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Research paper

Oropouche orthobunyavirus: Genetic characterization of full-length genomes and development of molecular methods to discriminate natural reassortments

Márcio Roberto Teixeira Nunes[a,b,*,1], William Marciel de Souza[c,d,1], Nazir Savji[e], Mário Luís Figueiredo[f], Jedson Ferreira Cardoso[a,g], Sandro Patroca da Silva[h], Clayton Pereira da Silva de Lima[a], Helena Baldez Vasconcelos[h], Sueli Guerreiro Rodrigues[h], W. Ian Lipkin[i,1], Pedro Fernando Costa Vasconcelos[h,1], Gustavo Palacios[j,1]

[a] *Center for Technological Innovations, Evandro Chagas Institute, Ministry of Health, Ananindeua, Pará, Brazil*
[b] *Center for Biodefense and Emerging Infectious Diseases, Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA*
[c] *Virology Research Center, School of Medicine of Ribeirao Preto of University of São Paulo, Ribeirao Preto, São Paulo, Brazil*
[d] *MRC-University of Glasgow Centre for Virus Research, Glasgow, Scotland, United Kingdom*
[e] *Department of Medicine, Massachusetts General Hospital, Boston, MA, USA*
[f] *Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, São Paulo, Brazil*
[g] *Posgraduate Program in Virology, Evandro Chagas Institute, Ministry of Health, Ananindeua, Brazil*
[h] *Department of Arbovirology and Hemorrhagic Fevers, Evandro Chagas Institute, Ministry of Health, Ananindeua, Brazil*
[i] *Center for Infection and Immunity, Mailman School of Public Health, Columbia University, NY, New York, USA*
[j] *The Center for Genome Science, US Army Medical Research Institute of Infectious Disease at Fort Detrick, MD, USA*

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ABSTRACT

Oropouche orthobunyavirus (OROV) has significant impact in public health in Amazon region. This arbovirus is one of the most common causes of febrile illness in Brazil, and is responsible for several epidemics since 1960's. In this study, we sequenced and characterized the complete coding sequences (S-, M- and L-RNA) of 35 OROV isolates from Brazil. Here, we classified 20 strains in genotype I from Pará and Maranhão states, nine as genotype II from Pará and Rondônia states confirmed, four classified into genotype III from Acre, Maranhão, Minas Gerais and Rondônia states and two genotype IV from Amazonas State. Also, we did not observe reassortment events involving the OROV isolates. In addition, we developed novel RT-PCR tools to identify reassortment events among OROV strains. These data will be useful to better understand the molecular epidemiology and diagnostic of OROV infections.

1. Introduction

Oropouche orthobunyavirus (OROV) is one of the most important arboviruses in the Amazon region. This virus is classified into Simbu serogroup, genus *Orthobunyavirus*, *Peribunyaviridae* family (Travassos Da Rosa et al., 2017). The prototype of OROV was isolated from a febrile patient in the rural village of Vega de Oropouche in 1955, Trinidad and Tobago (Anderson et al., 1961). In Brazil, this virus was first isolated in 1960 from the blood of a sloth (*Bradypus trydactylus*) captured during the construction of the Belém-Brasília Highway. In the last 60 years, > 30 outbreaks have been caused by OROV in Latin America, where approximately half million people have been infected (Travassos Da Rosa et al., 2017).

In the last decade, improvements in genome sequencing methods allowed several OROV strains to be completely or partially sequenced. Consequently, new insights into evolutionary history, epidemiology, reassortment events, and genotype classification of OROV species have been unearthed (Aguilar et al., 2011; Hontz et al., 2015; Navarro et al., 2016; Saeed et al., 2000; Tilston-Lunel et al., 2015b; Vasconcelos et al., 2011). However, the majority of studies have been limited to sequencing the S segment (Bastos et al., 2014; Cardoso et al., 2015; de Souza Luna et al., 2017; Gibrail et al., 2016). Viral taxonomy and evolutionary analysis are improved by the inclusion of multiple genetically distinct genomes, thereby ensuring a range of genetic diversity in phylogenetic

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[*] Corresponding author at: Center for Technological Innovation, Instituto Evandro Chagas, Ananindeua, Brazil.
E-mail address: marcionunes@iec.pa.gov.br (M.R.T. Nunes).

[1] These authors have contributed equally to this study.

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trees. Therefore, we sequenced all RNA segments from 35 OROV isolates from Brazil and investigated their taxonomic relationships within the OROV species. Also, we described a novel RT-PCR for identification of natural reassortments of OROV strains.

2. Materials and methods

2.1. Virus strains and RNA extraction

The OROV strains were obtained from the Department of Arbovirology and Hemorrhagic Fevers of the Evandro Chagas Institute and World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (Table 1 and Supplementary Table 1). The OROV strains used were low passage strains and were isolated and propagated in cultures of Vero cells (Ammerman et al., 2008). When viral cytopathic effect was apparent, the supernatant was collected and used for the extraction of viral RNA using QIAamp Viral RNA Mini Kit (Qiagen, USA) or Trizol LS® reagent (Invitrogen, USA) according to manufacturer's instructions. All samples were manipulated under biocontainment and biosafety rules using biosafety level-3 (BSL-3) laboratories.

