



Research Paper

Genomic characterization of orthobunyavirus of veterinary importance in America

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ABSTRACT

During 2013, in Argentina, three new isolates of serogroup Bunyamwera virus (genus *Orthobunyavirus*, family *Peribunyaviridae*) were recovered from two horses with encephalitis, and from an aborted equine fetus. In the present study, we report the complete genome sequence, genetic characterization, and phylogenetic analysis of three new strains isolated in Argentina to clarifying their relationship within the Bunyamwera serogroup virus and to investigate the evolutionary history of viruses with segmented genomes.

1. Introduction

The *Peribunyaviridae* family (Order *Bunyavirales*) includes arthropod-borne viruses composed of 53 viral species and classified into two genera, the *Orthobunyavirus* and *Herbevirus* genus (King et al., 2018). Orthobunyaviruses are lipid-enveloped viruses with three distinct segments negative-stranded RNA genomes (ssRNA), which are called as large (L), medium (M), and small (S) (Walter and Barr, 2011). *Orthobunyavirus* genus is composed of a wide range of viruses, including some viruses that cause human and veterinary diseases (Elliott and Schmaljohn, 2013). Based on serological relationships of complement fixing antibodies, as well as hemagglutinating assay results and neutralizing antibodies the majority of the orthobunyaviruses have been classified into 18 serogroups, and some viruses remain ungrouped based on serologic relatedness (Elliott and Schmaljohn, 2013).

Bunyamwera serogroup is one of the most important serogroups in *Orthobunyavirus* genus because of it can cause disease in both livestock animals, as well as the pose a potential threat to human public health (Noronha and Wilson, 2017; Dutuze et al., 2018). This group includes two viral species, the *Kairi orthobunyavirus* with only one subtype and the *Bunyamwera orthobunyavirus* species that according to the ICTV have many subtypes, such as Cache Valley (CCV), Fort Sherman (FSV),

Guaroa (GROV), Ilesha (ILEV), Maguari (MAGV), Playas (PLAV), Tensaw (TENV), Batai (BATV), Ngari (NRIV) virus among other. These viruses are spread in many countries in Africa, America, and Europe (Elliott and Schmaljohn, 2013; King et al., 2018). In Argentina, two strains related to the *Bunyamwera orthobunyavirus* were isolated: the Laguna Larga strain CbaAr426 isolated from *Ochlerotatus albifasciatus* mosquitoes collected in Cordoba province between 1964 and 1965 (Bianchini et al., 1968), and the Maguari strain AG83–1746 from *Psorophora varinervis* mosquitoes collected in Santa Fe province in 1982. Both viruses were classified by serology as Cache Valley virus (CVV), isolated by the first time in 1956 in Utah, United States of America (Mitchell et al., 1987).

In 2013, in Santa Fe province three new orthobunyaviruses were isolated, two from horses with encephalitis (SFCrEq231 and SFBzEq232), and another (SFABEq238) from an aborted equine fetus (Tauro et al., 2015). Phylogenetic analyses using the partial sequence of the S segment revealed that those viruses belongs to the Bunyamwera serogroup and are closely related to *Laguna Larga virus* strain CbaAr426 isolated from mosquitoes in Argentina and FSV strain 86MSP18 isolated from a patient with meningitis from Panama (Tauro et al., 2015). However, the taxonomy of orthobunyaviruses has been a complex issue, mainly due to the cross-reaction in serologic assays, as well as the

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occurrence of natural reassortant viruses, which may be more related to members of one group or another depending on the assay used (Elliott and Schmaljohn, 2013). Therefore, the aim of this study was to determine the complete genome sequences of the three new viruses of the Bunyamwera serogroup isolated in Argentina in 2013 in order to conduct genomic and phylogenetic analyses to elucidate the evolutionary and taxonomy aspects of these new isolates into the Bunyamwera serogroup based on previously established criteria for species demarcation.

