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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 morphological, 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 identity to Thogoto virus (segment 2; ~74% at the amino acid level) and a more distant relationship to Dhori virus, the two prototype viruses of the recognized species of the genus *Thogotovirus*. Despite sequence similarity, the coding potentials of UPOV and ABV differed from that of Thogoto virus, instead being like that of Dhori virus. Our findings suggest that the tick-transmitted viruses 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 this 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 are paramount in this molecular age to devise appropriate surveillance and diagnostics. Although more closely related to Thogoto virus by sequence, UPOV and ABV differ in their coding potentials by lacking a proposed pathogenicity factor. In this respect, they are similar to Dhori virus, which, despite the lack of a pathogenicity factor, can cause disease. These findings enable further studies into the evolution and pathogenicity of orthomyxoviruses.**

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

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

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

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TABLE 1 Properties of UPOV and ABV genome segments[a]

Virus	Segment	Segment length (nt)	5′ UTR length (nt)	ORF length (aa)	3′ UTR length (nt)	Predicted molecular mass (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	

[a] nt, nucleotides; aa, amino acids.

acidic subunit (PA), synthesizes negative-strand, viral genomic RNA (vRNA), positive-strand RNA (cRNA) complementary to vRNA, and capped polyadenylated mRNAs 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 has been assigned to PB1 (19–21), and cRNA and vRNA synthesis as well as cap cleavage have been assigned 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 a distant relationship to that of baculoviruses (27, 28), segment 5 encodes the nucleoprotein (NP), and segment 6 encodes the matrix protein (M) and in some species also encodes 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 the sequence of THOV than to that of DHOV, but both viruses have coding repertoires similar to that of DHOV and not that of THOV. Analyses of the increasing sequence diversity of thogotovirus genomes have begun 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, Galveston, TX. 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°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 were scraped, and pelleted cells were postfixed 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°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 with a Philips 201 transmission electron microscope at 60 kV.

Serologic tests. Viral antigens used in serologic tests were not inactivated and were 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°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 titer that gave a 3+/4+ fixation of complement; antibody titers of ≥1:8 were rated positive. Hemagglutination inhibition (HI) tests were also done in microtiter plates (31, 32). Nonspecific hemagglutinin inhibitors were removed by acetone extraction, sera were rehydrated in 0.05 M borate–0.12 M NaCl (pH 9), and naturally occurring agglutinins were 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 2-fold 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, reverse transcription-PCR, and rapid amplification of cDNA ends. Genomic sequences were generated by applying a combination of unbiased high-throughput sequencing (UHTS), subsequent consensus reverse transcription-PCR (RT-PCR), and rapid amplification of cDNA ends (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 with Superscript III (Invitrogen, Carlsbad, CA, USA) with random octamer primers linked to an

TABLE 2 Sequence conservation at the termini of genome segments[a]

Segment	3′ terminus (genomic orientation)	5′ terminus (genomic orientation)
UPOV		
1 (PB2)	3′-UCG UUA UCG UUC GUC AAAA GUA	5′-AGA GAU AUC AAA GCA G UUU UUU
2 (PB1)	UCG UUA UCG UCC GUC AAAA GUU	AGA GAU AUC AAG GCA G UUU UUU
3 (PA)	UCG UUA UCG UUC GUC AAAA GUU	AGA GAA AUC AAA GCA G UUU UUU
4 (HP)	UCG UUA UUG UCC GUC AAAA GUU	AGA GAA AUC AAG GCA G UUU UUU
5 (NP)	UCG UUU UCG UCC GUC AAAA GUU	AGA GAC AUC AAG GCA G UUU UUC
6 (M)	UCG UUA UUG UCC GUC AACA GAU	AGA GAU AUC AAG GCA G UUU UUU
ABV		
1 (PB2)	3′-UCG UUA UCG UUC GUC AAAG UGA	5′-AGA GAU AUC AAA GCA G UUU UUU
2 (PB1)	UCG UUA UCG UCC GUC AAAA AGU	AGA GAU AUC AAG GCA G UUU UUU
3 (PA)	UCG UUA UCG UUC GUC AAAA GUU	AGA GAA AUC AAA GCA G UUU UUC
4 (HP)	UCG UUU UCG UCC GUC AAAA GUU	AGA GAA AUC AAG GCA G UUU UUU
5 (NP)	UCG UUU UCG UCC GUC AAAA GUU	AGA GAU AUC AAG GCA G UUU UUU
6 (M)	UCG UUA UCG UCC GUC AAAG AAU	AGA GAU AUC AAG GCA G UUU UUU
THOV	consensus, **UCG UUU UUG UYC GYB WVCW KKK**	consensus, **AGA GAW AUC AAR GCR S UUU UUU**
1 (PB2)	3′-UCG UUU UUG UUC GCU ACCU GUC	5′-AGA GAA AUC AAG GCG A UUU UUC
2 (PB1)	UCG UUU UUG UCC GCG AGGU 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 AGG	NGA GAU AUC AAA GCA G UUU UUU
5 (NP)	UCG UUU UUG UCC GUC AGUU UUA	AGA GAA AUC AAG GCA G UUU UUU
6 (M)	UCA CCU UUG UCC GUC ACCU CUA	AGA GAA AUC AAG GCA G UUU UUU
DHOV		
1 (PB2)	3′-UCG UUU UUG UUC GUC AAAU CUG	5′-AGA GAA AUC AAA GCA G UUU UUC
2 (PB1)	UCG UUU UUG UUC GUC AACU GUC	AGA GAU AUC AAA GCA G UUU UUU
3 (PA)	UCG UUU UUG UUC GUC AAUG GUG	AGA GAA AUC AAA GCA G UUU UUU
4 (HP)	UCG UUU UUG UUC GUC AAUG CUA	AGA GAA AUC AAA GCA G UUU UUC
5 (NP)	UCG UUA UUG UUC GUC AAAG CUU	AGA GAU AUC AAA GCA G UUU UUU
6 (M)	UCG UUA UUG UUC GUC AUGA UCU	AGA GAA AUC AAA GCA G UUU UUU
JOSV		
1 (PB2)	3′-UCG UUU UUG UUC NUC AAAA GUU	5′-AGA GAA AUC AAA GCA G UUU UUU
2 (PB1)	UCG UUU UUG UCC GUC AAAG GGU	NA
3 (PA)	NA	guuucccaguaggucuc AGA GAU AUC AAG GCA G UUU UUU
4 (HP)	UCG UUU UUC UCC UCA AAAA CCU	AGA GAA AUC AAG GCA G UUU UUU
5 (NP)	UCG UUU UUC UCC UGU ACCU CGA	NA
6 (M)	g UCA CCU UUG UCC GUC AAAA GCU	AGA GAA AUC AAG GCA G UUU UUU