2.2. Genome sequencing, assembling and gene annotations

The cDNAs were prepared for high-throughput sequencing using a random amplification approach in the Ion Torrent sequencer that uses the ion semi conduction method (Thermo Fisher Scientific, USA) as previously described (Rothberg et al., 2011). A *de novo* assembly strategy to obtain the genomes was applied using the MIRA software v.4.9.2 (Chevreux et al., 2004). Contigs were considered if at least five reads were assembled. Quality inspection (base call quality > 20) was set as default to reconstruct the RNA segments. Subsequently, the annotations of putative ORF genes and motifs characteristics of orthobunyaviruses were predicted using the Geneious v.9.1.2 (Biomatters, New Zealand) using as the reference sequences the Brazilian prototype strain BeAn 19,991 (Genbank acession No. KP052852, KP052851, and KP052850).

2.3. Phylogenetic analysis and inspection of genome sequences

Maximum likelihood (ML) were reconstructed using 35 complete coding sequences here reported and OROV with complete coding sequences to all segments (S, M, and L available in GenBank database (http://www. ncbi.nlm.nih.gov/) until February 26, 2018. Multiple sequence alignments (MSA) based on nucleotide sequences were performed using the *E*-INS-i algorithm implemented in MAFFT version 7.158b program (Katoh and Standley, 2013). To identify potential reassortment events was used an MSA concatenated with all genes of each strain and analyzed using the RDP4 software employing RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan and 3Seq methods (Martin et al., 2015). ML phylogenies for all segments were inferred using IQ-TREE version 1.4.3 software with the best-fit model based on Bayesian Information Criterion. The best-fit substitutions models were GTR + I + G4 (for M and L segments) and TVMe + G4 for S segment (Nguyen et al., 2015). The potential reassortment identified by RD4 analysis, such as Iquitos virus (IQTV), Madre de Dios virus (MDDV) and

Table 1
Oropouche orhobunyavirus strains used in this study.

Identification	Strain	Host association	Year of isolation	GenBank access number		
				SRNA	MRNA	LRNA
AC02	BeH 543100	*Homo sapiens*	1996	MG747503	MG747504	MG747505
AM01	BeH 389865	*Homo sapiens*	1980	MG747506	MG747507	MG747508
AM03	BeH 390242	*Homo sapiens*	1980	MG747509	MG747510	MG747511
MA02	BeH 472433	*Homo sapiens*	1988	MG747512	MG747513	MG747514
MA03	BeH 472435	*Homo sapiens*	1988	MG747515	MG747516	MG747517
MA06	BeH 521086	*Homo sapiens*	1993	MG747518	MG747519	MG747520
MG01	BeAn 626990	*Callithrix sp.*	2000	MG747521	MG747522	MG747523
PA01	BeAr 19886	*Ochlerotatus serratus*	1960	MG747524	MG7475215	MG747526
PA03	BeH 29086	*Homo sapiens*	1961	MG747527	MG747528	MG747529
PA04	BeH 29090	*Homo sapiens*	1961	MG747530	MG747531	MG747532
PA05	BeH 121923	Homo sapiens	1967	MG747533	MG747534	MG747535
PA06	BeAr 136921	*Culex quinquefasciatus*	1968	MG747536	MG747537	MG747538
PA07	BeAn 206119	*Bradypus tridactylus*	1971	MG747539	MG747540	MG747541
PA08	BeAn 208402	*Bradypus tridactylus*	1971	MG747542	MG747543	MG747544
PA09	BeAn 208819	*Bradypus tridactylus*	1971	MG747545	MG747546	MG747547
PA14	BeH 355173	*Homo sapiens*	1978	MG747548	MG747549	MG747550
PA17	BeAr 366927	*Culicoides paraensis*	1979	MG747551	MG747552	MG747553
PA22	BeH 385591	*Homo sapiens*	1980	MG747554	MG747555	MG747556
PA25	BeH 532314	*Homo sapiens*	1994	MG747557	MG747558	MG747559
PA26	BeH 532422	*Homo sapiens*	1994	MG747560	MG747561	MG747562
PA27	BeH 532490	*Homo sapiens*	1994	MG747563	MG747564	MG747565
PA28	BeH 532500	*Homo sapiens*	1994	MG747566	MG747567	MG747568
PA29	BeH 541140	*Homo sapiens*	1994	MG747569	MG747570	MG747571
PA34	BeH 543629	*Homo sapiens*	1996	MG747572	MG747573	MG747574
PA38	BeH 543760	*Homo sapiens*	1996	MG747575	MG747576	MG747577
PA39	BeH 543857	*Homo sapiens*	1996	MG747578	MG747579	MG747580
PA41	PPS 522 H 669314	*Homo sapiens*	2003	MG747581	MG747582	MG747583
PA42	PPS 523 H 669315	*Homo sapiens*	2003	MG747584	MG747585	MG747586
PA43	PMOH 682426	*Homo sapiens*	2004	MG747587	MG747588	MG747589
PA44	PMOH 682431	*Homo sapiens*	2004	MG747590	MG747591	MG747592
PA47	BeH 708139	*Homo sapiens*	2006	MG747593	MG747594	MG747595
PA49	BeH 707287	*Homo sapiens*	2006	MG747596	MG747597	MG747598
PA50	BeH 708717	*Homo sapiens*	2006	MG747599	MG747600	MG747601
RO01	BeH 498913	*Homo sapiens*	1990	MG747602	MG747603	MG747604
RO05	BeH 505768	*Homo sapiens*	1991	MG747605	MG747606	MG747607

