RESEARCH ARTICLE

Revalidation and genetic characterization of new members of Group C (Orthobunyavirus genus, Peribunyaviridae family) isolated in the Americas

Márcio Roberto Teixeira Nunes1,2*, William Marciel de Souza3*, Gustavo Olszanski Acrani4*, Jedson Ferreira Cardoso1, Sandro Patroca da Silva1, Soraya Jabur Badra3, Luiz Tadeu Moraes Figueiredo3, Pedro Fernando da Costa Vasconcelos5*

1 Center for Technological Innovation, Evandro Chagas Institute, Ministry of Health, Ananindeua, Pará, Brazil, 2 Department of Pathology, University of Texas Medical Branch, Galveston, Texas, United States of America, 3 Virology Research Center, School of Medicine of Ribeirão Preto of University of São Paulo, Ribeirão Preto, São Paulo, Brazil, 4 Universidade Federal da Fronteira Sul, Campus Passo Fundo, Rio Grande do Sul, Brazil, 5 Department of Arbovirology and Hemorrhagic Fevers, Evandro Chagas Institute, Ministry of Health, Ananindeua, Pará, Brazil

☯ These authors contributed equally to this work.

* marcionunesbrasil@yahoo.com.br (MRT); pedrovasconcelos@iec.pa.gov.br (PFCV)

Abstract

Group C serogroup includes members of the Orthobunyavirus genus (family Peribunyaviridae) and comprises 15 arboviruses that can be associated with febrile illness in humans. Although previous studies described the genome characterization of Group C orthobunyavirus, there is a gap in genomic information about the other viruses in this group. Therefore, in this study, complete genomes of members of Group C serogroup were sequenced or re-sequenced and used for genetic characterization, as well as to understand their phylogenetic and evolutionary aspects. Thus, our study reported the genomes of three new members in Group C virus (Apeu strain BeAn848, Itaqui strain BeAn12797 and Nepuyo strain BeAn10709), as well as re-sequencing of original strains of five members: Caraparu (strain BeAn3994), Madrid (strain BT4075), Murucutu (strain BeAn974), Oriboca (strain BeAn17), and Marituba (strain BeAn15). These viruses presented a typical genomic organization related to members of the Orthobunyavirus genus. Interestingly, all viruses of this serogroup showed an open reading frame (ORF) that encodes the putative nonstructural NSs protein that precedes the nucleoprotein ORF, an unprecedented fact in Group C virus. Also, we confirmed the presence of natural reassortment events. This study expands the genomic information of Group C viruses, as well as revalidates the genomic organization of viruses that were previously reported.
Introduction

Group C viruses are antigenically characterized into the genus Orthobunyavirus, family Peribunyaviridae, order Bunyavirales [1]. This name is historically based on their serological characteristics, which makes them distinct from members of group A (Alphaviruses genus of the family Togaviridae) and group B (Flavivirus genus of the family Flaviviridae) antigenic groups [2]. Currently, the group C serogroup is composed of 15 distinct viruses isolated from humans, wild animals (mainly rodents, monkeys, marsupials, and bats), and mosquitoes. These viruses are present in tropical and subtropical areas of the Americas, including the United States, Mexico, Panama, Honduras, Guatemala, Trinidad, Brazil, Peru, Ecuador, Venezuela, and French Guiana [1, 3, 4].

Clinically, the human infections caused by Group C viruses are asymptomatic or characterized by unspecific febrile illness [1, 5, 6]. The percentage of asymptomatic Group C viruses infections are unknown, and apparently, the prevalence of antibodies against Group C viruses is directly related to people living nearby or maintaining close contact to forest or ecological niches in tropical areas [5].