2. Materials and methods

2.1. Virus propagation and RNA extraction

An aliquot of 0.1 mL of each the isolates SFCrEq231, SFBzEq232, and SFABCrEq238 were propagated in Vero cells (African green monkey kidney cells) monolayers. The infected cells were incubated for 5–7 days until the visualization of viral cytopathic effect. Culture suspensions were centrifuged 11,600 rtf for 15 min and treated with 50% polyethylene glycol 8000 and 23% NaCl for viral precipitation. After centrifugation, viral pellets were eluted in 60 ul of RNase-free water and used for RNA extraction. Samples were subjected to a TRIZOL separation step and, the aqueous phase was further processed using Qiampr Viral RNA Minikit (Qiagen, Germany) according to the manufacturer's instructions.

2.2. Genome sequencing and assembling

Viral genomes from the isolates SFCrEq231 and SFBzEq232 were recovered using the pyrosequencing method while the genome of the strain SFABCrEq238 was sequenced by the Ion semiconductor technique. Briefly, regardless the method used, the genomes were obtained based on the following steps: (a) Reverse Transcription for cDNA synthesis using random hexamer primers and the cDNA Synthesis kit (Roche Life Science); (b) library construction by a second-strand cDNA synthesis followed by emulsion PCR; and (c) nucleotide sequencing using the pyrosequencing implemented in the GS FLX 454 (Roche Life Science) as previously described (Margulies et al., 2005) and the ion semiconductor method (Gill et al., 2013) implemented in the Ion Torrent (Life Technologies). In addition, the 3' and 5' non-coding regions (3' and 5' NCRs) were obtained using both the 3' and 5' rapid amplification of cDNA ends (RACE) methods applying a specific set of primers (Table 1). The reads generated by both GS FLX 454 and Ion Torrent instruments were assembled by using a *De novo* DNA assembling method implemented in the MIRA software (Chevreux et al., 2004) was used. Adaptors were first trimmed from generated reads and

then assembled to generate contigs. Parameters were left at default. Sequences were evaluated for homopolymers before generating the final genome sequence.

2.3. Genomic characterization

The genome size, open reading frame (ORF) descriptions, 5' and 3' terminal NCRs, as well as conserved motifs, and cleavage sites were determined using the Geneious R9, 1.2 (Biomatters, New Zealand) and InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan5/>).

2.4. 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 Bunyamwera serogroup with complete coding sequences (S, M, and L) available in the GenBank database (<http://www.ncbi.nlm.nih.gov/>) until 16th of April of 2018. The multiple sequencing alignments (MSAs) were carried out using PROMALS and RevTrans 2.0 and inspected with Geneious v9.1.8 (Pei and Grishin, 2014; Wernersson and Pedersen, 2003). The phylogenies were inferred by IQ-TREE version 1.4.3 software using the best-fit model based on Bayesian Information Criterion (Kalyaanamoorthy et al., 2017; Nguyen et al., 2015), and the nucleotide substitution model used was TPM2u + I + G4 to S segment and TIM3 + I + G4 to M and L segments. To amino acids substitution model was used LG + G4 to nucleoprotein (N), FLU + F + I + G4 to glycoprotein precursor (GPC), and LG + F + I + G4 to RNA-dependent RNA polymerase (RdRp). Statistical supports for individual nodes were estimated using the bootstrap value using 1000 replicates. The phylogenetic trees were visualized using the FigTree software v.1.4.2. In addition, the nucleotides and amino acid distances among viruses of Bunyamwera serogroup were estimated with segment S, M, and L using the p-distance values. Standard error estimations were calculated by bootstrapping method (1000 replicates) using the MEGA v.6 program (Tamura et al., 2013).

2.5. Reassortment analysis

The potential reassortment events were identified based on evidence of distinct phylogenetic topologies of trees at the nucleotide level. The genes that encode N, GPC, and RdRp were concatenated in a single sequence and an MSA was performed using the program RevTrans 2.0, as previously described. Potential reassortment events were then analyzed using the RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan and 3Seq methods implemented in the RDP4 package (Gill et al., 2013). Default program settings for all methods were used to perceive

Table 1

Set of primers used for 5' and 3' non coding regions recovering and sequencing using the RACE method.

