50.1
THOV	73.8	73.6	71.9		63.7	51.6
DHOV	62.3	62.1	60.6	61.9		51.5
FLUAV	28.4	28.8	29.3	30.3	32.1	

S3 [nt] / PA [aa]	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
UPOV		79.0	63.1	59.5	55.4	48.1
ABV	86.0		63.8	60.7	55.5	48.5
JOSV	63.1	64.8		58.7	53.7	43.0
THOV	46.5	46.4	45.5		55.0	47.1
DHOV	39.4	39.1	40.6	35.1		49.5
FLUAV	22.0	23.5	23.2	22.4	24.3	

S4 [nt] / GP [aa]	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
UPOV		69.3	60.5	57.1	53.9	49.8
ABV	67.1		59.5	55.7	53.2	50.0
JOSV	52.9	51.9		55.5	54.6	44.8
THOV	42.4	43.6	42.9		53.6	47.0
DHOV	36.3	32.6	33.3	35.3		46.2
FLUAV	22.0	19.3	21.6	21.1	19.4	

S5 [nt] / NP [aa]	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
UPOV		79.9	57.4	58.8	55.9	39.1
ABV	89.1		59.2	59.6	56.7	48.1
JOSV	64.3	65.2		63.6	52.7	42.1
THOV	59.1	59.9	63.2		55.2	49.0
DHOV	40.7	41.3	43.6	42.1		49.5
FLUAV	22.4	23.0	21.2	21.0	23.0	

S6 [nt] / M [aa]	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
UPOV		81.9	64.4	60.0	50.3	49.7
ABV	95.9		64.7	59.2	50.7	50.2
JOSV	71.2	70.5		61.7	51.1	49.1
THOV	45.4	43.5	48.7		51.3	48.0
DHOV	27.9	28.3	28.5	23.4		47.4
FLUAV	18.1	16.3	19.7	18.3	23.3	

649 Pairwise sequence identities between Upolu virus (UPOV), Aransas Bay virus (ABV), Jos virus (JOSV),
650 Thogoto virus (THOV), Dhori virus (DHOV), and influenza A virus (FLUAV) at nucleotide level (nt) and
651 amino acid level(aa).
652

653

654 **Table 4**. Serologic analyses.

Hemagglutination inhibition	Antigen [4u]				
Antibody	ABV	UPOV	Araguari	DHOV	THOV
ABV	1280 *	40	nd	<10	160
UPOV	160	320	nd	<10	40
Araguari	<10	<10	640	<10	10
DHOV	<10	<10	<10	2560	10
THOV	1280	40	10	80	5120

Complement fixation	Antibody				
Antigen	ABV	UPOV	Araguari	DHOV	THOV
ABV	≥64 / ≥8 #	<8 / <8	<8 / <8	<8 / <8	32 / ≥8
UPOV	≥64 / ≥Φ	≥64 / ≥Φ	<8 / <8	<8 / <8	<8 / <8
Araguari	<8 / <8	<8 / <8	≥64 / ≥8	<8 / <8	<8 / <8
DHOV	<8 / <8	<8 / <8	<8 / <8	≥64 / ≥8	<8 / <8
THOV	32 / ≥8	<8 / <8	<8 / <8	<8 / <8	≥64 / ≥8

655 * Reciprocal of serum dilution giving complete inhibition of agglutination with 4 units of antigen;
656 nd, not done.
657 # Reciprocal of serum dilution/antigen dilution resulting in fixation of complement (2 units guinea
658 pig complement).
659

660

661 **Figure Legends**

662

663 **Figure 1**. Ultrastructure of Upolu virus (**A**) and Aransas Bay virus (**B**) in infected Vero E6 cell
664 cultures. Size bar = 100 nm.

665

666 **Figure 2.** Potential base pairing of UPOV and ABV segments terminal bases.