[a] GenBank accession numbers are NC_006504, NC_006506 to NC_006508, NC_006495, and NC_006496 for THOV; GU969308 to GU969313 for DHOV; and HM627170 to HM627175 for JOSV. Consensus sequences are in bold. Underlining indicates bases deviating from the consensus. NA, not available.

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 of >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 and then clustered and assembled into contiguous fragments (contigs) for comparison to data in the GenBank database by using the Basic Local Alignment Search Tool (blast) (35) at the nucleotide (blastn) and deduced amino acid (blastx) levels.

Various specific primer sets for the 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 are available upon request). Gaps between contigs were filled, and the completed draft genomes were resequenced 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 using RACE kits (Invitrogen). For 5′-RACE, first-strand cDNA was synthesized from total RNA by using custom gene-specific primer 1 (GSP1) and Superscript III. After purification using SNAP 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 RNA (corresponding to the 3′ end of antigenomic RNA) or the antigenomic RNA were determined. Products were cloned into the 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 an 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 and 5′-GCT GAT

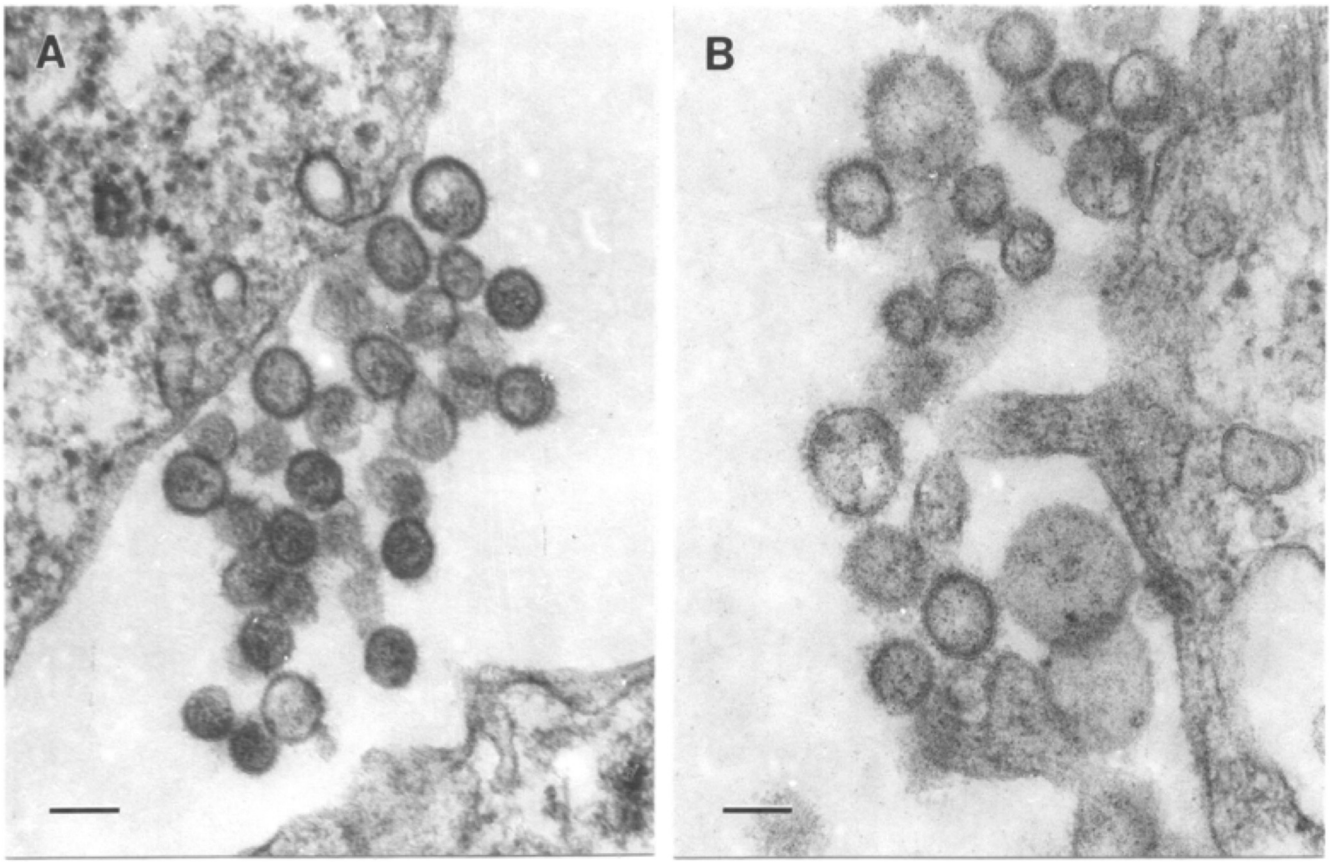

FIG 1 Ultrastructure of Upolu virus (A) and Aransas Bay virus (B) in infected Vero E6 cell cultures. Bar = 100 nm.

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 ATT AAA AT for UPOV and 5′-ATG CGG CCG CTT TTT TTT TTT TTT TTT TAA CAC C for JOSV) and p3 (5′-ccg ccA GAG ATA TCA AGG CA for UPOV and 5′-gcc gcc AGA GAA ATC AAG GCA for JOSV; lowercase indicates nonviral bases added to raise primer binding temperature). Nucleic acid extracts for amplification were generated from crude cell homogenates (cellular RNA) or nuclease-treated (8 ng/μl RNase A [Ambion] for 15 min at room temperature and 0.3 U/μl Benzonase nuclease [Qiagen] and 0.06 U/μl Turbo DNase [Ambion] for 45 min at room temperature, followed by 8 ng/μl RNase A and 0.08 U/μl RNase H [Invitrogen] for 2 h at 37°C) cell culture supernatants (genomic RNA) obtained from virus-infected HEK 293 cells harvested at 72 h postinfection. PCR products were analyzed by agarose gel electrophoresis and visualization by GelGreen staining (Biotium, Hayward, CA, USA).

Sequence analyses. Sequence assembly and analysis were done by employing programs of the Wisconsin GCG Package (version 10.3; Accelrys Inc., San Diego, CA), MEGA 5 (36), Geneious 5.5 (37), and Newbler-Assembler 2.4. Identities of nucleotide and amino acid sequences were calculated with the Needleman-Wunsch algorithm, applying an EBLOSUM62 substitution matrix (gap open/extension penalties of 12/2 for nucleotide and 6/1 for amino acid 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 (http://www.cbs.dtu.dk/services); Phobius (http://phobius.sbc.su.se); and Phyre2 (http://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.

Nucleotide sequence accession numbers. The GenBank accession numbers for the segments of Upolu virus and of Aransas Bay virus are KC506156 to KC506161 and KC506162 to KC506167, respectively.

RESULTS

Recognition of UPOV and ABV as orthomyxoviruses. The failure to obtain amplification products from nucleic acids of UPOV or ABV by reverse transcription-PCR (RT-PCR) using a panel of degenerate bunyaviral consensus primers led us to pursue UHTS. Sequence libraries were prepared from total RNA extracted from an ABV stock. Sequencing on the Roche GS-FLX platform yielded 94,835 reads with a mean length of 222 bases (range, 29 to 382 bases) 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%; 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 (Tables 1 and 2).

Morphology of UPOV and ABV virions. 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