The genomes of members of Group C viruses presents the typical organization of other orthobunyaviruses, which are a tri-segmented negative-sense RNA named small (SRNA), medium (MRNA) and large (LRNA) segments. The SRNA encodes a nucleocapsid protein (N protein) and a non-structural protein (NSs), while the MRNA encodes a polyprotein precursor that after a post-cleavage process gives rise to two envelope glycoproteins (Gc and Gn) and a non-structural protein (NSm). LRNA segment encodes a large RNA-dependent RNA polymerase (RdRp) [7]. So far, previous studies have described the genomic characteristics S and M RNA segments of members of Group C viruses, but many sequences generated by Sanger sequencing approach were divergent, despite using the same strains of group C viruses [1, 7–9]. Therefore, in this study, we combined high-throughput sequencing (HTS), rapid amplification of cDNA ends (RACE), and comprehensive phylogenetic analysis of complete coding sequences of Apeu virus (APEUV) strain BeAn848, Itaqui virus (ITQV) strain BeAn12797, and Nepuyo virus (NEPV) strain BeAn10709 and re-sequencing of Caraparu virus (CARV) strain BeAn3994, Madrid virus (MADV) strain BT4075, Murucutu virus (MURV) strain BeAn974, Oriboca virus (ORIV) strain BeAn17, Marituba virus (MTBV) strain BeAn15.

Material and methods

Viruses and propagation in culture cells

Viruses strains used in this study were propagated into VERO cells (ATCC® CCL-81™), as previously described [10]. The infected cells were incubated for 3 to 5 days until visualization of viral cytopathic effect. Table 1 provides the names, strains, year, sources and local of isolation, as well as the GenBank accession numbers.

RNA extraction, construction of RNA library, sequence assembly and RACE assay

Viral RNAs were extracted from the supernatant of infected VERO cells using the PureLink Viral RNA Mini Kit (Invitrogen, USA) following the manufactures instructions. Then, the strands of the RNA synthesis were performed using the kit cDNA Synthesis System and 400 μM Roche “random” Primer according to the manufacturer’s instructions. The cDNAs were prepared for HTS on a GS FLX+ pyrosequencer (Roche, 454 Life Sciences) at the Center for Technological Innovation at the Evandro Chagas Institute, Ministry of Health, Brazil. The de novo assembling strategy applied to obtain the genomes was used with program Newbler v.
Additionally, sequences for terminal untranslated regions (UTRs) were determined by 5'/3' rapid amplification of cDNA ends (RACE) sequencing (S1 Table) [12].

**Genomic characterization**

Virus genomes were evaluated regarding it sizes, annotations of putative open reading frame (ORF), 5’ and 3’ non-coding regions (NCRs) and conserved motifs with Geneious 9.1.2 (Biomatters, New Zealand), for identification of transmembrane regions and signal peptide we used the TOPCONS web server [13] and for identification of N-glycosylation sites we used NetNglyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The annotations of protein domains were performed with InterPro 60.0 [14]. The presence of potential characteristic motifs for orthobunyaviruses were identified on multiple sequence alignments (MSA) based on amino acids sequences, which were carried out using Muscle v.3.7 [15], and visualized in Geneious 9.1.2 (Biomatters, New Zealand).

**Phylogenetic analysis and genetic distance**

Maximum likelihood (ML) phylogenetic trees were constructed using nucleotide and amino acids sequences of viruses reported in our study and additional sequences of members of Group C viruses with complete coding sequences (S, M, and L) available in the GenBank database (http://www.ncbi.nlm.nih.gov/) until 10th of April of 2018. The MSAs were carried out using Muscle v.3.7 [15]. The phylogenies were inferred by IQ-TREE version 1.4.3 software using the best-fit model based on Bayesian Information Criterion, and the GTR+I+G4 nucleotide substitution model was used to all RNA segments, and LG+G4, LG+I+G4, and LG+G4 amino acids substitution model to S, M, and L, respectively. Statistical supports for individual nodes were estimated using the bootstrap value using 1,000 replicates [16]. The phylogenetic trees were visualized using the FigTree software v.1.4.2. In addition, the nucleotides and amino acid distances among viruses of Group C were estimated with segment S, M, and L using the p-distance values. Standard error estimations were calculated by bootstrapping method (1,000 replicates) using the MEGA v.6 program [17].

