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Detection of *Orthobunyavirus* in mosquitoes collected in Argentina

L. B. TAURO[1], G. P. BATALLAN[1,2], M. E. RIVAROLA[1], A. VISINTIN[3,4], C. I. BERRÓN[1], E. C. SOUSA Jr.[5], L. A. DIAZ[1,6], W. R. ALMIRON[4,6], M. R. NUNES[7] and M. S. CONTIGIANI[1]

[1]Laboratorio de Arbovirus, Instituto de Virología Dr J. M. Vanella, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [2]Departamento de Ciencias Básicas y Tecnológicas, Instituto de Ambiente de Montañas y Regiones Áridas, Universidad Nacional de Chilecito, Chilecito, Argentina, [3]Cátedra de Biología Animal, Departamento de Ciencas Exactas, Físicas y Naturales, Universidad Nacional de La Rioja, La Rioja, Argentina, [4]Cátedra de Entomología, Centro de Investigaciones Entomológicas de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina, [5]Seção Virologia, Instituto Evandro Chagas, Ministério da Saúde, Belem, Brazil, [6]Instituto de Investigaciones Biológicas y Tecnológicas (IIByT–CONICET), Córdoba, Argentina and [7]Centro de Inovações Tecnológicas, Instituto Evandro Chagas, Ministério da Saúde, Belem, Brazil

Abstract. Bunyamwera virus (BUNV) (Bunyaviridae, genus *Orthobunyavirus*, serogroup Bunyamwera) is considered an emerging pathogen for humans and animals in American countries. The CbaAr-426 strain of BUNV was recovered from mosquitoes *Ochlerotatus albifasciatus* (Diptera: Culicidae) collected in Córdoba province (Argentina), where serological studies detected high seroprevalences in humans and animals. Molecular detection of *Orthobunyavirus* was performed in mosquitoes collected in Córdoba province. Seventeen mosquito pools of *Oc. albifasciatus*, *Ochlerotatus scapularis* and *Culex quinquefasciatus* (Diptera: Culicidae) showed positive results; four of these positive pools, all of *Oc. scapularis*, were sequenced. All amplicons grouped with BUNV in the Bunyamwera serogroup. The findings highlight the circulation of BUNV in Córdoba province and represent the first report of BUNV-infected *Oc. scapularis* mosquitoes in Argentina.

Key words. Bunyaviridae, *Orthobunyavirus*, Bunyamwera virus, mosquitoes, Argentina, Córdoba.

Introduction

Bunyamwera virus (BUNV) is a mosquito-transmitted virus of the Bunyamwera serogroup of the genus *Orthobunyavirus* (Bunyaviridae) widely distributed throughout both hemispheres (Plyusnin *et al.*, 2012). Originally, BUNV was isolated from *Stegomyia* spp. (Diptera: Culicidae) from Uganda (Africa) (Smithbum *et al.*, 1946); in America, the first *Orthobunyavirus* closely related to BUNV was Cache Valley virus (CVV), isolated in 1956 from *Culiseta inornata* (Diptera: Culicidae) (Holden & Hess, 1959). Since 1998, according to the classification of the International Committee of Taxonomy of Viruses, CVV has been considered as a strain or isolation of BUNV (Van Regenmortel *et al.*, 2000). Several strains of BUNV are responsible for nervous and febrile syndromes in humans and teratogenic effects and abortions in domestic animals, mainly ruminants (Chung *et al.*, 1990; Tauro *et al.*, 2012; Nguyen *et al.*, 2013). All viruses in the genus *Orthobunyavirus* possess a tripartite, single-stranded and negative-sense RNA genome (Schmaljohn & Nichol, 2007). The three genomic segments are designated as, respectively, small (S), medium (M) and large (L). The S segment encodes for the nucleocapsid and a non-structural

Correspondence: Laura B. Tauro, Laboratorio de Arbovirus, Instituto de Virología Dr J. M. Vanella, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Enfermera Gordillo Gómez S/N Ciudad Universitaria, Córdoba CP X5016 GRA, Argentina. Tel.: +54 351 4334022; E-mail: laurataur o@gmail.com

protein, NSs. The M segment encodes for the glycoproteins Gn and Gc, and the L segment encodes the viral polymerase.

