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Short Communication

Genomic and antigenic characterization of Jos virus

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Summary

Jos virus (JOSV), originally isolated in Jos, Nigeria in 1967, has remained unclassified despite cultivation in tissue culture, development of animal models of infection and implementation of seroprevalence surveys for infection. Here we report genetic, ultrastructural and serologic evidence that JOSV is an orthomyxovirus distinct from but phylogenetically related to viruses of the genus *Thogotovirus*.

The family *Orthomyxoviridae* comprises viruses with 6 to 8 segments of linear, negative sense, single strand RNA genomes. These viruses are currently assigned to six genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus* ,*Thogotovirus* (Kawaoka *et al.*, 2005) and the recently proposed *Quarjavirus*, (Presti *et al.*, 2009). The influenzaviruses and isavirus (infectious salmon anemia virus) are transmitted to their vertebrate hosts by aerosol or through water (Kawaoka *et al.*, 2005) and the thogotoviruses and quarjaviruses are transmitted by ticks (Da Silva *et al.*, 2005; Kawaoka *et al.*, 2005; Presti *et al.*, 2009).

The genus *Thogotovirus* currently consists of three named viruses; Thogoto (THOV), Dhori (DHOV), and Araguari (ARAV) (Da Silva *et al.*, 2005; Kawaoka *et al.*, 2005). THOV and DHOV are widely distributed in Africa, southern Europe and Central Asia and are associated with ticks and mammals (Hubalek & Halouzka, 1996; Institut Pasteur da Dakar, 2001; Karabatsos, 1985). Two natural human infections have been reported for THOV with one fatality (Moore *et al.*, 1975), and five accidental infections with DHOV have been reported (Butenko *et al.*, 1987). All seven cases presented fever and encephalitis or meningoencephalitis. ARAV was isolated from a marsupial (opossum) collected in northern Brazil (Da Silva *et al.*, 2005); its pathogenic potential is unknown.

Jos virus (JOSV) was originally isolated from cow serum (*Bos indicus*) in Jos, Nigeria in 1967 (Lee *et al.*, 1974). Thereafter, the virus was repeatedly isolated from *Amblyomma* and *Rhipicephalus (Boophilus*) ticks collected in Ethiopia, Guinea, Central African Republic, Nigeria, Ivory Coast, and Senegal (Institut Pasteur da Dakar, 2001; Wood *et al.*, 1978). Studies of the field infection rate in ticks in the Central African

Republic and Ethiopia found the prevalence to be 3% and 1%, respectively (Sureau *et al.*, 1976; Wood *et al.*, 1978). Initial efforts to characterize the pathology of JOSV in infected suckling mice showed acute cell necrosis in the liver, lymph nodes, bone marrow and spleen (Fagbami & Ikede, 1978; Lee *et al.*, 1974). Given the lack of genetic information or a serologic relationship with other arboviruses, JOSV has remained unclassified.

Here we report genetic, ultrastructural and serologic evidence that JOSV is an orthomyxovirus distinct from but phylogenetically related to viruses of the genus *Thogotovirus*.

The virus strains used were THOV strain ITAL Ar 126; DHOV strain IAr-611313 and ARAV strain BeAn-174214. The JOSV prototype strain (IBAn-17854) was isolated in newborn mice inoculated with the original infected bovine serum (Lee *et al.*, 1974). The virus killed newborn and 10-day old mice within 4-5 days when inoculated intracerebrally or intraperitoneally (Fagbami & Ikede, 1978). All virus stocks were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch. Methods used to prepare antigens for the complement-fixation (CF) tests and for making immune ascitic fluids have been described (Beaty *et al.*, 1989; Travassos da Rosa *et al.*, 1983; Xu *et al.*, 2007). Antigens and antibodies were both prepared in mice. CF titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement. Titers of 1:8 were considered positive. Hemagglutination inhibition (HI) tests were done in microtiter plates as described (Travassos da Rosa *et al.*, 1983). HI tests were performed with 4 hemagglutination units of virus at the optimal pH (5.75) against serial two-fold antiserum dilutions starting at

1:20. HI titers of 1:20 were considered positive. Results obtained in CF tests with ARAV, DHOV, THOV and JOSV are summarized in **Supplementary Table 1**. All antisera were robustly reactive with their cognate antigens. Antisera to JOSV were modestly cross-reactive with the ARAV, DHOV and THOV; however, JOSV was recognized by antisera to THOV but not antisera to ARAV, or DHOV.