**Reassortment events analysis**

Potential reassortment events were analyzed by distinct phylogenetic topologies based on the depicted trees at the nucleotide level. In addition, all genes were concatenated in a single sequence, and an MSA was performed using the program Muscle 3.7 [15]. Potential reassortment events were then analyzed using the RDP, GENECONV, Bootscan, MaxChi, Chimaera,
SiScan and 3Seq methods implemented in RDP4 [18]. Common program settings for all methods were used to perceive sequences as linear, to require phylogenetic evidence, to refine breakpoints and to check alignment consistency. The highest acceptable p-value was set as 0.05, after considering Bonferroni correction for multiple comparisons. All method-specific program settings remained at their default values.

Results and discussion

Genome organization of Group C viruses

Our results showed that the complete SRNA segments of Group C viruses ranged from 1,003 to 1,111 nucleotides (nt) and presents the open reading frame (ORF) of the nucleocapsid (N) protein, with a conserved size of 235 amino acids (aa) and 26.72 to 27.03 kDa for all viruses (Fig 1). The second ORF in the S segment in these viruses encodes a putative non-structural protein (NSs). Interestingly, the ORF to NSs gene starts at an ATG codon -38 nucleotides downstream of the N ORF start codon, which potentially encodes an NSs protein of 318 nt

Fig 1. Genome organization of Group C viruses. (a) Apeu, (b) Caraparu, (c) Itaqui, (d) Madrid, (e) Marituba, (f) Murucutu, (g) Nepuyo and (h) Oríboca.

https://doi.org/10.1371/journal.pone.0197294.g001
(105 aa) with a molecular mass of ~11.8 kDa (Fig 1). The NSs protein is common in most orthobunyaviruses that have been isolated from vertebrates, such as the members of Group C viruses [19]. Previously studies have been reported that Caraparu and Madrid from Group C viruses encode an NSs protein with 83 amino acids, and also a 62 aa NSs, predicted to be truncated in the N-terminus of Marituba and Oriboca viruses, and consequently, could not be expressed [6, 7, 9]. Indeed, our analysis demonstrates that possibly the members of Group C viruses present a pre-N coding strategy, as recently described for the Brazoran and Enseada orthobunyaviruses [20, 21]. Therefore, some S segment sequences previously reported for Group C viruses are possibly incomplete, as shown in Fig 2. Whereas that NSs protein has been demonstrated to play crucial roles in virulence and pathogenesis of Orthobunyaviruses [19], further studies using reverse genetics may elucidate the biological importance of NSs protein found in viruses of Group C.

The complete mRNA segments ranged from 4,534 to 4,613 nt in length, which encodes the glycoprotein precursor (GPC), ranging from 1,428 to 1,435 aa in length (159.94 to 161.87 kDa) with a similar topology to other members of the Orthobunyavirus genus (Fig 1). The GPC is proteolytically cleaved to yield two structural glycoproteins, Gn (204 to 211 aa and 34.01 to 35.13 kDa) and Gc (942 to 947 aa and 100.45 to 102.8 kDa), and a nonstructural protein (NSm) with 281 or 282 nt (24.99 to 26.27 kDa) (Fig 1). The GPC contains the specific N-terminal signal peptide that is common to all members of the genus, which is responsible for delivering the nascent polypeptide to the endoplasmic reticulum [22]. The conserved Zinc finger region (amino acids position 257 to 295) and fusion peptide (amino acids position 1068 to 1091) in the GPC protein were also identified, which is an important RNA binding site and is probably involved in viral assembly [23]. Furthermore, the GPC was predicted to contain five transmembrane regions (TMDs): a single TMD in Gn (close to the C-terminus); three TMDs in NSm; and a single one, close to the C-terminus in Gc (Fig 1). The TMDs in Gn and Gc have been shown to play crucial roles in membrane fusion, assembly, and morphogenesis [24].