In Argentina, CbaAr-426 strain (previously classified as CVV subtype Laguna Larga) was the first BUNV isolated from *Ochlerotatus albifasciatus* mosquitoes in Córdoba province during 1964 and 1965 (Bianchini *et al*., 1968). Subsequently, in 1981 the AG83-1746 strain (previously classified as CVV subtype Maguari), which is closely related but not identical to CbaAr-426 BUNV, was isolated from *Psorophora varinervis* (Diptera: Culicidae) in Santa Fé province (Mitchell *et al*., 1987). Recently, the first case of human febrile syndrome attributable to BUNV (CbaAr-426 strain or a closely related strain) was detected in Córdoba city (Tauro *et al*., 2012). Another *Orthobunyavirus* that circulates in Argentina is Kairi virus (KRIV). Two strains were isolated, one from *Stegomyia* spp. in 1966, at the same place in Córdoba from which BUNV (CbaAr-426) had been recovered in the previous year (Sabattini *et al*., 1998), and the other from a febrile horse in Carmen de Patagones, Buenos Aires province (Calisher *et al*., 1988). KRIV shares epitopes with BUNV (CbaAr-426), which leads to cross-reactions (Casals & Whitman, 1960). Specific antibodies against BUNV and KRIV have been detected in humans, domestic animals and wildlife in Córdoba province (Cámara *et al*., 1990; Tauro *et al*., 2009a, 2009b). Therefore, the aim of this study was to investigate the circulation of *Orthobunyavirus* in mosquito populations collected in the central area of Argentina.

Materials and methods

Collection sites

Mosquito collection was carried out in Córdoba province (Argentina), particularly in Córdoba city (31°24′0′′S; 64°11′0′′W) and also in Altos de Chipión (30°54′0′′S; 62°18′0′′W) and La Para (30°53′35′′S; 63°00′00′′W). Both towns are located on the southern coast of Mar Chiquita lagoon in the northeast of the province (Fig. 1). Córdoba city is located in the central region of Argentina at 450 m a.s.l. and has a population of 1 330 000 inhabitants. It is characterized by a mixture of landscapes, including urban, suburban and rural areas. The climate is temperate, with a dry season in winter. Mean annual precipitation levels range between 750 mm and 800 mm (Jarsun *et al*., 2003). The collections were made at different sites around the city, which were selected to represent different levels of urbanization, from fully urbanized sites to others with more natural environmental characteristics. By contrast, Mar Chiquita lagoon is located in the northeast area of the Province of Córdoba and has a semi-arid subtropical monsoon climate with a warm temperature and relatively scarce periods of concentrated rainfall in the warm season (October–April) (Cabido & Zak, 1999). The southern coast of the lagoon represents a transition zone between the dry Chaco forest and the thorn forest. During recent years, the native forest has been substantially reduced by the advancement of agriculture and livestock, which represent the main economic activities in the area.

Sample collection

Mosquitoes were sampled with Centers for Disease Control (CDC) light traps baited with dry ice as a source of carbon dioxide (CO_2) and transported to the laboratory under cold conditions. Individuals were identified based on their morphological characteristics (Darsie, 1985) on a chill table and pooled into groups of up to 50 unengorged females according to species, collection date and site. Each pool was triturated in a mortar with minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% gentamicin. Samples were then stored at −70 °C until processing and afterwards centrifuged at 11 400 ***g*** for 30 min. Supernatants were stored at −70 °C until molecular detection was performed. Details related to field sampling activities are shown in Table 1.

Orthobunyavirus molecular detection and sequencing analyses

Viral RNA was extracted using Trizol reagent (Invitrogen BRL, Life Technologies, Inc., Rockville, MD, U.S.A.) according to the manufacturer's instructions. Reverse-transcriptase polymerase chain reaction (RT-PCR) for *Orthobunyavirus* (Bunyamwera and California serogroups) detection was performed, amplifying a fragment of 251 bp of the S segment using the primers BCS82C (5′ATGACTGAGTT GGAGTTTCATGATGTCGC3′) and BCS332V (5′TGTTCC TGTTGCCAGGAAAAT3′), as described by Kuno *et al*. (1996). Polymerase chain reaction products of the expected size were purified using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, U.S.A.) and sequenced in both directions using an ABI automatic sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.). The fragments obtained were edited using the program Geneious Pro Version 5.6 (Biomatters Ltd, Auckland, New Zealand; http://www.geneious.com/default.28.downloads.sm). Subsequently, the consensus sequences obtained were subjected to a BLASTn 2.2.19 (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov) (Zhang *et al*., 2000) to identify the degree of homology with data for other viruses of the *Orthobunyavirus* genus available in GenBank. Then, alignment of all sequences in the database was performed using MEGA Version 4.0 (Center for Evolutionary Functional Genomics, Tempe, AZ, U.S.A.) by means of the neighbour-joining method and p-distance model with bootstrapping 1000 times (Tamura *et al*., 2007).

Results

A total of 14 498 mosquitoes (478 pools) collected and identified in Córdoba province were analysed by RT-PCR (Table 1). Of a total of 17 mosquito species, *Culex interfor*, *Cx. quinquefasciatus*, *Culex saltanensis*, *Culex apicinus*, *Oc. albifasciatus* and *Ochlerotatus scapularis* were the most abundant. During the study period, 17 mosquito pools were found to be positive: 14 of these came from Córdoba city and 3 from La Para (Table 2).