Results obtained in HI tests are summarized in **Supplementary Table 2**. JOSV, ARAV, DHOV and THOV had high titer HI activity against their cognate antigens. THOV antisera had HI activity against both JOSV and DHOV (1:640 vs. 1:80, respectively). Similarly, JOSV antisera had HI activity against both DHOV and THOV antigens (1:320 vs 1:40, respectively). Although DHOV antigen was recognized by both JOSV and THOV antisera, DHOV serum only recognized its cognate antigen.

Transmission electron microscopy was performed as previously described by Popov et al (Popov *et al.*, 1995). Briefly, Vero cells were infected with 0.01 TCID50 per cell, harvested 5 days post infection, fixed with formaldehyde/glutaraldehyde, post-fixed with 1% OsO_4 and stained with uranyl acetate. Ultrathin sections were stained with lead citrate and examined in a transmission electron microscope at 60 kV. Pleiomorphic ovoid virions 85-120 nm in diameter were observed in the cytoplasm of infected cells. In some instances virions could also be seen budding from the cell surface (**Figure 1**).

For genome sequencing JOS viral stocks were pyrosequenced as previously described (Cox-Foster *et al.*, 2007; Margulies *et al.*, 2005; Palacios *et al.*, 2008). Sequence gaps were completed by PCR and posterior Sanger sequencing, using primers based on pyrosequencing data. For 3' termini of each segment, two primers (one for segments 1-5; a second for segment 6) with the 13 nucleotide (nt) conserved

104 THOV sequence were used for a specific reverse transcription with additional arbitrary
105 nt on the 5’ end. This primer is designed to bind to the 3’ end of the genomic RNA. For
106 the termini of each segment, we used the Clontech SMART RACE kit (Clontech,
107 Mountain View, CA, USA) for the 5’ termini and3' RACE kit (Clontech, Mountain View,
108 CA) for 3’ termini. The sequence of the different segments was verified by Sanger
109 sequencing using primers designed to create products of 1,000 basepairs (bp) with 500
110 bp overlap from the draft sequence. For sequence assembly and analysis Geneious
111 4.8.3 (Biomatters Inc., New Zealand) was used.
112 The assembled data resembled a classical *Thogotovirus* genus like genome
113 (GenBank Accession numbers HM627170-HM627175). Sequence analysis of JOSV
114 indicates the presence of at least 6 RNA segments coding for 7 open reading frames
115 (ORF) corresponding to the polymerase basic protein 2 (PB2, segment 1); polymerase
116 basic protein 1 (PB1, segment 2); acidic polypeptide (PA, segment 3), glycoprotein (GP,
117 segment 4), nucleoprotein (NP, segment 5) and matrix (M) and its long isoform (ML)
118 (segment 6). All remaining contigs and singletons in the pyrosequenced data were
119 properly identified. No additional non-matched data was observed. This was interpreted
120 as an indication that JOSV was composed of at least 6 segments.
121 The conserved terminal sequences of the viral RNA (vRNA) are partially
122 complementary like those of THOV and influenzaviruses. Indeed, the conserved
123 terminal sequences of JOSV vRNA are identical to those of THOV: 5’-
124 AGAGAUAUCAAGGC and 3’-UCGUUUUUGUCCG (segments 1-5) or 3’-
125 UCACCUUUGUCCG (segment 6). Priming of viral mRNA synthesis in influenzaviruses
126 occurs by stealing capped fragments of 10-13 nt from the host (Lamb & Krug, 2001).

Although THOV virus mRNA is capped, 5' RACE analysis indicates that THOV mRNAs do not contain heterogeneous sequences (Weber *et al.*, 1996; 1997). Similarly JOSV mRNAs do not contain heterogeneous sequences (data not shown). In contrast, 5'RACE of mRNA from the novel QRFV identified 9-11 nt that are heterogeneous among the different products, a finding consistent with cap stealing (Presti *et al.*, 2009; Weber *et al.*, 1996; 1997).