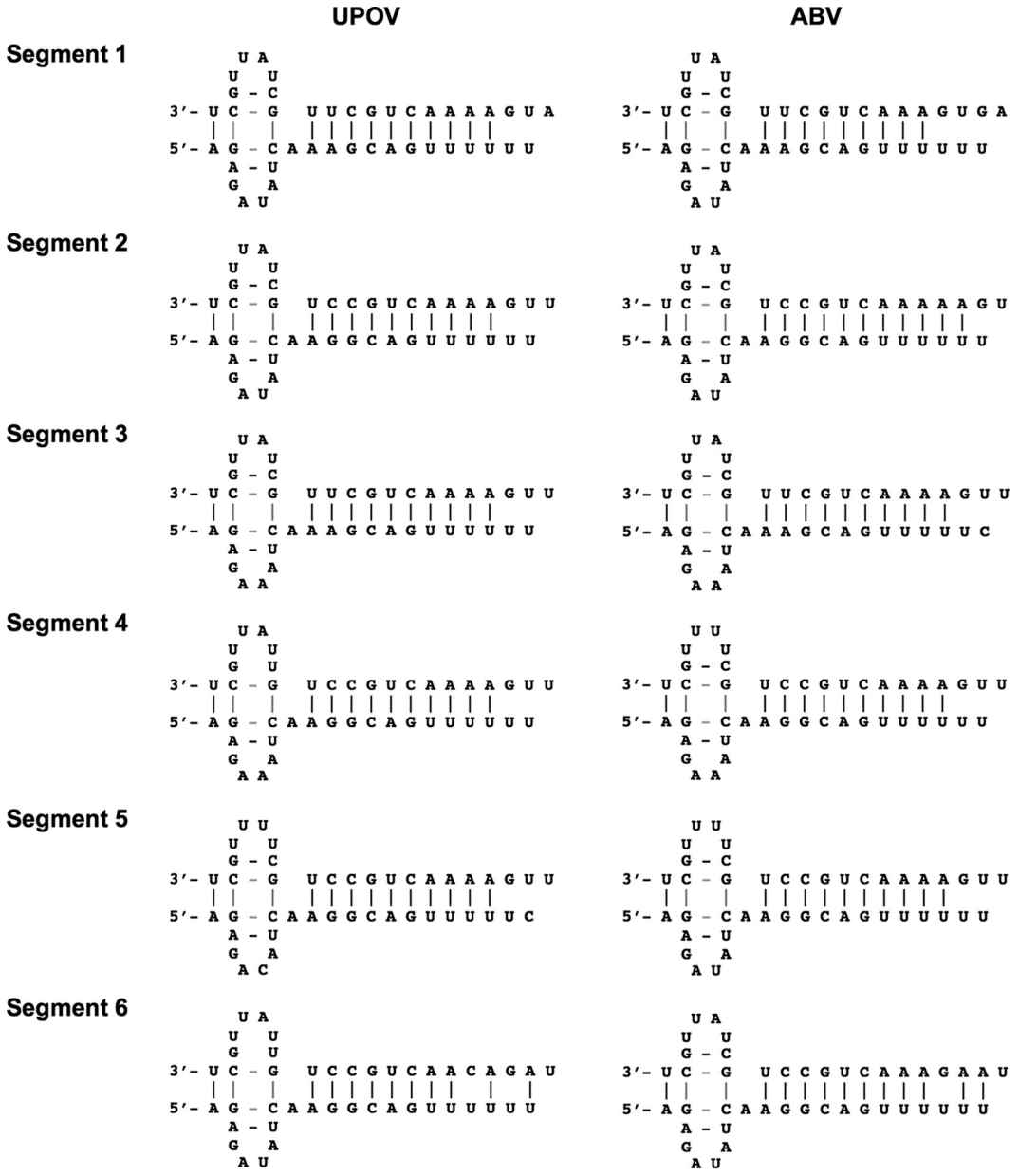

FIG 2 Potential base pairing of UPOV and ABV segment terminal bases.

to 95 nm, or slightly oval, with sizes ranging from 75 by 85 nm to 105 by 120 nm (Fig. 1A). Virions of ABV were more polymorphic and partly larger, ranging from 75 by 85 nm up to 105 by 130 and 120 by 140 nm (Fig. 1B).

Genetic and serologic characterization of UPOV and ABV. UPOV and ABV display terminal sequences that are semicomplementary 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 position 6 ("A," similar to DHOV segments 5 and 6), position 8 ("C," similar to influenza viruses), and positions 16 to 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 that of all other segments. No specific difference in the 3′-terminal sequence of segment 6 was found for UPOV and ABV, similar to DHOV. Analogous to influenza virus, the formation of a forked terminal panhandle has been shown to be essential for promoter function in THOV although with potential differences in the intrastrand base pairing of vRNA and cRNA "hook" structures (44–47). Compared to this, the changes in the terminal sequences of UPOV and ABV are located either at the unpaired fork region (3′/5′ position 6) (Fig. 2), with no compensating base change at the opposite terminus, or in the paired panhandle region, with compensating mutations at the opposite terminus (3′ position 11/5′ position 12; genomic orienta-

tion). 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 the intrastrand pairing of 3′ C2/G9-5′ G2/C9 and the interstrand pairing of 3′ C2/5′ G2-3′G9/5′ C9 may provide options for "breathing" of the structure (Fig. 2). Termination of mRNA transcripts occurred at a conserved oligo(U)$_{5-6}$ signal located 17 nucleotides (nt) from the 5′ end of vRNA templates, as indicated by RACE with oligo(dT) priming. The level of coding sequence similarity between individual segments of UPOV and ABV, and to corresponding segments of other orthomyxoviruses, was variable (Table 3). Phylogenetic analysis indicated that the evolutionary relationship for all segments is consistently closest between UPOV and ABV and that both viruses are more closely related 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 (Pfam accession number PF00604; "Flu_PB2" [http://pfam.sanger.ac.uk/]) (Tables 1 and 3). Although the PB2 sequence is the least conserved among orthomyxoviral polymerase subunits, UPOV and ABV sequences closely match those of JOSV and THOV, with DHOV being more distantly related, particularly in the C-terminal portion. Only a few amino acid motifs (D_{89}LG, R_{149}KPV, W_{225}LP, and I_{314}CRVALG in UPOV) are conserved with respect to influenza viruses outside an N-terminal motif (F_{40} to 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 was 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 also previously reported 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 of 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 (Pfam accession number PF00602; "Flu_PB1" [http://pfam.sanger.ac.uk/]) (Tables 1 and 3) and show conservation of the polymerase motifs pre-A, A, B, C, D, and E (20, 54–56). Conservation was also noted for amino acids maintained between THOV, DHOV, and influenza viruses 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 to that of influenza viruses. No conservation was obvious in the region of the FLUAV bipartite NLS (60), as is also the case for THOV and DHOV.

Segments 3 of UPOV and ABV encode a PA-like protein (Pfam accession number PF00603; "Flu_PA" [http://pfam.sanger.ac.uk/]) (Tables 1 and 3). The endonuclease motif PDX$_n$(D/E) described previously for FLUAV (24, 25) corresponds to a P_{96}HX$_{16}$D motif in UPOV and ABV that is not surrounded by additional characteristic primary or secondary sequence conservation as reported for FLUAV. An elevated level of conservation was noted for the C-terminal part of the sequence (around Q_{426}-F_{452} in UPOV), which has been implicated in interactions with PB1 in FLUAV (61).