Method	Segment	Primer	Sequence 5'-3'	Tm ^a	Position	
5' RACE	SRNA	GSp1-SRNA	TCTCAAGTAGGTACCGGGCA	60	384–365	
		GSp2-SRNA	GTTCCGGTTGCCAGGAAAAT	59	314–295	
		GSp3-SRNA	GCCCCAAGGTTAAGCGTAA	60	269–250	
	MRNA	GSp1-MRNA	GAAGTCAGGGTTGTGCCAGA	60	466–447	
		GSp2-MRNA	TCTGAGCCTCCTCTTTGTCT	57	408–389	
		GSp3-MRNA	TGGTACTGGGTTGCAAAGCT	59	293–274	
	LRNA	GSp1-LRNA	GGATGGGTGGTTAACTCTGGA	59	662–642	
		GSp2-LRNA	CACCATGGTGTGTCAATGT	58	629–610	
		GSp3-LRNA	GCGATCTCAGTAGGGATCCC	50	428–408	
	3' RACE	SRNA	GSp1-SRNA	GAGGCAGAGATACATGGGCT	58	611–630
			GSp2-SRNA	ATGGACAGTCAGCAAGGTGA	58	644–663
		MRNA	GSp1-MRNA	AGTCTGCTCTGTGAGGGAC	59	3890–3909
GSp2-MRNA			TTGCAAGACAAAGCCAGGAC	59	3977–3996	
LRNA		GSp1-LRNA	TTGCAATGCTATTGGAGGGC	58	6482–6501	
		GSp2-LRNA	CATGTTCTCCTTGCCATCGG	59	6519–6539	

^a Tm: temperature of melting; GSp: gene specific primer.