Phylogenetic analysis was performed using a set of orthomyxovirus sequences (16 for the nucleoprotein segment, and 15 for the PB1 segment) comprising all sequences available from GenBank (January 2011). Additionally, the phylogeny of each of the 6 segments of the members of the *Thogotovirus* and *Quarjavirus* genera was analyzed with the purpose of clarifying the origin of the segments and for identifying recombination events. All sequences were aligned using the CLUSTAL algorithm (as implemented in the MEGA package Version 3) at the nt and amino acid (aa) level with additional manual editing to ensure the highest possible quality of alignment. Neighbor-joining analysis at the aa level was performed due to the observed high variability of the underlying nt sequences of members of the family *Orthomyxoviridae*. The statistical significance of tree topology was evaluated by bootstrap re-sampling of the sequences 1,000 times. Phylogenetic analysis were performed using MEGA software (Kumar *et al.*, 2004). Neighbor-joining analysis at the nt level was performed using the Kimura-2 parameter and was evaluated by bootstrap re-sampling of the sequences 1,000 times.

In the phylogenetic analysis of the more conserved ORFs at the family level (nucleoprotein and PB1), major nodes that represent viruses belonging to the same genus were clearly distinct and confirmed previously reported topologies (Presti *et al.*,

2009). JOSV was clearly associated with *Thogotovirus* and the proposed genus *Quarjavirus* (**Figure 2**).

Analysis of the 6 segments at the nt level confirmed the clustering of JOSV with thogotoviruses. Distance similarities of JOSV with other thogoto-, quarja- and other member of the family are shown in **Supplementary Table 3**. Branching inconsistencies were detected when ARAV was compared to JOSV and THOV (**Supplementary figure 1**). This may reflect the paucity of sequences used for analysis; only partial sequences of the segment 4 and 5 of Araguari are available (575 nt for HA; 526 nt for NP). No evidence of reassortment was found using the Recombination Detection Program (RDP, Darren Martin) (Martin D, 2000) and the algorithms Bootscan (Salminen *et al.*, 1995), MaxChi (Smith, 1992), Chimaera (Posada & Crandall, 2001), LARD (Holmes, 1998) and Phylip Plot (Felsenstein, 1989) (data not shown).

Finally, topology and targeting predictions were generated by employing SignalP, NetNGlyc, TMHMM (http://www.cbs.dtu.dk/services), TopPred2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), and integrated predictions in Geneious (Bendtsen *et al.*, 2004; Claros & von Heijne, 1994; Kahsay *et al.*, 2005; Kall *et al.*, 2004; Krogh *et al.*, 2001). The program PHYRE was used to predict structural similarity of the predicted Open Reading Frame (ORF) against known protein structures (Kelley & Sternberg, 2009). The algorithm WWIHS was used to predict areas of likely interaction between viral proteins and cell membrane proteins (Wimley & White, 1996).

ORFs analysis showed that the JOSV viral RNA-dependent RNA-polymerase (PB1) contains the pre-A, A, B, C, D, and E motifs found in the catalytic domain of negative strand RNA viruses (Delarue *et al.*, 1990; Müller *et al.*, 1994; Poch *et al.*, 1989;

Vieth *et al.*, 2004) (**Supplementary Figure 2**). The influenzavirus PB1 has two nuclear localization domains, not found in thogotoviruses and JOSV; however, a nuclear localization signal was predicted in JOSV by using PredictNLS (Nair & Rost, 2005) (K_{754}RREAEEAIEEMTKRRK) (**Supplementary Figure 2**).

Comparison of JOSV with THOV PB2 showed regions of high similarity at the 5' end, suggesting that their conservation is under selection pressure (data not shown). This region is implicated in the interaction of the PB1 and PB2 subunits of influenza A virus (Perales *et al.*, 1996).