The putative glycoproteins (GPs) of UPOV and ABV are encoded by segment 4 (Tables 1 and 3). Instead of showing conservation with respect to influenza virus-like orthomyxoviral GPs, the overall structures of UPOV and ABV GPs are similar to those of the corresponding proteins of THOV and the "baculovirus gp64 envelope glycoprotein family" (Pfam accession number

TABLE 3 Percent sequence identities[a]

Sequence and virus	% sequence identity					
	UPOV	ABV	JOSV	THOV	DHOV	FLUAV
PB2 (aa)/S1 (nt)						
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	
PB1 (aa)/S2 (nt)						
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	
PA (aa)/S3 (nt)						
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	
GP (aa)/S4 (nt)						
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	
NP (aa)/S5 (nt)						
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	
M (aa)/S6 (nt)						
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	

[a] Pairwise sequence identities between Upolu virus (UPOV), Aransas Bay virus (ABV), Jos virus (JOSV), Thogoto virus (THOV), Dhori virus (DHOV), and influenza A virus (FLUAV) at the nucleotide (nt) level (values above the diagonal) and at the amino acid (aa) level (values below the diagonal). S1, segment 1.

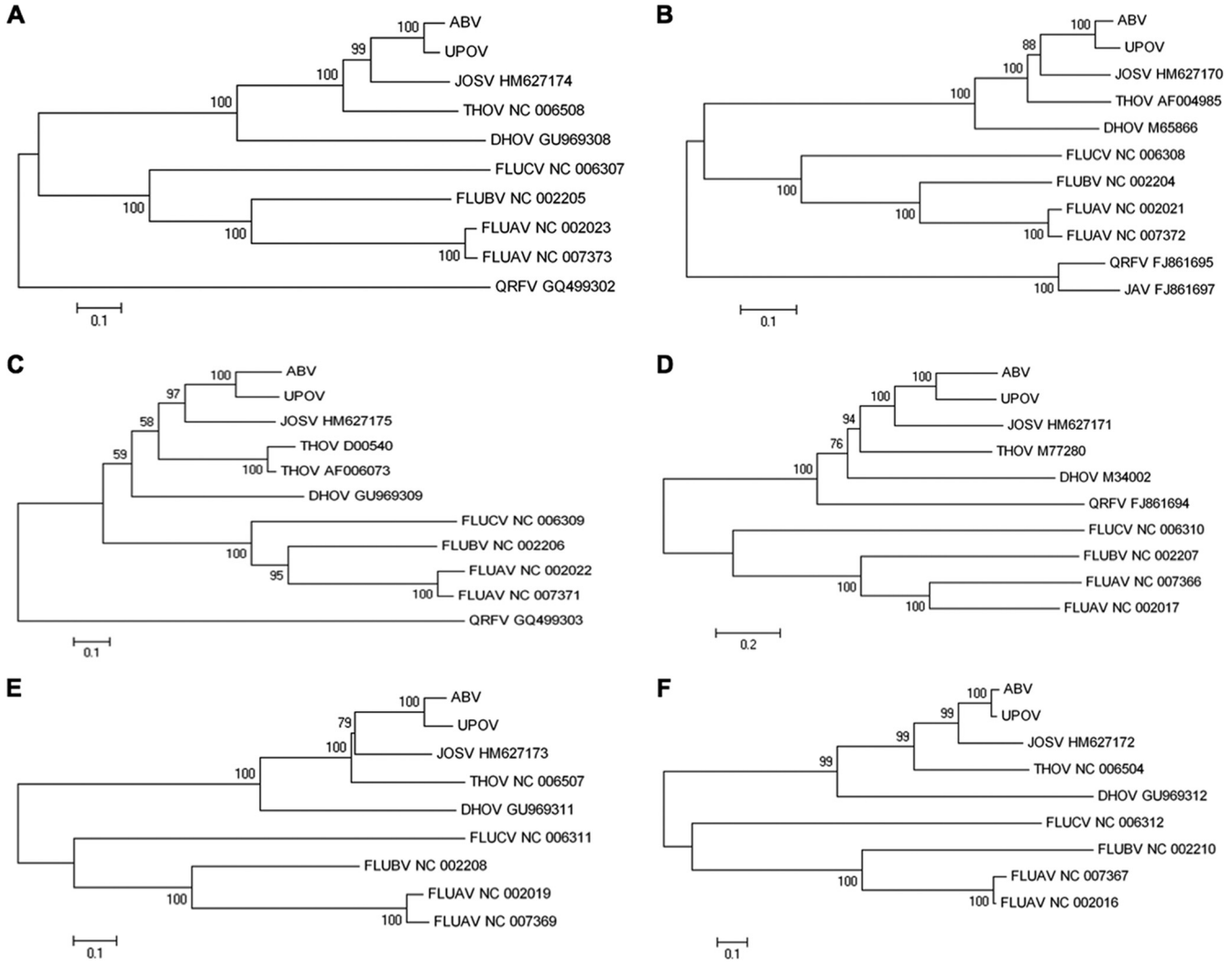

FIG 3 Phylogenetic analysis of deduced amino acid sequences of UPOV and ABV in comparison to those of other selected orthomyxoviruses, indicated by their GenBank accession number and abbreviation (UPOV, Upolu virus; ABV, Aransas Bay virus; JOSV, Jos virus; THOV, Thogoto virus; DHOV, Dhori virus; FLUCV, influenza C virus; FLUBV, influenza B virus; FLUAV, influenza A virus; QRFV, Quaranfil virus; JAV, Johnston Atoll virus). Neighbor-joining trees were constructed under a Jukes-Cantor model, running 1,000 pseudoreplicates; bootstrap values of >50% are indicated at the respective nodes; and scale bars indicate substitutions per site. (A) PB2 (segment 1); (B) PB1 (segment 2); (C) PA (segment 3); (D) GP (segment 4); (E) NP (segment 5); (F) M (segment 6).