667

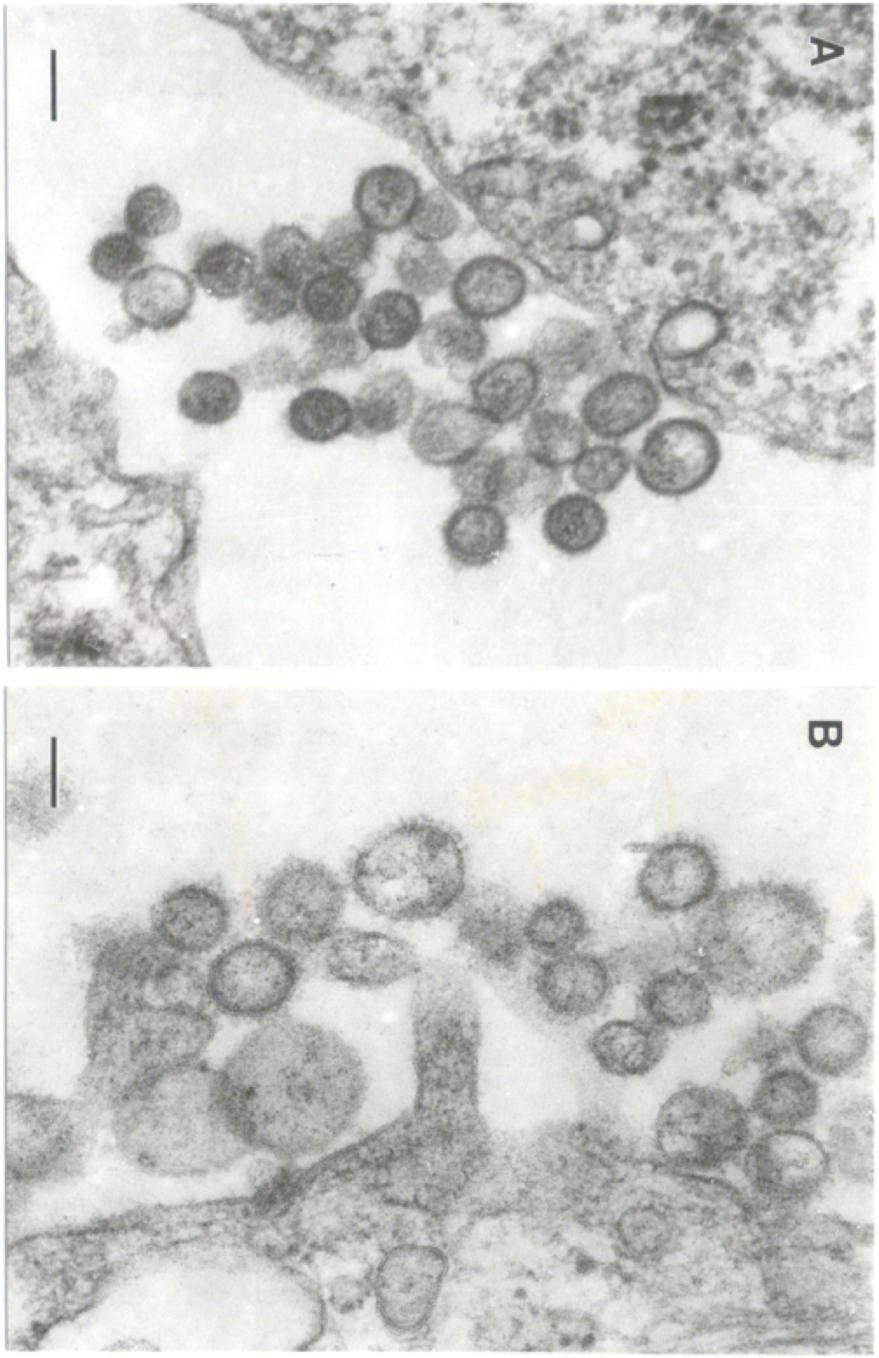
668 **Figure 3.** Phylogenetic analysis of deduced aa sequences of UPOV and ABV in comparison to
669 those of other selected orthomyxoviruses indicated by their GenBank accession no. and
670 abbreviation: UPOV, Upolu virus; ABV, Aransas Bay virus; JOSV, Jos virus; THOV, Thogoto
671 virus; DHOV, Dhori virus; FLUCV, influenza C virus; FLUBV, influenza B virus; FLUAV, influenza
672 A virus; QRFV, Quaranfil virus; JAV, Johnston Atoll virus. Neighbor-joining trees were
673 constructed under a Jukes-Cantor model, running 1000 pseudo replicates; bootstrap values
674 >50% are indicated at the respective nodes; scale bars indicate substitutions per site. **A**: PB2
675 (S1); **B**: PB1 (S2); **C**: PA (S3); **D**: GP (S4); **E**: NP (S5); **F**: M (S6).