The NP of *Orthomyxoviridae* is the major structural protein that associates with the genomic RNA segments to form the ribonucleoprotein particles. JOSV NP has many protein domains in common with the NPs of influenza viruses, although the aa sequence similarity is only 14.6; 16.4; and 17.3% with FLUCV, FLUBV and FLUAV, respectively. Interestingly, four separate highly conserved short regions (14 to 30 aas long), initially identified for DHOV by Fuller et al. (Fuller *et al.*, 1987), were detected (**Supplementary Figure 3**). They may represent critical domains for conserved functions of this protein family; in fact, one of them includes the nuclear accumulation sequence as defined by Davey et al (Davey *et al.*, 1985) (**Supplementary Figure 4**). A bipartite nuclear localization signal similar to the one demonstrated in THOV (Weber *et al.*, 1998) was detected in JOSV NP (positions 174-175; 185-188). Moreover, a second putative bipartite nuclear localization signal was found at position 367-381. This sequence contains an upstream (KR) and a downstream (KGKR) cluster of basic aa that are separated by a stretch of 8 aa. It is predicted to have surface exposure. Using a similar approach, a similar motif can be also predicted in THOV. This putative signal

overlaps partially with the fourth highly conserved regions mentioned above and corresponds with the tail loop of the FLUAV NP. No sequence conservation was found when comparing JOSV sequence with the regions responsible for influenzavirus RNA binding (Albo *et al.*, 1995; Kobayashi *et al.*, 1994). Nonetheless, the predicted secondary structure (consisting of two alpha helices connected by a loop-beta sheet-loop domain) of the RNA-binding domain described by Albo et al (Albo *et al.*, 1995) is conserved and the c-terminal region of the nucleoprotein has structural similarity to the influenzavirus NP as predicted by the program Phyre (Kelley & Sternberg, 2009) (**Supplementary Figure 4**). Taken together, these data suggest that while the NP gene derives from a common ancestor among orthomyxoviruses, it followed a separate evolutionary path for the tick borne viruses.

As previously predicted for THOV (Garry & Garry, 2008), the fourth largest RNA segment of JOSV encodes a putative glycoprotein (GP) that is similar to the corresponding proteins of ARAV, THOV and baculovirus GP64 with respect to the N-terminal signal sequence, pre-transmembrane and transmembrane domains, cysteine links, sequences with propensity to interface with a lipid bilayer (as identified with by Wimley-White interfacial hydrophobicity scale (WWIHS, (Wimley & White, 1996)) and areas of N-glycosylation (**Supplementary Figure 5**). Furthermore, the alignment shows five areas of high conservation, three of them corresponding to the predicted fusion domain for AcMNPV and THOV (Garry & Garry, 2008)(**Supplementary Figure 5**). Thus, based on this structural similarity, the GP of JOSV should be classified as a class III penetrene.

JOSV segment 6 is homologous to THOV segment 6 at the aa and nt levels. THOV segment 6 encodes two transcripts: a spliced RNA that encodes the M protein and an unspliced RNA that encodes a C-terminally extended M protein termed ML (matrix protein long) (Hagmaier *et al.*, 2003). RT-PCR analysis of Vero cells infected with JOSV using primers spanning the putative intron revealed the presence of the two expected RNA isoforms. The splicing of JOSV segment 6 transcript results in the formation of an UAA stop codon that terminates the ORF at nt position 813, where the UA originates from the 5' splice site and the A from the 3' splice site (**Figure 3A**). Moreover, we observed a time-dependent expression of these two isoforms. Whereas ML is expressed as early as 1 h after cell infection, M is expressed only after 12 h post-infection (**Figure 3B**). The THOV ML protein has been described as an interferon-antagonist (Jennings *et al.*, 2005) and the early expression of the JOSV protein ML is consistent with it serving a similar role. Moreover, the expression of the M isoform late during the infection is in agreement with it putative role as the major component of the virus particle, like the THOV M protein is (44).