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 was observed for the N-terminal region containing a potential fusion peptide cleavage site (V_{59}GY-WGS$_{116}$ in UPOV, homologous to the A_{61}GY-WGS$_{118}$ sequence proposed for THOV [28]) and for the motifs W_{155}RCGV, upstream of the only strictly conserved glycosylation site, N_{183}GS; S_{351}LSKIDERLIG, S_{391}NC, D_{401}GRW, and G_{444}VIE DEEGWNF. There are significant differences in the cytoplasmic tail regions of GPs of the various orthomyxovirus species (Fig. 4). Serologic analyses by hemagglutination inhibition (HI) tests 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 being involved in HI epitopes (possibly including 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}, which includes indel regions, in UPOV).

The nucleoprotein (NP) of orthomyxoviruses represents the main type-specific antigen recognized in complement fixation (CF) tests (Table 4) and has been widely used to assess phylogenetic relationships. The open reading frame (ORF) encoded by segments 5 of UPOV and ABV is conserved with respect to "influenza virus nucleoprotein" (Pfam accession number PF00506 [http://pfam.sanger.ac.uk/]) (Tables 1 and 3). Although only a low level of conservation was observed for the characterized N-terminal NLS in the NP of FLUAV (62, 63), which is also the case for THOV and DHOV, greater conservation was noted for the second half of a region that has been proposed for RNA interactions 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 was also evident in the previously characterized internal NP regions 2 to 5 (66). This includes a sequence in region 4 that corresponds to