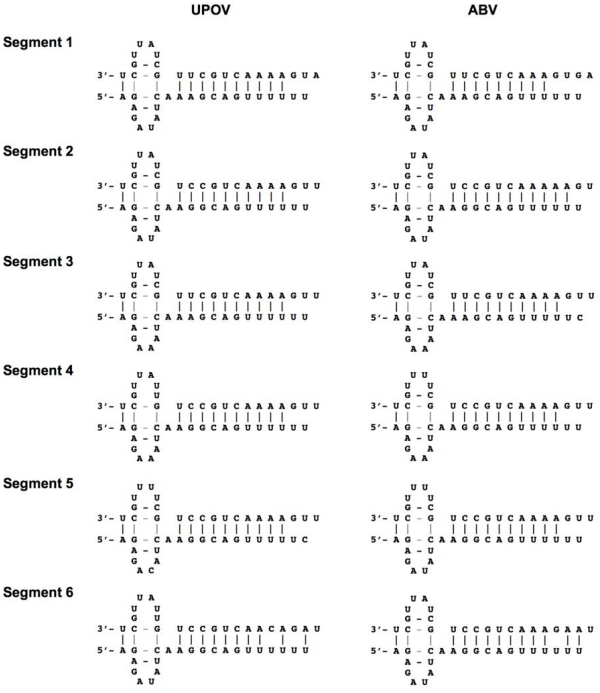
676

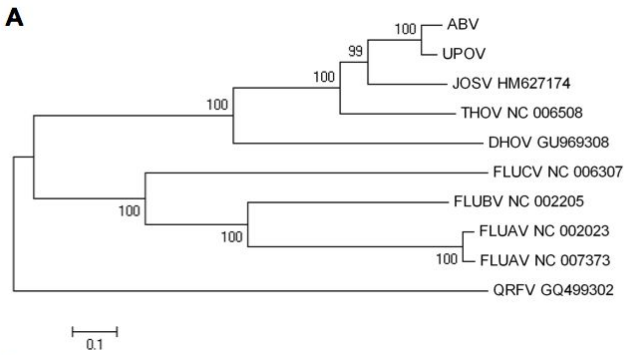
677 **Figure 4.** Schematic of glycoprotein alignment including tick-borne orthomyxoviruses Upolu
678 (UPOV), Aransas Bay (ABV), Jos (JOSV), Thogoto (THOV), Dhori (DHOV), and Quaranfil
679 (QRFV), as well as influenza A virus (FLUAV) and the insect *Autographa californica* multicapsid
680 polyhedrosis virus (AcMNPV), showing signal peptide (Signal); motifs of a potential fusion
681 peptide cleavage site proposed for THOV (Fusion-pep); cysteine (C) residues conserved in all
682 orthomyxoviruses, or in the tick-borne orthomyxoviruses and AcMNPV ◊7, in tick-borne viruses
683 and AcMNPV except DHOV ◊6, in thogoto- and dhoriviruses or in thogotoviruses and AcMNPV ◊5,
684 conserved glycosylation sites surrounding positions 183 (N_{183}GS/N_{183}GT, and N_{197}VT in
685 AcMNPV) and 415/428 (NxT/S, including $N_{415/412/410}$XT/S in UPOV, ABV, JOSV and

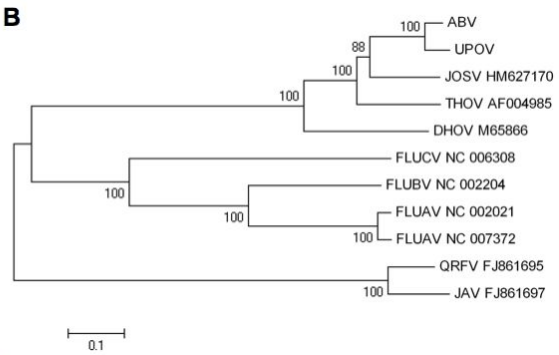
$N_{428/427/423/416}$XT/S in UPOV, ABV, JOSV, THOV as well as N_{378}NT in THOV, N_{396}HS in DHOV, N_{422}VS in QRFV, and N_{384}NS/N_{426}TT in AcMNPV); the trans-membrane anchor (TM); and aa of the cytoplasmic tail region (Cyt-tail).