The molecular characterization of JOSV in addition with its similarity with DHOV and THOV, supports the possibility that JOSV should be considered a potential human pathogen. Fagbami et al reported that intracerebral, intraperitoneal or subcutaneous inoculation of newborn mice with JOSV caused a fatal illness within 5 or 6 days with acute hepatocellular necrosis (Fagbami & Ikede, 1978; Mateo *et al.*, 2007). Similar findings have been reported in mice experimentally infected with DHOV (Mateo *et al.*, 2007) and with highly pathogenic influenza viruses (Kawaoka, 1991; Lu *et al.*, 1999) suggesting a common pathogenesis for all of these orthomyxoviruses. Besides being

241 genetically related to THOV, JOSV shares similar temporal and geographic distribution
242 to the pathogenic THOV(Causey *et al.*, 1969) .Since JOSV is a tick borne virus,
243 seroprevalence studies on domestic animals could provide information on the level of
244 circulation. Because of their structural and biochemical similarities to the influenza
245 viruses, their abundance and wide geographic distribution, the ability of
246 orthomyxoviruses to undergo reassortment and the emergence of new virus strains,
247 JOSV and other thogotoviruses may deserve more attention. Their disease potential for
248 humans, livestock and poultry may be overlooked.
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Supplementary Table 1. Results of complement fixation tests with JOSV and three other thogotoviruses.

Antigens	Complement Fixation test Antibody JOSV	ARAV	DHOV	THOV
JOSV	**≥64** **≥8**	0	0	16 ≥8
ARAV	8 ≥8	**≥64** **≥8**	0	0
DHOV	8 ≥8	0	**≥64** **≥8**	0
THOV	8 ≥8	0	0	**≥64** **≥8**

* CF tests expressed as the highest antibody dilution/highest antigen dilution. 0 = <8/8

Supplementary Table 2. Results of hemagglutination-inhibition tests with JOSV and three other thogotoviruses

Serum Samples	Hemagglutination Inhibition test			
	Antigens 4 HA units			
	JOSV	**ARAV**	**DHOV**	**THOV**
JOSV	1:2540	*	1:40	1:320
ARAV	0	≥1:640	0	1:10
DHOV	0	0	≥1:640	1:10
THOV	1:640	1:10	1:80	1:5120

*No hemagglutinating antigen available.

Values expressed as the highest positive antibody dilution. 0 = <1:20.

Supplementary Table 3. Distance similarity of JOSV with other members of Orthomyxoviridae. P-distance at the aminoacid level was calculated using MEGA.

ORF	Segment	THOV	ARAV	DHOV	FLAV	ISAV	QUARV
		RefSeq	BeAn 174214	1313/61	A/Puerto Rico/8/1934	CCBB	EG T 377
NP	**S5**	35.4	52.6	57.5	83.2	85.1	NA
PB1	**S2**	26.9	NA[1]	37.5	73.0	81.9	75.7
PB2	**S1**	39.6	NA	65.2	NS[2]	NS	87.2
PA	**S3**	51.0	NA	65.5	NS	NS	91.9
HA	**S4**	59.5	76.5	69.2	NS	NS	77.9
ML	**S6**	49.0	NA	74.4	NS	NS	NA

[1]NA; not available for comparison

[2]NS; No similarity found; not aligned.

Figure Legends

Figure 1. JOSV as observed in the cytoplasm of an infected Vero cell in ultrathin section. Arrow above indicates a virion budding from the cell surface. Bar = 100 nm.

Figure 2. Phylogenetic analysis of the NP and PB1 ORFs from all orthomyxoviruses.

Figure 3. Transcripts of JOSV segment 6 modified by splicing. (A) Bars represent unspliced and spliced transcripts of segment 6. The black arrows show the position and orientation of PCR primers spanning the putative intron. (B) Time course analysis of the expression of mRNA coding for JOSV ML and M (Two biologic replicates for each time point). Expected product size for ML and M transcript isoforms were 161 and 86 bp, (arrow) respectively.

Supplementary Figure 1. Phylogenetic analysis of all six ORFs with other *Thogotovirus* and *Quarjavirus* genus members. Neighbor-joining analysis at the aa level was performed due to the observed high variability of the underlying nt sequences.

Supplementary Figure 2. *Orthomyxoviridae* PB1 conserved regions. The white arrows represent the conserved regions of the polymerase module (pre-A, A, B, C, D, E) and the purple arrows represent the influenzavirus nuclear localization domains.