The complete LRNA segments ranged from 6,907 to 6,979 nt in length, with an ORF that encodes an RNA-dependent RNA polymerase (RdRp) of 2,248 aa, with a predicted molecular weight of 255.64 to 262.13 kDa. The unique exception is Nepuyo virus strain BeAn 10709 with a RdRp of 2,249 aa. This protein contains the conserved polymerase activity domains consisting of Pre-Motif A and Motifs A through E in position 951 to 1230 aa, as well as the N-terminal endonuclease motifs H, PD, and DxE (Fig 1). These domains are highly conserved in negative
sense RNA viral polymerases as well as other members of the Bunyavirales order and are directly involved in the polymerase function activity [25]. In addition, we observed two mismatches in the 8th and 9th nucleotide of the S segments, and another mismatch in the 9th nucleotide of the L segments, but we did not found mismatches in the UTR of M segments (S2 Fig).

**Nucleotide, protein sequence conservation analysis and identification of variable regions**

MURV strain BeAn974, ORIV strain BeAn17, MTBV strain BeAn15, CARV strain BeAn3994 and MADV strain BT4075 viruses present 99.96 to 100% nucleotide identities with the corresponding partial genomic sequences that were previously reported (Fig 3D–3F and Parts D–F of S1 Fig) [4, 7, 8]. On the other hand, the new viruses that we have been sequenced the complete coding sequences shared higher nucleotide identity with partial genomes, such as Apei strain BeAn 848, which shared 99% nucleotide identity in all segments with partial sequences.
of same strains previously described [9]. In addition, the amino acid substitutions were observed as follow: CARV strain BeAn3994 (S344P and K605N) on GPC. MTBV strain BeAn15 (Q335R); (L728Q); (Q1073K) and (K1345R) on RdRp and (G456R and D626N) in GPC. MURV strain BeAn974 (F949S) in RdRp. However, we did not observe any amino acid substitutions in the nucleoprotein sequence of the viruses that were submitted to the re-sequencing protocol. On the other hand, the identified amino acids substitutions observed in GPC and RdRp of our results compared with previously sequences reported to same strains. Therefore, we suspected that this fact is probably due to serial passages in cultured cells [26]. Collectively, our results are consistent with the same viral strains sequenced previously [4, 7–9], but the results of segment S suggested that some complete coding sequences previously reported for Group C viruses are possibly are incomplete.

**Evolutionary relationship of Group C viruses**

To better understand the genetic relationships among group C viruses, we conducted the ML trees based on the complete coding sequences in nucleotide and amino acids level for all segments (Fig 3 and S1 Fig). These viruses were clustered in a unique monophyletic clade with different topologies for each segment. The S and L segments present two major clades, namely of clade A and B (Fig 3A–3C). The clade A was subdivided in three: subclade Ia, composed by MURV, ORIV, and Restan (RESV); subclade Iia, with MTBV and APEUV; subclade IIa comprised by NEPV, Gumbo Limbo (GLV). The clade B was split into two subclades: subclade Ib, represented by CARV strains, ITQV, Itaya (ITYV) strain IQT9646 and FSL2923 and MADV and the subclade IIb with Bruconha virus (BRUV) (Fig 3A–3C).

The topology of the ML tree for the M segment reveals four phylogenetic groups that were well supported (bootstrap > 80%). These groups were named following previous serological classifications based on complement fixation, neutralization, and hemagglutination inhibition tests: Marituba, Caraparu, Madrid and Oriboca complexes (Fig 3B) [1, 27]. The Caraparu complex includes CARV, APEUV and BRUV, Madrid complex comprises MADV and ITYV, Marituba complex comprises MTBV, MURV, RESV, NEPV and GLV, and the Oriboca complex was composed by ORIV and ITQV [1]. Unfortunately, there are no complete sequences available to Vinces and Ossa viruses, but probably these viruses will be clustered into Caraparu complex as previously showed by serological assays [28]. Possibly, the concordance observed between the phylogeny of the M segment and the serological classification occurs because the M segment encodes the glycoproteins, which are the primary antigenic determinants recognized by the neutralization and hemagglutination inhibition tests [1, 29].