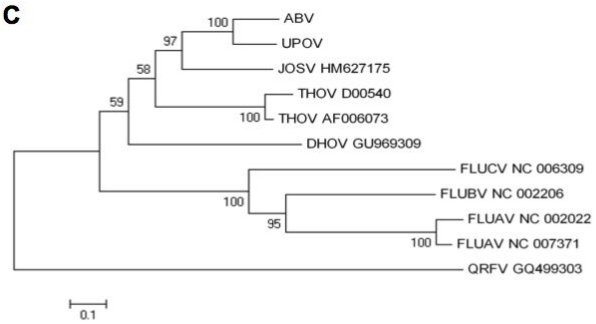
Figure 5. Segment 6 coding strategies. (**A**) RNA extracts obtained from 293 cells infected with Upolu (UPOV) or Jos virus (JOSV)(cellular RNA; cR) or from DNase and RNase treated supernatants (genomic RNA; gR). cDNA was amplified with primers located upstream of a potential splice region (p1) and downstream at a mRNA polyadenylation signal (p2), or at the segment terminal sequence (p3). M indicates molecular size markers. Only a single size amplification product was observed with UPOV template (bands 1, 2), whereas differently sized products were generated with JOSV template (bands 3, 4 and 5). (**B**) Relevant sequences obtained from the respective bands shown in (A). (**C**) Schematic of segment 6 coding strategies of Thogoto virus (THOV) and JOSV, and Dhori virus (DHOV), Aransas Bay virus (ABV) and UPOV, indicating locations of primers p1, p2 and p3, and ML or M ORF termination codons (ochre, opal, amber), and splice sites.