AC: Acre State; AM: Amazonas state; MA: Maranhão State; MG: Minas Gerais State; PA: Pará State; RO: Rondônia State.

Table 2
Specific set of primers used to detect the MRNA of OROV and reassortant species by the RT-PCR and RT-Nested PCR assays.

Virus	Assay	Primer	Sequence	T_M °C	Product size (bp)
OROV	RT-PCR and Nested	M575F	TCAGTAAAATTCCATGCATG	52–55	
		M1005R	TCTAAAAGCTGGTCATTCAC	52–55	427
		M716R*	TTGCAGCAAAGATATACAATGT	52-55	135
	RTqPCR	OROV F	ACATTGTATATCTTTGCTGCAA	52–55	
		OROVR	TTTTCCGTAAAGCCAGGTTA	52–55	
		Probe	ACTCCTACCATTGTTTTATCCGG	58.2	118
IQTV	RT-PCR and Nested	M661F	CATATGTGTGCAAACCTAGAG	52–55	
		M1150R	ATGCAAAGATCAATATATAATC	52–55	480
		MN1125*	CTATGAAGACTGAAGTAACA	52-55	397
		IQTVF	CATATGTGTGCAAACCTAGAG	52–55	
	RTqPCR	IQTVR	AAATACATAAGTTAATGGGTA	52–55	
		Probe	ATATATCTTTACCGCATTGGTG	58.3	135
MDDV	RT-PCR and Nested	M639F	GACCATGTGCGTTAACCTT	52–55	
		M1139R	GAAGAAAGCTATTAACAACT	52–55	500
		M1077R*	AAGCGGCGAAAGGATGTATAAG	52-55	399
		MDDVF	GACCATGTGCGTTAACCTT	52–55	
	RTqPCR	MDDVR	TATAGAACCTAAAATATATG	52–55	
		Probe	TCTTCTGCCGGTTTTTTATCCGA	57.8	147
PERDV	RT-PCR and Nested	M645F	TATTGTTATGGTGGAATCAC	52–55	
		M1137R	AGTAGATATAATCAATTATA	52–55	495
		M1092R*	ATAAATTATCTGAACTACCA	52-55	435
		PERDVF	CATCACATATATTTTTGTGTGCC	52–55	
	RTqPCR	PERDVR	TATGCATATGTGAAAGGGTA	52–55	
		Probe	AGCTTACCTACTAATACCTGTATT	58.1	137

* Reverse (R) primers used for semi-Nested PCR assay. Forward (F) primers; OROV: Oropouche virus; IQTV: Iquitos virus; MDDV: MDDV: Madre de Dios virus; PERDV: Perdoes virus. Probes are labeled at 5` and 3` ends with FAM (fluorophore) and BHQ (black hole quencher), respectively. Bp: base pairs.

Perdões virus (PERDV) were excluded in phylogenetic trees. Statistical supports for individual nodes were estimated using bootstrap value. The phylogenetic trees were visualized using FigTree software v.1.4.2.

The Neighbour-Joining tree was obtained with S sequences reported in our study in combination with all complete nucleotide sequences that encode the N protein available in GenBank database until February 26, 2018. This tree was inferred using MEGA v.6.06 software, employing maximum composite likelihood model with among-site rate heterogeneity parameter (γ) as the substitution model (Tamura et al., 2013). Bootstrap analyses were performed using 1000 bootstrap replications. The phylogenetic tree was visualized using iTOL v.3.4. (Letunic and Bork, 2016).

2.4. Design of specific primer for OROV isolates detection

Divergent regions were identified along the MRNA ORF genome segment to determine the best region for specific OROV set of primers and probes able to differentiate OROV isolates of OROV reassortments, such as IQTV, MDDV and PERDV (Aguilar et al., 2011; Navarro et al., 2016; Tilston-Lunel et al., 2015b). Table 2 describes the primers and probes used for specific genome detection of OROV isolates.