**Reassortment events in Group C viruses**

Reassortment is an important evolutionary mechanism of segmented RNA viruses in which co-infection of a host cell with multiple viruses may result in the shuffling of genomic segments [30]. This fact has been shown to be involved in virus emergence and interspecies transmission, which include Schmallenberg and Oropouche virus [31–33]. In Group C viruses, a classic and informative study using standard antigenic tests showed cross-reactivity in Marituba and Murutucu by hemagglutination-inhibition and neutralization, Murutucu and Oriboca by complement-fixation, Oriboca and Itaqui by hemagglutination-inhibition and neutralization, Itaqui and Caraparu by complement-fixation, Caraparu and Apeu by hemagglutination-inhibition and neutralization and Apeu and Marituba by complement-fixation [27]. However, some these viruses cross-reacted completely by one test, but not by another test and still are related. Thus, these and other data indicated that the Group C viruses include many natural reassortants [34]. To elucidate this point, we compared the topology of both S
and L trees with the phylogenetic tree generated using the M segment sequences and observed some discrepancies, which were supported by higher bootstrap values and significantly different likelihood scores, which suggested natural reassortment. Also, after combining these branching inconsistencies in the phylogenetic trees with RDP4 analyses of concatenated segments, we confirmed four reassortment events with different genome segment organization.

Fig 4. Reassortment events in Group C viruses. (a) Apeu, (b) Itaqui, (c) Oriboca and (d) Summary of RDP4 analysis to determine potential reassortants.

https://doi.org/10.1371/journal.pone.0197294.g004
The first reassortment event was identified in APEUV strain BeAn848 contains S and L segments from MTBV strain BeAn15 and an M segment that is probably unique. Also, ITQV strain BeAn 12797 has S and L segments from CARV strain BeAn3994 and a possibly unique M segment. Interestingly, based on the classification of International Committee on Taxonomy of Viruses (ICTV), the reassortment events described in this study occurs between different viral species, as the Apeu virus that is classified into Caraparu orthobunyavirus, but the S and L of this virus is from Marituba orthobunyavirus. Also, the Itaqui and Oriboca viruses are classified into Oriboca orthobunyavirus, but the S and L segments of both viruses are from viruses of Caraparu orthobunyavirus. Despite, previous studies based on serological tests and partial genome sequences indicated that APEUV and ITQV are potential reassortments, the first complete coding sequences for three segments were reported in this study [1, 7, 9, 34]. The other reassortment event was observed in ORIV strain BeAn17, which possesses the S and L segments from MURV strain BeAn974, and a unique M segment (Fig 4). Interestingly, all reassortments observed in this study have been reported cross-reactions only by the complement-fixation method [27]. This fact, suggests that cross-reactivity observed by complement-fixation test was determined by S segment because serologic makers identified by the L segment remains unidentified, as well as hemagglutination-inhibition, and neutralization assay that is determined by M segment, therefore, these viruses probably are unique [34]. Also, we confirmed the reassortments in ITYV identified in Amazon Region of Peru in 1999, both strains of this virus have S and L segments from Caraparu virus and a unique M segment [4]. On the other hand, MURV and RESV were previously reported to be reassortments, but we did not find any evidence to support this phenomenon [1]. Additional studies, as well as other strains of Group C viruses, may help to clarify this point.

In summary, our study provides a better understanding and clarifies the genome sequences, genomic characterization and reassortment events, as well as the evolutionary relationship of Group C serogroup. Thus, our results may be helpful to better understand the evolution and diversity of these viruses, as well as can be used in further molecular epidemiological, evolutionary studies, and development of molecular methods to diagnostic of infection by Group C viruses.

**Nucleotide sequence accession number.** The nucleotide sequence determined in this study have been deposited in GenBank under the accession number MG029269 to MG029292.