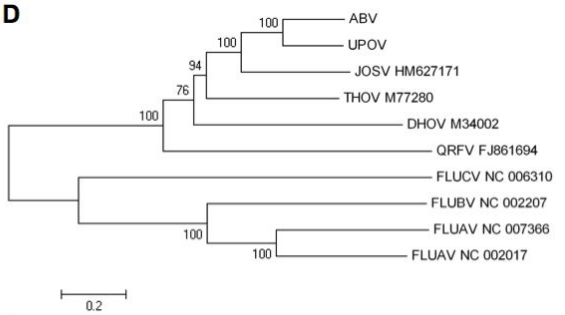


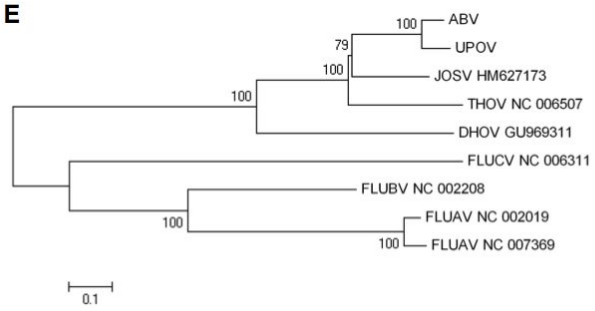


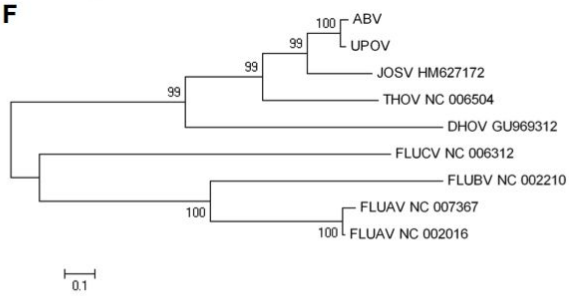


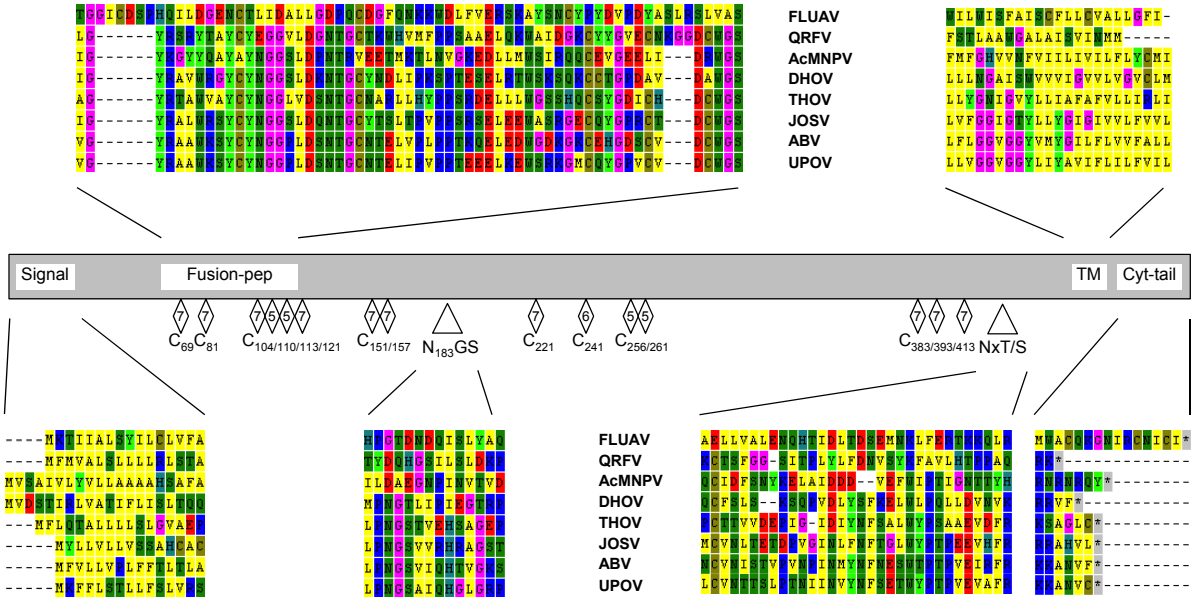


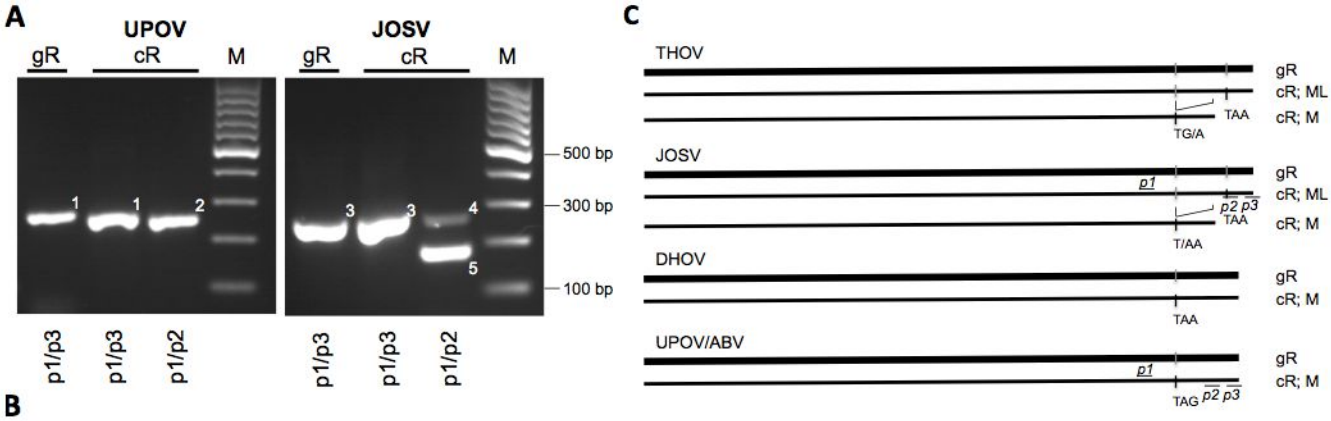












B

1 AGGGATAGTATCTAAAAATGCAGCAGAGACTCTGGAGGCCCCGCTCTTGCTGGCTTAAAGAAGGTCATACTCATGCTTCTGTGCTTTTTCTGCTGTTGAAATATATTGTTATTTT.....AATAAAAAACTGCCTTGATATCTCTggcggc

2 AGGGATAGTATCTAAAAATGCAGCAGAGACTCTGGAGGCCCCGCTCTTGCTGGCTTAAAGAAGGTCATACTCATGCTTCTGTGCTTTTTCTGCTGTTGAAATATATTGTTATTTT.....AATAAAAAAAAAAAAAAAAAAgcggccgcat
R D S I *

3 CGAGAAAGCATATGTAAGTGTATTTTCTTAAGAGTCCTATTGAGACGTTTTTTTACTACAATAACTTTGCTGATGGACATTTTATTTGTTCATTTTCAGAATCTGACCCGGAGGTTCGTCTGGTGTTAAAAAACTGCCTTGATTTCTCTggcggc

4 CGAGAAAGCATATGTAAGTGTATTTTCTTAAGAGTCCTATTGAGACGTTTTTTTACTACAATAACTTTGCTGATGGACATTTTATTTGTTCATTTTCAGAATCTGACCCGGAGGTTCGTCTGGTGTTAAAAAAAAAAAAAAAAAAgcggccgcat
... F V W C *

5 CGAGAAAGCATAT.....AATCTGACCCGGAGGTTCGTCTGGTGTTAAAAAAAAAAAAAAAAAgcggccgcat
R E S I *