2.5. RT-nested PCR and RT-qPCR methods

Briefly, RT-nested-PCR was developed using external primers to non-reassortant OROV isolates (M575F and M1005R) in a one-step RT-PCR, followed by a semi nested-PCR step using the inner reverse primer (M716R) (Table 2). Also, RT-qPCR was developed using a specific set of primers and probes to OROV and OROV reassortants (Table 2). Protocol conditions are available in Supplementary material (Appendix). The limit of detection for both methods was assessed using a standard synthetic RNAs commercially prepared flanking the targeted region of amplification (Thermo Scientific, USA) for each used virus in the following concentration for a reaction volume of 25 μL: i) OROV: 5,9 × 10^6 copies/μL or 147,5 × 10^6 copies/reaction; ii) IQTV: 4,01 × 10^6 copies/μL or 102,5 × 10^6 copies/reaction; iii) MDDV: 6,1 × 10^5 copies/μL or 152,5 × 10^6 copies/reaction; and iv) PERDV: 3,71 × 10^6 copies/μL or 92,75 × 10^6 copies/reaction. The standard RNAs were tested in eight different ten-fold serial dilutions from 10^{-1} to 10^{-8} and the limit was determined for the highest dilution where positive results were considered under the Ct threshold values ≤38 cycles. Sensitivity (Se) and specificity (Sp) were determined using a panel of 78 samples divided into three groups (35 OROV isolates, 3 OROV reassortant strains, and 40 non-OROV orthobunyaviruses) (Supplementary Table 2). Then, it was applied the following formula: $Se = a/a + b$; $Sp = d/c + d$, where $a =$ positive; $b =$ false negative; $c =$ false positive; $d =$ negative. Values are expressed in percentage under a 95% confidence interval.

3. Results

3.1. Genome characterization of 35 OROV isolates

Complete coding sequences of all segments of 35 OROV isolates were determined with mean coverage to each segment, including 89× to S segment, 56× to M segment and 67× to L segment based on quality higher than 20. The S segment has two ORFs, one with 696 to 699 nucleotides (nt) and another with 276 to 288 nt in length, which encodes nucleoprotein (N) with 232 and 233 amino acids (aa) and the nonstructural protein (NSs) with 92 and 96 aa, respectively. However, the OROV strain PA01 possesses a longer ORF for the NSs with 339 nt (113 aa) in length. Resequencing of the N region using specific N OROV primers (OROV N and ORONR) described by (Saeed et al., 2000), confirmed the largest NSs region in strain PA01.

M segment contained a single ORF of 4257 to 4263 nt, which encodes a glycoprotein precursor (GPC) of 1418 to 1420 aa. The L segment has a unique ORF of 6753 to 6759 nt, which encodes the RNA-dependent RNA polymerase (RdRP) of 2252–2250 aa. Conserved motifs characteristic of orthobunyaviruses were identified in the OROV isolates, which include peptide fusion and zinc finger in the GPC, endonuclease and the Pre-A, A, B, C, D, and E motifs in the RdRP (Reguera et al., 2010; Shi et al., 2009).