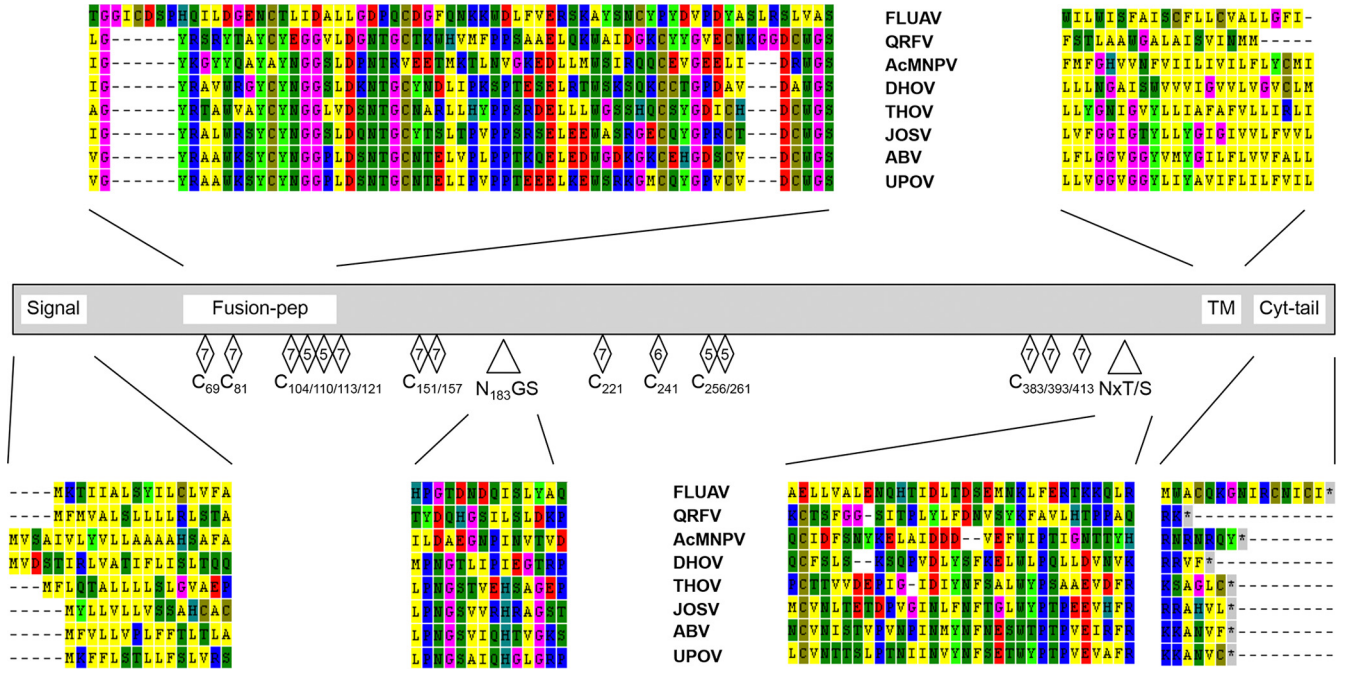

FIG 4 Schematic of glycoprotein alignment including the tick-borne orthomyxoviruses Upolu virus (UPOV), Aransas Bay virus (ABV), Jos virus (JOSV), Thogoto virus (THOV), Dhori virus (DHOV), and Quaranfil virus (QRFV) as well as influenza A virus (FLUAV) and the insect *Autographa californica* multicapsid polyhedrosis virus (AcMNPV), showing the signal peptide (Signal); motifs of a potential fusion peptide cleavage site proposed for THOV (Fusion-pep); cysteine (C) residues conserved in all orthomyxoviruses or in the tick-borne orthomyxoviruses and AcMNPV ◇(7), in tick-borne viruses and AcMNPV except DHOV ◇(6), or in thogoto- and dhoriviruses or in thogotoviruses and AcMNPV ◇(5); conserved glycosylation sites surrounding position 183 (N_{183}GS/N_{183}GT; N_{197}VT in AcMNPV) and position 415/428 (NxT/S, including $N_{415/412/410}$XT/S in UPOV, ABV, and JOSV; $N_{428/427/423/416}$XT/S in UPOV, ABV, JOSV, and THOV; 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 amino acids of the cytoplasmic tail region (Cyt-tail).

a proposed nuclear accumulation motif of FLUAV (S_{329}AGEDL GLLS in UPOV) (67, 68) and a motif in region 5 that is 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 for THOV and FLUAV is conserved ($K_{195}RX_9$ KTKR in UPOV) (69).

Segments 6 of UPOV and ABV show no homology to entries in the protein family database. The nucleotide sequences align only with the segment 6 sequence of JOSV and the C-terminal quarter of that of THOV but not with that of DHOV or the influenza viruses (Tables 1 and 3). Limited conservation with respect to DHOV was discernible at the deduced amino acid level for a short motif (A_{249}KGVSYQVL in UPOV) and strictly conserved amino acids E_{175}, N_{181}T, E_{212}, Y_{224}D, G_{232}, E_{236}, and I_{240} located in the

TABLE 4 Serologic analyses

Serologic analysis	Titer				
	ABV	UPOV	Araguari virus	DHOV	THOV
Hemagglutination inhibition					
Antibody	Antigen (4 U)[a]				
ABV	1,280	40	ND	<10	160
UPOV	160	320	ND	<10	40
Araguari virus	<10	<10	640	<10	10
DHOV	<10	<10	<10	2,560	10
THOV	1,280	40	10	80	5,120
Complement fixation					
Antigen	Antibody[b]				
ABV	≥64/≥8	<8/<8	<8/<8	<8/<8	32/≥8
UPOV	≥64/≥1	≥64/≥1	<8/<8	<8/<8	<8/<8
Araguari virus	<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

[a] Reciprocal of the serum dilution giving complete inhibition of agglutination with 4 units of antigen. ND, not done.
[b] Reciprocal of the serum dilution/optimal antigen dilution resulting in fixation of complement (2 units of guinea pig complement).