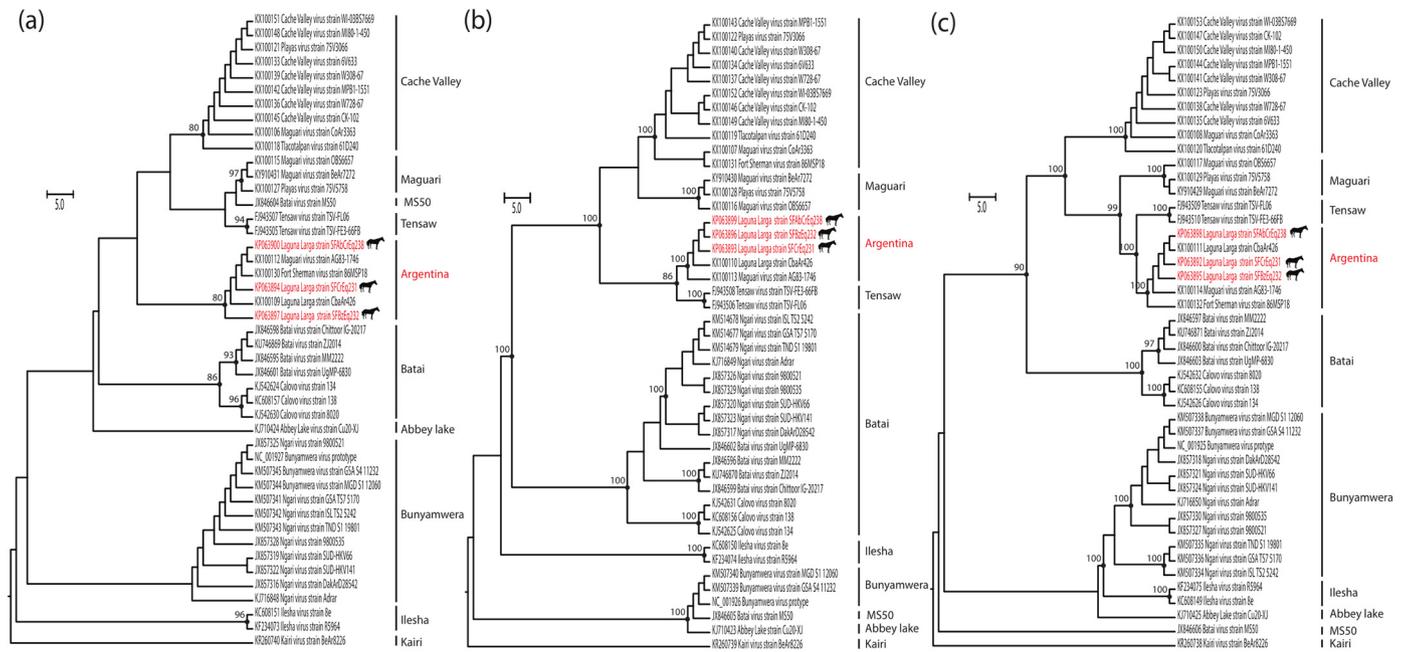


Fig. 2. Maximum likelihood phylogenetic trees based on alignments of amino acids sequences of Bunyamwera group. (a) The nucleoprotein was based on LG + G4 amino acids substitution model. (b) The Glycoprotein precursor was based on FLU + F + I + G4 amino acids substitution model. (c) The RNA-dependent RNA polymerase was based on LG + F + I + G4 amino acids substitution model. Phylogenies are midpoint rooted for clarity of presentation. The scale bar indicates evolutionary distance in numbers of substitutions per amino acids substitutions/site, and the principal bootstrap support levels were indicated. The branches were highlighted according to viral lineages. Viruses strains sequenced in this study are highlighted with red color and horse silhouette. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sequences as linear, to require phylogenetic evidence, to refine break-points and to check alignment consistency. The highest acceptable *P* value was set at 0.05, after considering Bonferroni correction for multiple comparisons. All method-specific program settings remained at their default values.

3. Result

The genome sequences obtained in this study were deposited in the GenBank database under the following accession numbers (S segment, M segment, and L segment): KP063894, KP063893, and KP063892 (SFCrEq231); KP063897, KP063896, and KP063895 (SFBzEq232); KP063900, KP063899, and KP063898 (SFAbCrEq238), and the obtained genomes possess a typical organization of orthobunyaviruses.

The SRNA segments showed to be 948 nt in length with an Open Reading Frame (ORF) of 702 nucleotides (nt), which encodes the N protein with 233 amino acids (aa) and NSs protein in frame +1 with 101 aa. The MRNA segments were determined in 4453 nt in length with a single ORF of 4311 nt that encodes a GPC protein with 1436 aa. The LRNA segments were 6883 nt with a single ORF of 6717 nt in length that encodes RdRp protein of 2238 aa. Also, we have identified in RdRp the conserved polymerase activity domains consisting of Pre-Motif A and Motifs A through E, which are domains highly conserved in RdRp in *Bunyavirales* order (Gill et al., 2013). Evidence that the complete sequences were recovered for each of the three RNA segments was confirmed by the presence of the eight conserved complementary terminal sequences (5'-AGTAGTGTA and [C/T] AACTACTT) AACT ACT-3') using the RACE 5' and RACE 3' protocol.

Based on the alignments and phylogenetic reconstructions at amino acids levels using the complete coding sequences for all segments of the isolates SFCrEq231, SFBzEq232, and SFAbCrEq238 and selected viruses of the Bunyamwera serogroup, 10 different groups were assigned.

For the S segment nucleoprotein, the isolates SFCrEq231, SFBzEq232, and SFAbCrEq238 clustered with MAGV strain CbaAr426 (KX100111), also named Laguna Larga, MAGV strain AG83–1746

(KX100114), and FSV strain 86MSP18 (KX100132) sharing 99.1 to 100% amino acids identity (Fig. 1).