3.2. Phylogenetic analysis, genotyping and reassortment events of OROV

Phylogenetic analysis of the complete coding sequences of S

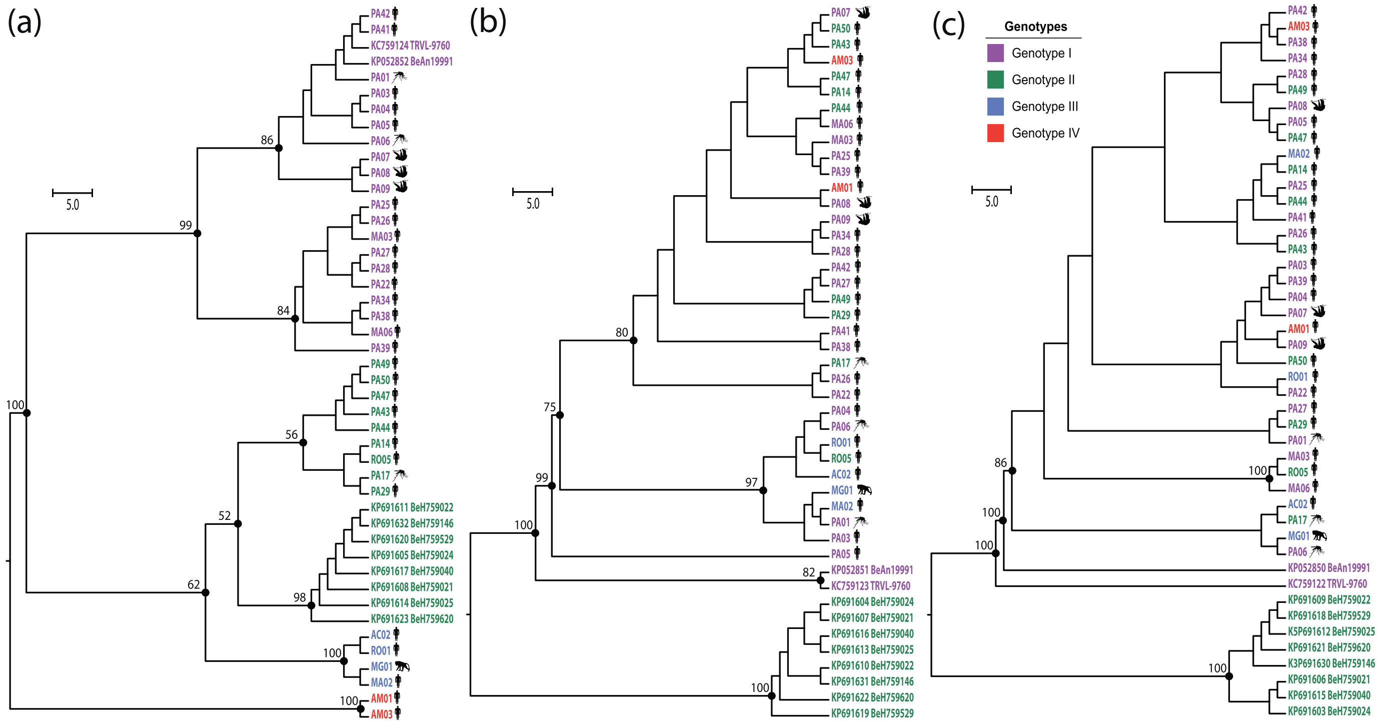

Fig. 1. ML phylogenetic trees of OROV strains based on nucleotide sequences of S segment (a) M segment (b) and L segment (c). Phylogenies are midpoint rooted for clarity of presentation. The scale bar indicates evolutionary distance in numbers of nucleotides substitutions per site, and the principal bootstrap support levels were indicated. Branches are colored according to genotype. OROV strains sequenced in this study are highlighted with silhouette host.

segment of 45 OROV strains allowed clustering into four genotypes, named genotypes I, II, III and IV (Fig. 1a) as previously described (Vasconcelos et al., 2011). Genotype I was divided into two subclades. The first subclade was constituted only by isolates of patients from Pará State, Brazil (isolates PA42, PA41, PA06, PA09, PA08, PA07, PA01, PA05, PA04, PA03), and OROV prototypes (strains TRV-9760 and BeAn19991). The second subclade was composed only by strains reported in our study from Pará State (isolates PA39, PA38, PA34, PA26, PA25, PA22, PA28, and PA27) and two isolates from Maranhão State (isolates MA03 and MA06) (Fig. 1a).

Genotype II was divided into two subclades, the first subclade was formed only by isolates reported in our study, including eight strains from Pará State (isolates PA43, PA44, PA47, PA49, PA50, PA14, PA17, and PA29) and another isolate from Rondônia State (RO05). The second subclade of genotype II was composed by eight strains from patients in Mazagão town, Amapá State, Brazil. This group showed a very high similarity score among strains with pairwise nucleotide similarities over 99.8% (Tilston-Lunel et al., 2015b)(Fig. 1a). The genotypes III and IV were made up only by isolates reported in this study. The genotype III was formed by strains isolated from patients from Acre (isolate AC02), Rondônia (isolate RO01) and Minas Gerais (isolates MG01 and MA02) states. Finally, genotype IV was formed by a well-supported cluster of two isolates from Amazonas State (isolates AM01 and AM03) (Fig. 1a).

Surprisingly, the M and L sequences were distributed in two well-supported clusters (> 99% bootstrap) with a different topology than the tree based on S segments. One clade was constituted with 37 strains classified as genotypes I to IV, which includes all sequences obtained in our study. The second cluster was formed by eight strains of patients from Mazagão town, Amapá State, Brazil that were reported previously (Tilston-Lunel et al., 2015) (Fig. 1b and c). However, based on the nucleotide phylogenies for discordances in clade clustering between coding sequences combined with RDP4 analyses, we did not observe any reassortment events in OROV strains sequenced in our study.

3.3. Geographical distribution of genotypes of OROV

To confirm the genotyping and geographical distribution of OROV, we performed NJ phylogeny with 106 OROV sequences, which showed topologies with four genotypes (I, II, III and IV) similar to those presented in Fig. 1a, and as previously described (Vasconcelos et al., 2011). Currently, genotype I have the largest number of available sequences, 53 strains including 20 strains reported in the present study. Genotype II contains 43 strains including nine strains described here. Genotype III has eight strains including four new strains, and genotype IV possesses only the two strains previously reported (Fig. 2). Despite, the partial genomes these strains have been previously described, here we reported the geographical distribution of complete coding sequence to all three segments, which includes strains of genotype I from Pará and Maranhão States. Furthermore, we described the nearly complete sequences of strains of genotype II from Central-North of Pará and Rondônia States. Also, strains of genotype III were reported in Rondônia, Acre, Maranhão, and Minas Gerais states. Finally, we described nearly complete sequences of strains classified into genotype IV from Amazonas State (Fig. 3).

3.4. RT-nested PCR and Real-Time RT-PCR assay

To discriminate reassortment events among OROV species, we standardized an RT-nested-PCR and RT-qPCR adapted from previous protocols using a specific set of primers and probes to M segment of OROV non-reassortment and reassortment. The specific amplification for the viruses were obtained by RT-nested-PCR (Supplementary Fig. 1).