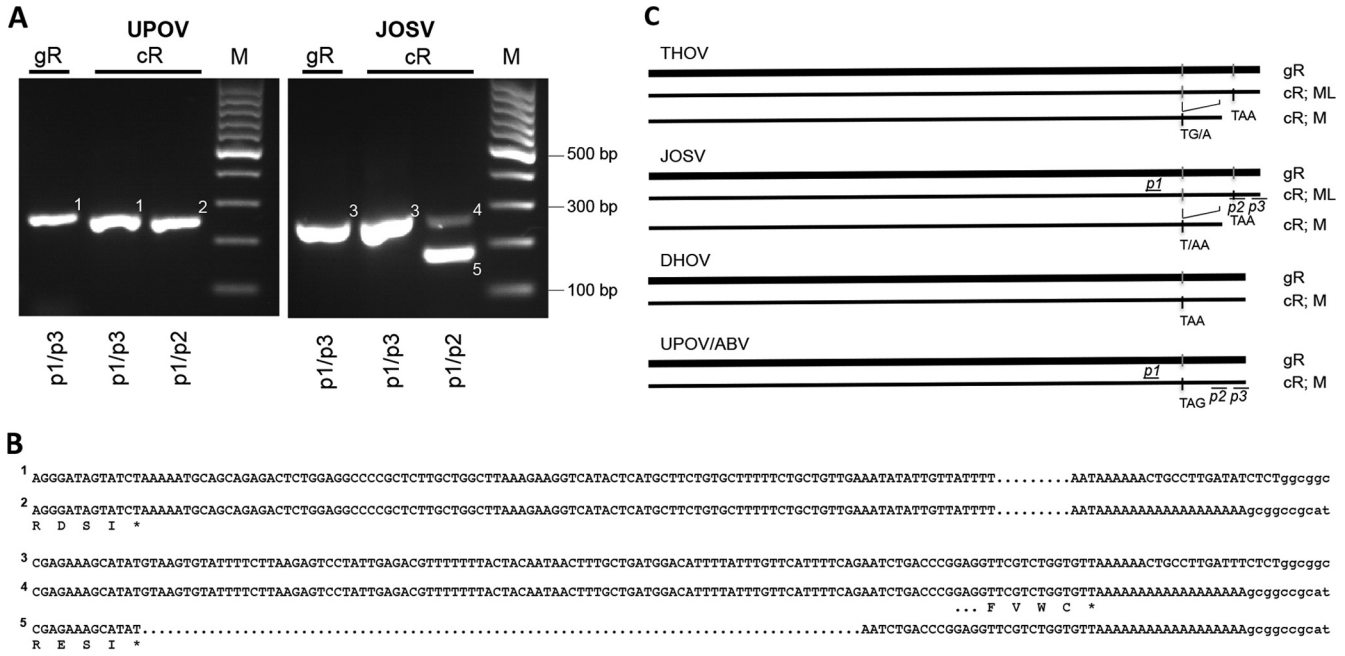

FIG 5 Segment 6 coding strategies. (A) RNA extracts obtained from HEK 293 cells infected with Upolu virus (UPOV) or Jos virus (JOSV) (cellular RNA [cR]) or from DNase- and RNase-treated supernatants (genomic RNA [gR]). cDNA was amplified with primers located upstream of a potential splice region (p1) and downstream at an 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 the UPOV template (bands 1 and 2), whereas differently sized products were generated with the JOSV template (bands 3 to 5). (B) Relevant sequences obtained from the respective bands shown in panel A. (C) Schematic of segment 6 coding strategies of Thogoto virus (THOV) and JOSV, and of Dhori virus (DHOV), Aransas Bay virus (ABV), and UPOV, indicating locations of primers p1, p2, and p3; ML or M ORF termination codons (ochre, opal, and amber); and splice sites.

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 untranslated regions (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 a position analogous to that of the putative M ORFs of UPOV and ABV, THOV and JOSV are known to generate two products through splicing (72); ML is generated from nonspliced transcripts, resulting in a UTR of 20 nt (73), while M is generated from a spliced transcript by the 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, the level of sequence conservation between UPOV and ABV is highest for segment 6, and this segment's sequence is also one of the closest to those of JOSV and THOV (Table 3), despite the observed differences in cod ing potential.

DISCUSSION

Analyses of the genome sequences of UPOV from Australia and ABV from North America show that they are up to ~75% identical at the amino acid level (~68% identical at the nucleotide level) (Table 3) to viruses in the family *Orthomyxoviridae*. The genetic distances of these viruses are shortest with respect to JOSV and THOV, ranging from approximately 76% amino acid and 68% nucleotide (PB1) to 52% amino acid and 60% nucleotide (GP) identity with JOSV and from approximately 74% amino acid and 68% nucleotide (PB1) to 43% amino acid and 56% nucleotide (GP) identity with THOV. However, the coding strategy of segments 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 approximately 97% and 90% amino acid (87% and 80% nucleotide) identity among their available partial NP and GP sequences, respectively. In comparison, DHOV and THOV share only between 42% and 35% amino acid (55% and 54% nucleotide) 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 for the inclusion of 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 by HI tests, whereas by CF tests there are differences in their cross-reactivities to THOV and to each other when ABV antigen/UPOV antibody is tested. These serologic results, combined with the <60% amino acid (<60% nucleotide) sequence identity of their NP or GP sequences to that of THOV or DHOV and the observed differences in coding capacity, suggest that UPOV and ABV should be considered separate species within the genus *Thogotovirus*, distinct from the species *Dhori virus* and *Thogoto virus*. In