Supplementary Figure 3. Orthomyxoviridae NP conserved regions. Four highly conserved regions originally identified by Fuller et al. (orange arrows), the region of structural similarity based on Phyre analysis (pink arrow), and RNA binding domains (turquoise arrows) are shown.

Supplementary Figure 4. Orthomyxoviridae NP c-terminal domain. Structural conservation of JOSV NP compared with the known structure of the influenzavirus nucleoprotein as reported by Phyre. Above, structure of FLUAV NP (right, PDB: 2IQH) and PHYRE2 predicted structure of JOSV NP (left); coiled, strand and helix are depicted in blue, yellow and coiled blue, respectively; red areas depict Fuller et al conserved regions 1 to 4; green areas correspond with putative NLS regions.

Supplementary Figure 5. Thogotovirus and Quarjavirus glycoprotein conserved regions. The signal sequences (pink arrow), cysteines (gold rectangle), pre-transmembrane domains (purple arrows), transmembrane domains (red arrows), N-linked glycosylation sites (purple triangles), sequences with propensity to interface with a lipid bilayer (orange arrows), and highly conserved regions (white arrows) and fusion domain are all shown.

Figure 1

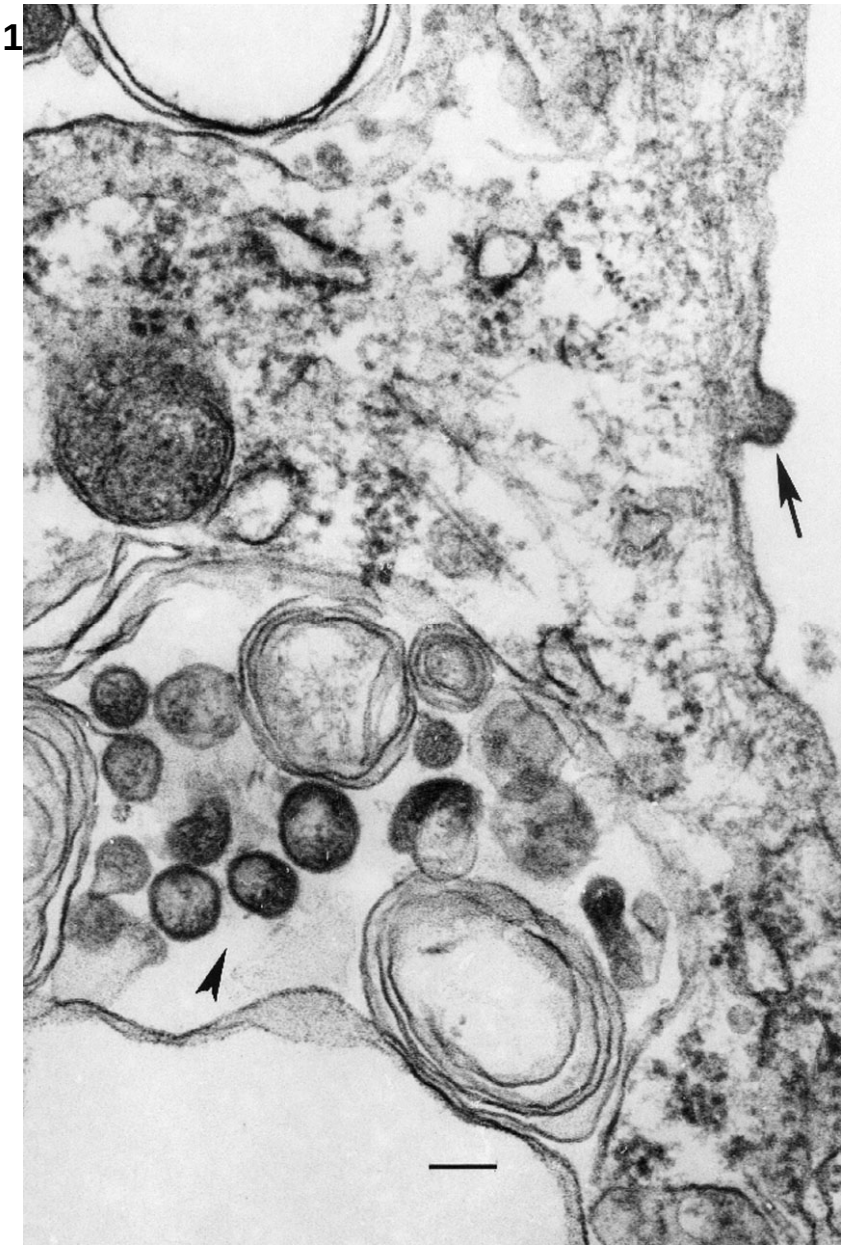
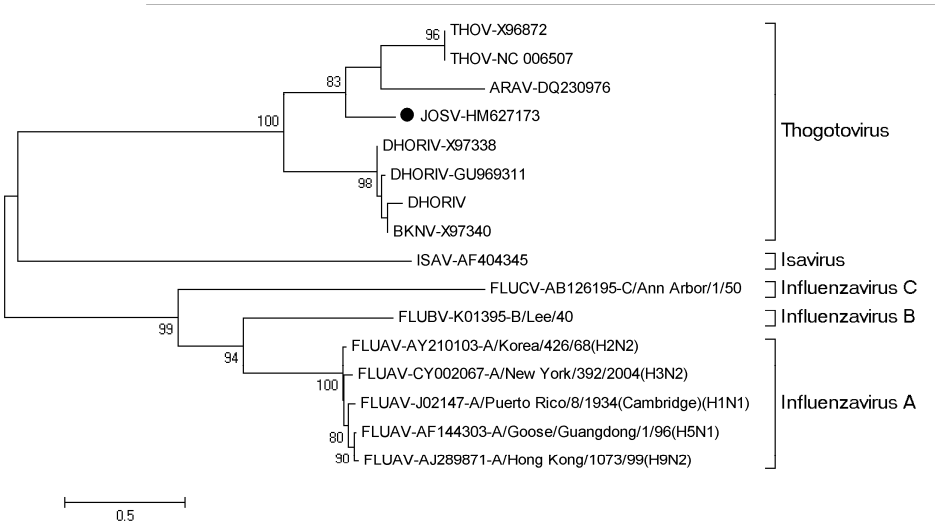