The limits of detection were determined considering a reaction volume of 25 μL as follow: (i) many OROV strains (non-reassortment) were 5.9×10^{2} copies/μL (14,750 copies/reaction; dilution 10^{-4}) in RT-PCR, 5.9 copies/μL (147.5 copies /reaction; dilution 10^{-6}) in nested-PCR and 0.59 copies/μL (14.75 copies/reaction; dilution 10^{-7}) in RT-qPCR, (ii) MDDV strain TVP 19255 were 6.1×10^{2} copies/μL (15,250 copies/reaction; dilution 10^{-3}) in RT-PCR, 6.1 copies/μL

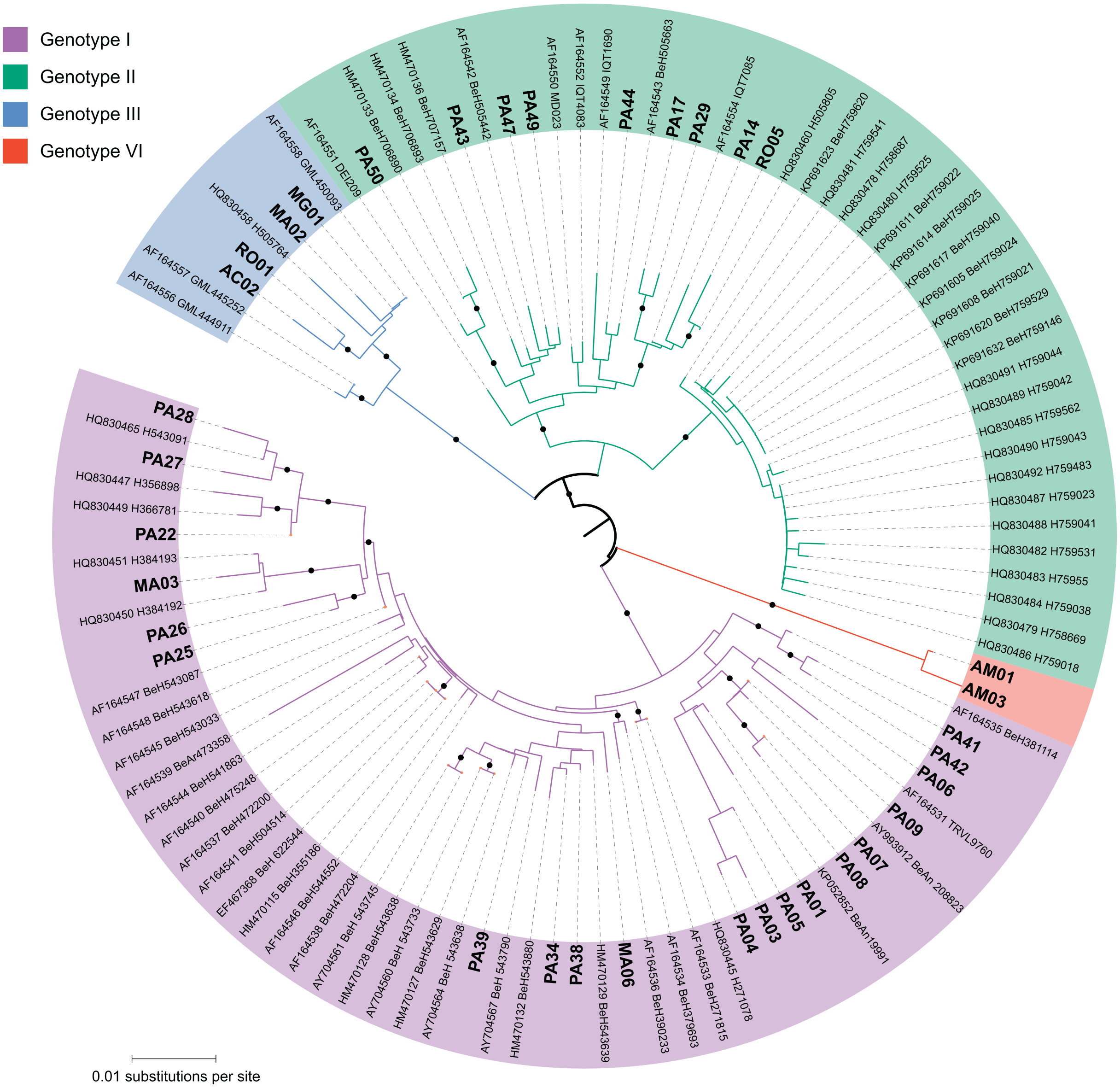

Fig. 2. NJ tree based on the complete nucleotide sequences of the S segment of 106 OROV strains. Phylogeny tree is midpoint rooted for clarity of presentation. The scale bar indicates evolutionary distance in numbers of nucleotides substitutions per site, and the principal bootstrap support levels were indicated with black circle. Branches are color-coded according to genotype. OROV strains sequenced in this study are highlighted with name bold.