**Supporting information**

**S1 Fig.** ML phylogenetic trees based on alignments of amino acids sequences of Group C viruses. (a) Nucleoprotein (b) Glycoprotein precursor and (c) RNA-dependent RNA polymerase. Phylogenies are midpoint rooted for clarity of presentation. The scale bar indicates evolutionary distance in numbers of substitutions per nucleotides substitutions/site, and the principal bootstrap support levels were indicated. Branches are color-coded according to group. Viruses strains sequenced in this study are highlighted with red color. The Bimiti, Guama, Catu and Mahogany hammock were used as outgroup. Pairwise distance based on alignments of amino acids sequences of Group C viruses with (d) Nucleoprotein (e) Glycoprotein precursor and (f) RNA-dependent RNA polymerase. (EPS)

**S2 Fig.** Untranslated regions of Group C viruses obtained by RACE assay. (TIFF)

**S1 Table.** Primers used to RACE assay. (XLSX)
Author Contributions

Conceptualization: Márcio Roberto Teixeira Nunes, William Marciel de Souza, Pedro Fernando da Costa Vasconcelos.

Data curation: Sandro Patroca da Silva.

Formal analysis: William Marciel de Souza, Gustavo Olszanski Acrani, Sandro Patroca da Silva.

Funding acquisition: Márcio Roberto Teixeira Nunes, Pedro Fernando da Costa Vasconcelos.

Investigation: William Marciel de Souza.

Methodology: Márcio Roberto Teixeira Nunes, William Marciel de Souza, Gustavo Olszanski Acrani, Jedson Ferreira Cardoso, Sandro Patroca da Silva, Soraya Jabur Badra, Luiz Tadeu Moraes Figueiredo.

Project administration: Márcio Roberto Teixeira Nunes, Pedro Fernando da Costa Vasconcelos.

Resources: Márcio Roberto Teixeira Nunes.

Supervision: Márcio Roberto Teixeira Nunes, Luiz Tadeu Moraes Figueiredo, Pedro Fernando da Costa Vasconcelos.

Validation: Jedson Ferreira Cardoso, Sandro Patroca da Silva, Soraya Jabur Badra.

Visualization: Márcio Roberto Teixeira Nunes, William Marciel de Souza.

Writing – original draft: Márcio Roberto Teixeira Nunes, William Marciel de Souza, Gustavo Olszanski Acrani.

Writing – review & editing: Márcio Roberto Teixeira Nunes, William Marciel de Souza, Gustavo Olszanski Acrani, Jedson Ferreira Cardoso, Pedro Fernando da Costa Vasconcelos.

References

1. Nunes MR, Travassos da Rosa AP, Weaver SC, Tesh RB, Vasconcelos PF. Molecular epidemiology of group C viruses (Bunyaviridae, Orthobunyavirus) isolated in the Americas. Journal of virology. 2005; 79(16):10561–70. https://doi.org/10.1128/JVI.79.16.10561-10570.2005 PMID: 16051848.

2. Casals J, Whitman L. Group C, a new serologic group of hitherto undescribed arthropod-borne viruses. Immunological studies. The American journal of tropical medicine and hygiene. 1961; 10:250–8. PMID: 13691233.

3. Scherer WF, Madalengoitia J, Flores W, Acosta M. The first isolations of eastern encephalitis, group C, and Guama group arboviruses from the Peruvian Amazon region of western South America. Bulletin of the Pan American Health Organization. 1975; 9(1):19–26. PMID: 238693.

4. Hontz RD, Guevara C, Halsey ES, Silvas J, Santiago FW, Widen SG, et al. Itaya virus, a Novel Orthobunyavirus Associated with Human Febrile Illness, Peru. Emerging Infectious Diseases. 2015; 21(6):781–8. https://doi.org/10.3201/eid2105.141368 PMID: 25898901.

5. Pinheiro FP. Arboviral zoonoses in South America. In: Steele JH, Beran GW, editors. CRC Handbook Series in Zoonoses Section B Viral Zoonoses: CRC Press; 1981.

6. Forshey BM, Guevara C, Laguna-Torres VA, Cespedes M, Vargas J, Gianella A, et al. Arboviral etiologies of acute febrile illnesses in Western South America, 2000–2007. PLoS neglected tropical diseases. 2010; 4(8):e787. https://doi.org/10.1371/journal.pntd.0000787 PMID: 2076626.