By the segment M polyprotein, 10 groups were also assigned. This time, the isolates SFCrEq231, SFBzEq232, and SFAbCrEq238 clustered only with Laguna Larga strain CbaAr426 (KX100111) and MAGV strain AG83–1746 (KX100113) sharing 99.1% to 100% amino acid identities. FSV clustered with CCV isolates (Fig. 1).

By the segment L polyprotein, the isolates SFCrEq231, SFBzEq232, and SFAbCrEq238 grouped as observed in phylogenetic tree of nucleoprotein, and sharing 99.1% to 100% amino acid identities with Laguna Larga strain CbaAr426 (KX100111), MAGV strain AG83–1746 (KX100114) and FSV strain 86MSP18 (KX100132) (Fig. 1).

The phylogenetic analysis using complete ORF's showed that the strains SFCrEq231, SFBzEq232, and SFAbCrEq238 are clustered with Laguna Larga strain CbaAr426 and Maguari strain AG83–1746 in all three RNA segments (Fig. 2). Collectively, the data suggest that the SFCrEq231, SFBzEq232, SFAbCrEq238, Laguna Larga strain CbaAr426 and Maguari strain AG83–1746 shared the same common ancestor, as well as are strains of the same lineage within the Bunyamwera serogroup, hereafter named as Argentina lineage. In addition, we have combined the phylogenetic analysis with RDP4 analyses (Martin et al., 2015) and confirmed that strains reported in this study are not resultant of reassortment phenomenon.

4. Discussion

In this study, we showed that the strains associated to encephalitis and fetus abortion in equine in Argentina in 2013 are genetically closely related with the viruses that were described in *Ps. varinervis* and *Ae. albifasciatus* mosquitoes collected in Argentina since 1965 (Elliott and Schmaljohn, 2013; Sexton et al., 1997). Interestingly, the strains SFCrEq231, SFBzEq232, and SFAbCrEq238 presents a putative ORF to encode the NSs protein. The NSs protein of orthobunyaviruses is interferon antagonist, which can result in increased of virulence and pathogenesis in mammal infections as observed in horse infections by

this virus in 2013 (Walter and Barr, 2011). So far, the unique members of Bunyamwera serogroup that have been reported to cause infection in horses are CCV and Northway virus (NORV)(Campbell et al., 1990; McLean et al., 1987).

Recently, the International Committee on Taxonomy of Viruses (ICTV) have been endorsed the used of HTS approach associated with robust framework for viral taxonomy as described in this study (Simmonds, 2015; Simmonds et al., 2017). However, based on current taxonomy proposal for *Bunyavirales* order (Maes et al., 2019), only *Bunyamwera orthobunyavirus* and *Kairi orthobunyavirus* are recognized at the viral species level by ICTV in Bunyamwera serogroup (King et al., 2018). However, our results and previous study have been shown the higher divergence identity and abundance of lineages within the Bunyamwera orthobunyavirus specie, which suggests the presence of at least ten different lineages based on phylogenetic analysis (Sexton et al., 1997). A recent study has named the Laguna Larga strain CbAr 426 and CCV strain AG83–1745 as MAGV, which including the FSV constituted the Córdoba lineage (Groseth et al., 2017). However, these viruses have a high amino acids divergence related to the MAGV and CVV lineages (> 7%), as well as to the strains described in this study that clustered with Laguna Larga strain CbaAr 426 and CCV strain AG83–1745. Therefore, we proposed that Laguna Larga strain CbAr426 and CCV strain AG83–1745 are not called of MAGV, since > 7% amino acid divergence was demonstrated in this study. Also, this group has been initially named Cordoba lineage, however to avoid misleading with a previous named Córdoba negevirus (Nunes et al., 2017) we proposed the name of Argentina lineage.

In sum, the obtaining of complete sequences for viruses into the Argentina lineage could help in the future to develop or improve methods for diagnosis, as well as it appropriate classification as a possible new lineage within the Bunyamwera serogroup. Our results also highlight the importance of full-genome analyses to drive correct taxonomy classification and to investigate the evolutionary history of viruses with segmented genomes.

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Conflict of interest

The authors declare that they have no competing interests.

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