(152.5 copies/ reaction) to nested-PCR and 0.61 copies/μL (15.25 copies/reaction; dilution 10^{-5})in the RT-qPCR; (iii) IQTV strain TVP 19261 were 4.01×10^{2} copies/μL (10,025 copies/reaction; dilution 10^{-4}) in RT-PCR, 4.01 copies/μL (100.25 copies/ reaction) in nested-PCR and 0.4 copies/μL (10.25 copies/ reaction) in RT-qPCR; (iv) PERDV strain BeAn 789,726 were 3.71×10^{2} copies/μL (9275 copies/ reaction; dilution 10^{-4}) in RT-PCR, 3.71 copies/μL (92.75 copies/reaction; dilution 10^{-6}) to nested-PCR and 0.371 copies/μL (9.28 copies/ reaction; dilution 10^{-7}) in the RT-qPCR. The sensitivity and specificity to nested-PCR and RT-qPCR were calculated using samples listed in Supplementary Table 2. Nested PCR, 33 out 35 true positives were detected (2 false negatives) and 42 out 43 were negatives (one false positive). On another hand, all true positives were detected by RT-qPCR, and no false positive results were observed within the true negative samples. Based on these results the sensitivity and specificity were 94.3% and 97.6% to Nested-PCR, and 100% to RT-qPCR, respectively.

4. Discussion

In this study, complete coding sequences of 35 OROV isolates from Brazil were obtained with relatively low coverage in comparison to data generated by NGS (mean of 89× to SRNA, 56× to MRNA and 67 x to LRNA). However genomes assembled with coverage over 30× are acceptable and can be assumed as accurate enough to avoid assembling errors (Ajay et al., 2011). Thus, we used these genomes for phylogenetic and genomic analysis, and no major genomic differences were observed with those previously described (Travassos Da Rosa et al., 2017). The unique exception was found in the genome of isolate PA01, which has an atypical ORF to NSs with 339 nt (113 aa) in length, including 17 amino acids longer than other OROV strains. This result was confirmed by specific PCR amplification and Sanger sequencing shown the accuracy of the assembled genome. Currently, NSs protein is described as important in interferon antagonism in Oropouche infection (Tilston-Lunel et al., 2015a). Therefore, further studies using reverse genetics could be directed to elucidate the role of this atypical protein.