Figure 2

Nucleoprotein



PB1

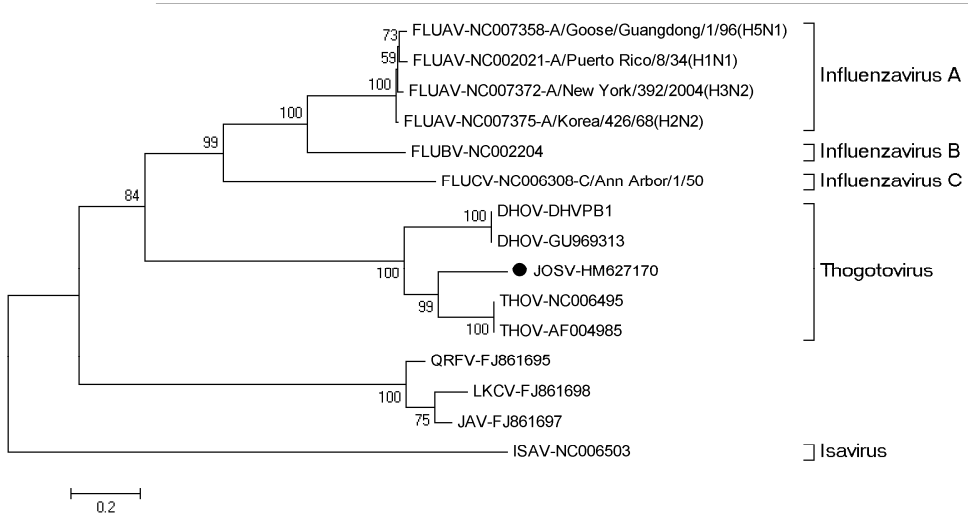


Figure 3