addition, amino acid sequence identities between UPOV and ABV of as little as 86% (78% nucleotide identity), and even less for the immunoreactive GP, combined with the serologic differences observed between them, may justify their classification as two separate species.

Due to their distinct structures, the GPs of the tick-infecting orthomyxoviruses have been classified as class 3 penetrenes, distinct from the class 2 penetrenes in alphaviruses and flaviviruses and the class 1 penetrenes in the influenza viruses (28). Furthermore, it has been hypothesized, based on sequence homologies, that GPs of viruses in the genus *Thogotovirus* may have been derived from a common ancestor with insect baculoviruses (27, 28). Thus, the tick-infecting orthomyxoviruses represent an evolutionary lineage distinct from that of the influenza viruses, and an ancestral relationship of either orthomyxoviral line to the other is not apparent from available data (Fig. 3). Nonetheless, the tick-adapted orthomyxoviral GPs are compatible with mammalian receptors, as exemplified by previous reports of human THOV and DHOV infections. In central Africa and regions of southern Europe, THOV has also been isolated from various ruminant species (61, 74). The geographic distribution of DHOV includes primarily India and eastern Russia but also East Africa, Egypt, and other Mediterranean countries, where serologic data indicate circulation in ruminants as well as waterfowl (61, 75–78). Migratory waterfowl are also reservoirs of influenza A viruses (78, 79). Cases of natural human infection have been reported for THOV from Africa (77), and accidental laboratory infections with DHOV indicate that this virus can also act as a human pathogen (76), despite the lack of an ML protein (71, 73). UPOV and ABV productively infect BHK, Vero, or HEK 293 cells and are lethal to newborn mice after intracerebral inoculation (1, 2, 61), suggesting that mammalian pathogenicity is also conceivable for UPOV and ABV.

In FLUAV, reassortment of genome segments is a well-known phenomenon that leads to sudden genetic shifts that can result in dramatic changes in pathogenicity. Reassortment in arthropod and vertebrate hosts has also been demonstrated for THOV in experimental settings (80, 81). The dissemination of genetically related tick-transmitted orthomyxoviruses over large distances by migratory birds (74) may support genome segment reassortment culminating in the emergence of novel genotypes with altered pathogenicity and host range. Indeed, the recent implication of other tick-borne orthomyxoviruses of the proposed genus *Quaranjavirus* in human febrile illness (82) and the discovery of variants with high bird pathogenicity (83, 84) reinforce the need for comprehensive surveillance and characterization of this growing group of viruses to closely monitor their potential as emerging pathogens.

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