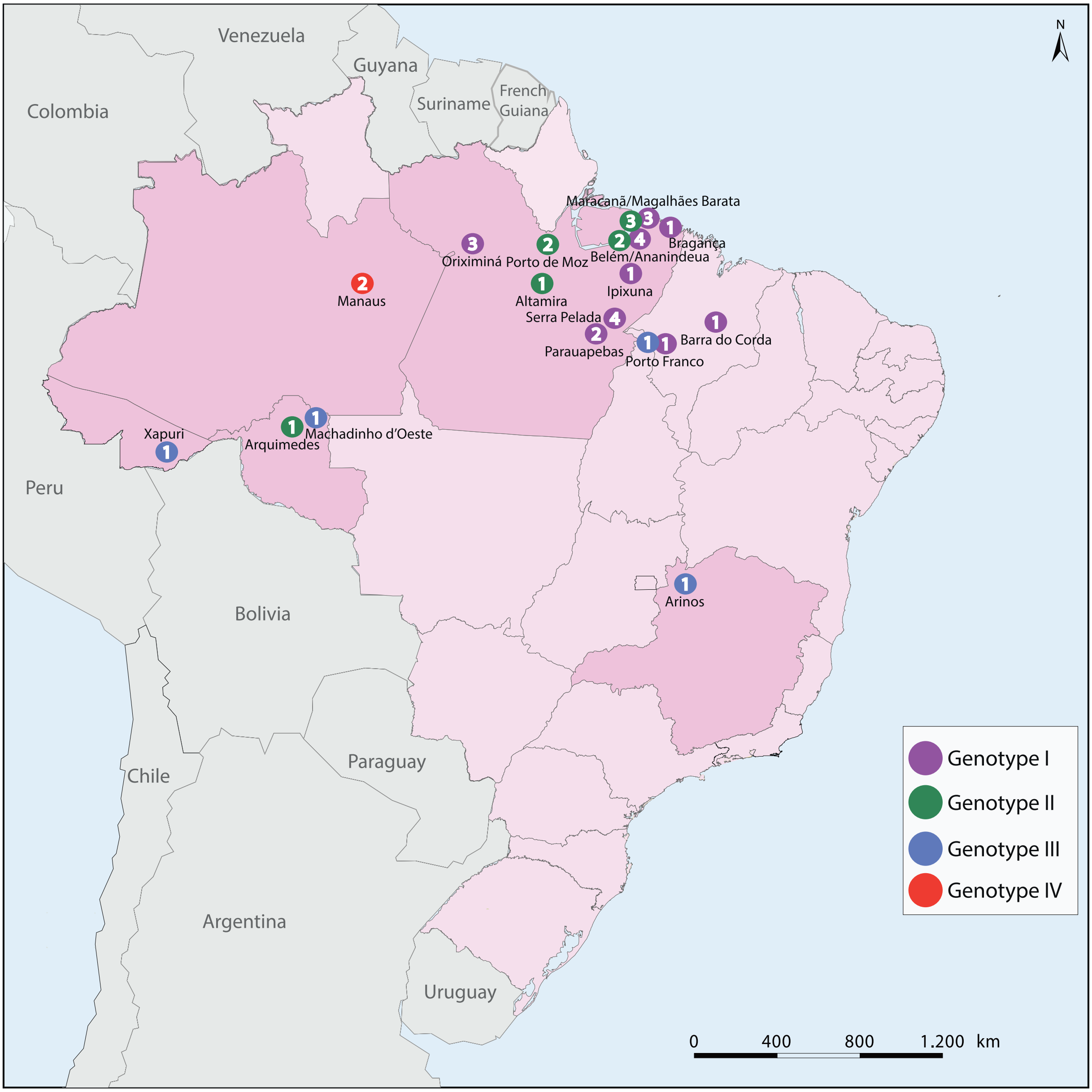

Fig. 3. Geographical distribution of genotypes of 35 strains OROV sequenced. The numbers inside the circles represents the number of isolates sequences within each genotype in the region showed.

Phylogenetic analysis based on S segment with complete coding sequences of nearly full genomes, or only the strains that have the S segments sequenced, confirms that OROV strains are split into four genotypes, as previously reported (Vasconcelos et al., 2011). The OROV have been described in Brazil, Trinidad and Tobago, Panama, Venezuela, and recently in Argentina (Azevedo et al., 2007; Nunes et al., 2005; Travassos da Rosa et al., 2017; Vasconcelos et al., 2009). Here, our results described the complete coding sequences of strains with wide broad geographical distribution; (i) genotypes I and II in Maranhão State; (ii) genotype III in Acre and Minas Gerais States; (iii) genotype IV in Amazonas State. These results support the previously published data regarding OROV circulation outside the states of the Brazilian Amazon, such as Maranhão and Minas Gerais States, as previously described (Tilston-Lunel et al., 2015b; Vasconcelos et al., 2011). The presence of arboviruses or viral genotypes produces a high impact on public health on a naïve population in new areas, such as *Dengue virus*, *Chikungunya virus*, and more recently *Zika virus* in Latin America (Guzman et al., 2010; Musso and Gubler, 2016; Nunes et al., 2015). Therefore, epidemiological surveillance should be carried out to evaluate the impact of the occurrence of novel genotypes or reassortment viruses in these regions, mainly in areas where OROV has not been previously described or where the population is naïve for this virus.

The genotyping of OROV strains has been performed based on complete coding sequences of S segment (Vasconcelos et al., 2011), and the presence of four genotypes were confirmed. Phylogenetic analyses were also performed with complete coding sequences of M and L segments, and although they have phylogenetic signal, it was noted that results using these segments could not currently discriminate the OROV genotypes. Thus, based on this observation, we assumed that the genotyping of OROV species could be based only on complete coding sequences of S segments, as previously described (Vasconcelos et al., 2011). However, the complete coding sequences of all genomes of OROV species is essential to discriminate potential reassortment events, which are frequently observed among members belonging to the genus *Orthobunyavirus*, such as IQTV in Peru, PERDV in Brazil and MDDV in Venezuela (Aguilar et al., 2011; Navarro et al., 2016; Tilston-Lunel et al., 2015b). Also, we did not find evidence of reassortment events in the OROV strains sequenced in this study. However, further studies may help clarify the frequency of these events.

Currently, OROV, MDDV, and IQTV have been reported as causing human disease, and unique MRNA segments have been reported to these viruses (Aguilar et al., 2011; da Rosa et al., 2017; Navarro et al., 2016). Therefore, we describe novel approaches to RT-PCR, RT-nested-PCR, and RT-qPCR to discriminate these unique RNA types with specific sets of primers targeting the M segment of these viruses. The limit of detection was calculated from 59 RNA copies per ml to 5.9 RNA copies per ml in RT-nested-PCR and RT-qPCR, respectively. The sensitivity results are similar to other arboviruses, such as *Yellow fever* and *Dengue virus* (Lanciotti et al., 1992; Nunes et al., 2011) while showing high specificity and no other orthobunyavirus was amplified by any of the assays. Only two known positive samples were negatives by RT-PCR, and one known negative sample was positive in the nested assay. These suggest that RT-PCR assay can fail due to its highest limit of detection in comparison to RT-nested and RT-qPCR, as well as that possible contamination can occur during the nested assay if multiple samples are handled at the same time. On the other hand, RT-qPCR showed 100% sensitivity and 100% specificity. In conclusion, our study provides a better understanding of the genomic and epidemiologic of OROV in Brazil while providing a new set of standardized tools to maintain an active surveillance of it.

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