7. Hang J, Forshey BM, Yang Y, Solorzano VF, Kuschner RA, Halsey ES, et al. Genomic characterization of group C Orthobunyavirus reference strains and recent South American clinical isolates. PloS one. 2014; 9(3):e92114. https://doi.org/10.1371/journal.pone.0092114 PMID: 24633174.

8. Forshey BM, Castillo RM, Hang J. Group C orthobunyavirus genomic sequences require validation. Journal of virology. 2014; 88(5):3052–3. https://doi.org/10.1128/JVI.03295-13 PMID: 24505105.
9. de Brito Magalhaes CL, Drumond BP, Novaes RF, Quinan BR, de Magalhaes JC, dos Santos JR, et al. Identification of a phylogenetically distinct orthobunyavirus from group C. Archives of virology. 2011; 156(7):1733–84. https://doi.org/10.1007/s00705-011-0976-1 PMID: 21465087.

10. Rhim JS, Schell K, Creasy B, Case W. Biological characteristics and viral susceptibility of an African green monkey kidney cell line (Vero). Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine. 1969; 132(2):670–8. PMID: 4982209.

11. Miller JR, Koren S, Sutton G. Assembly algorithms for next-generation sequencing data. Genomics. 2010; 95(6):315–27. https://doi.org/10.1016/j.ygeno.2010.03.001 PMID: 20211242.

12. Rapid amplification of 5' complementary DNA ends (5' RACE). Nature methods. 2005; 2(8):629–30. PMID: 16145794.

13. Tsurigos KD, Peters C, Shu N, Kall L, Elofsson A. The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic acids research. 2015; 43(W1):W401–7. https://doi.org/10.1093/nar/gku1243 PMID: 25428371.

14. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research. 2004; 32(5):1792–7. https://doi.org/10.1093/nar/gkh340 PMID: 15034147.

15. Nguyen LT, Schmidt HA, von Haeseler A, Minh BO. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular biology and evolution. 2015; 32(1):268–74. https://doi.org/10.1093/molbev/msu197 PMID: 25371430.

16. Shi X, Botting CH, Li P, Niglas M, Brennan B, Shirran SL, et al. Bunyamwera orthobunyavirus glycoprotein precursor is processed by cellular signal peptidase and signal peptide peptidase. Proceedings of the National Academy of Sciences of the United States of America. 2016; 113(31):8825–30. https://doi.org/10.1073/pnas.1603364113 PMID: 27438677.

17. Plassmeyer ML, Soldan SS, Stachelek KM, Roth SM, Martin-Garcia J, Gonzalez-Scarano F. Mutagenesis of the La Crosse Virus glycoprotein supports a role for Gc (1066–1087) as the fusion peptide. Virology. 2007; 358(2):273–82. https://doi.org/10.1016/j.virology.2006.08.050 PMID: 17027056.

18. Shi X, Kohl A, Li P, Elliott RM. Role of the cytoplasmic tail domains of Bunyamwera orthobunyavirus glycoproteins Gn and Gc in virus assembly and morphogenesis. Journal of virology. 2007; 81(18):10151–60. https://doi.org/10.1128/JVI.00573-07 PMID: 17609275.

19. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular evolutionary genetics analysis. 2013; 30(12):2725–9. https://doi.org/10.1093/molbev/ mst197 PMID: 24132122.

20. de Souza WM, Acrani GO, Romeiro MF, Reis O Jr., Tolardo AL, da Silva SP, et al. Molecular characterization of Capim and Enseada orthobunyaviruses. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2016; 40:47–53. https://doi.org/10.1016/j.igme.2016.02.024 PMID: 26921797.

21. Lanciotti RS, Kosoy OI, Bosco-Lauth AM, Pohl J, Stuchlik O, Reed M, et al. Isolation of a novel orthobunyavirus from group C. Archives of virology. 2011; 156(7):1733–84. https://doi.org/10.1007/s00705-011-0976-1 PMID: 21465087.

22. Shi X, Botting CH, Li P, Niglas M, Brennan B, Shirran SL, et al. Bunyamwera orthobunyavirus glycoprotein precursor is processed by cellular signal peptidase and signal peptide peptidase. Proceedings of the National Academy of Sciences of the United States of America. 2016; 113(31):8825–30. https://doi.org/10.1073/pnas.1603364113 PMID: 27438677.

23. Liu XB, Fang SG, Tay FP, Liu DX. Acquisition of cell-cell fusion activity by amino acid substitutions in spike protein determines the infectivity of a coronavirus in cultured cells. PLoS one. 2009; 4(7): e6130. https://doi.org/10.1371/journal.pone.0006130 PMID: 19572016.

24. Rhim JS, Schell K, Creasy B, Case W. Biological characteristics and viral susceptibility of an African green monkey kidney cell line (Vero). Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine. 1969; 132(2):670–8. PMID: 4982209.

25. Reguera J, Weber F, Cusack S. Bunyaviridae RNA polymerases (L-protein) have an N-terminal, influenza-like endonuclease domain, essential for viral cap-dependent transcription. PLoS pathogens. 2010; 6(9):e1001101. https://doi.org/10.1371/journal.ppat.1001101 PMID: 20862319.

26. Shope RE, Causey OR. Further studies on the serological relationships of group C arthropod-borne viruses and the application of these relationships to rapid identification of types. The American journal of tropical medicine and hygiene. 1962; 11(2):283–90. PMID: 13912242.

27. Calisher CH, Gutierrez E, Francy DB, Alava A, Muth DJ, Lazucik JS. Identification of hitherto unrecognized arboviruses from Ecuador: members of serogroups B, C, Bunyamwera, Patois, and Minatitlan. The American journal of tropical medicine and hygiene. 1983; 32(4):877–85. PMID: 6309029.
29. Palacios G, Tesh R, Travassos da Rosa A, Savji N, Sze W, Jain K, et al. Characterization of the Candiru antigenic complex (Bunyaviridae: Phlebovirus), a highly diverse and reassorting group of viruses affecting humans in tropical America. Journal of virology. 2011; 85(8):3811–20. https://doi.org/10.1128/JVI.02275-10 PMID: 21289119.

30. Vijaykrishna D, Mukerji R, Smith GJ. RNA Virus Reassortment: An Evolutionary Mechanism for Host Jumps and Immune Evasion. PLoS pathogens. 2015; 11(7):e1004902. https://doi.org/10.1371/journal.ppat.1004902 PMID: 26158697.

31. Yanase T, Kato T, Aizawa M, Shuto Y, Shirafuji H, Yamakawa M, et al. Genetic reassortment between Sathuperi and Shamonda viruses of the genus Orthobunyavirus in nature: implications for their genetic relationship to Schmallenberg virus. Archives of virology. 2012; 157(8):1611–6. https://doi.org/10.1007/s00705-012-1341-8 PMID: 22588368.

32. da Rosa JF, de Souza WM, de Paula Pinheiro F, Figueiredo ML, Cardoso JF, Acrani GO, et al. Oro-pouche Virus: Clinical, Epidemiological, and Molecular Aspects of a Neglected Orthobunyavirus. The American journal of tropical medicine and hygiene. 2017. https://doi.org/10.4269/ajtmh.16-0672 PMID: 28167595.

33. Aguilar PV, Barrett AD, Saeed MF, Watts DM, Russell K, Guevara C, et al. Iquitos virus: a novel reassortant Orthobunyavirus associated with human illness in Peru. PLoS neglected tropical diseases. 2011; 5(9):e1315. https://doi.org/10.1371/journal.pntd.0001315 PMID: 21949892.

34. Briese T, Calisher CH, Higgs S. Viruses of the family Bunyaviridae: are all available isolates reassortants? Virology. 2013; 446(1–2):207–16. https://doi.org/10.1016/j.virol.2013.07.030 PMID: 24074583.