Fig. 1. Sampling locations in the *Orthobunyavirus* investigation conducted in Córdoba province, Argentina.

Only four pools (PB-785, LP1-012, LP1-074, LP1-087) contained enough DNA load for sequencing (Table 1). According to the phylogenetic analysis, the sequences belonging to BUNV clustered with the Bunyamwera serogroup (Fig. 2). All *Orthobunyavirus* detected in the region of Mar Chiquita showed 100% nucleotide identity among themselves and 98% with the sequence obtained from mosquitoes of the same species caught in Córdoba city. The new sequences showed 95% nucleotide identity with the CbaAr-426 strain isolated in Córdoba province during the 1960s (Bianchini *et al*., 1968).

Discussion

By means of an RT-PCR technique generic for *Orthobunyavirus* (Bunyamwera and California serogroups) developed by Kuno *et al*. (1996), 17 pools of infected mosquitoes collected in Córdoba city (during the years 2003, 2009 and 2010) and on the southern coast of the Mar Chiquita lagoon (in 2008) were amplified. Infected mosquito species were *Oc. albifasciatus*, *Oc. scapularis* and *Cx. quinquefasciatus*. Only *Oc. albifasciatus* had been previously demonstrated to be infected with BUNV in Argentina (Bianchini *et al*., 1968). With reference to *Oc. scapularis*, several BUNV strains and other *Orthobunyavirus* such as KRIV have been recovered from this species in Brazil, Ecuador and Trinidad and Tobago (Anderson *et al*., 1960; Causey *et al*., 1961). It is important to note that other *Orthobunyavirus* included within the California serogroup have been detected in different species of *Culex* mosquitoes in several countries in South America (Newhouse *et al*., 1963). For instance, the AG83-497 California encephalitis virus strain was isolated from *Culex* spp. in Santa Fé province, Argentina, in 1985 (Mitchell *et al*., 1987).

Table 1. Summary of mosquitoes collected from Córdoba city and the southern coast of the Mar Chiquita lagoon (Altos de Chipión and La Para).

Place	Year	Months	Mosquitoes collected,*n*	Mosquitoes tested, *n*	Pools tested, *n*	Positive pools, *n*	Positive pools, %
Córdoba	2002	Nov and Dec	753	258	23	0	0
	2003	Jan–Apr	3468	3084	82	5	6.09
	2007	Dec	281	25	4	0	0
	2008	Jan–May and Aug–Dec	4960	322	58	0	0
	2009	Jan–May and Aug–Dec	7014	2380	113	8	7.07
	2010	Jan	1031	400	13	1	7.69
Altos de Chipión	2004	Dec	21.268	2077	46	0	0
	2006	Jan	24.711	3352	87	0	0
La Para	2008	Nov	4989	2600	52	3	5.76

Table 2. Pools of mosquitoes collected in Córdoba province and found positive by reverse-transcriptase polymerase chain reaction generic to *Orthobunyavirus.*

Code	Place	Month/year	Species	GenBank accession
2-103	Córdoba city	January/2003	*Culex quinquefasciatus*	—
2-169	Córdoba city	February/2003	*Culex quinquefasciatus*	—
2-171	Córdoba city	February/2003	*Ochlerotatus albifasciatus*	—
2-419	Córdoba city	April/2003	*Ochlerotatus albifasciatus*	—
2-434	Córdoba city	April/2003	*Ochlerotatus albifasciatus*	—
PB-418	Córdoba city	January/2009	*Culex quinquefasciatus*	—
PB-420	Córdoba city	January/2009	*Culex quinquefasciatus*	—
PB-553	Córdoba city	March/2009	*Ochlerotatus scapularis*	—
PB-749	Córdoba city	October/2009	*Ochlerotatus scapularis*	—
PB-785	Córdoba city	November/2009	*Ochlerotatus scapularis*	KM234272
PB-769	Córdoba city	November/2009	*Ochlerotatus scapularis*	—
PB-753	Córdoba city	November/2009	*Ochlerotatus albifasciatus*	—
PB-763	Córdoba city	November/2009	*Ochlerotatus albifasciatus*	—
PB-943	Córdoba city	January/2010	*Culex quinquefasciatus*	—
LP1-012	La Para	November/2008	*Ochlerotatus scapularis*	KM234269
LP1-074	La Para	November/2008	*Ochlerotatus scapularis*	KM234270
LP1-087	La Para	November/2008	*Ochlerotatus scapularis*	KM234271

To be considered a vector, mosquitoes are required to fulfil several ecological and biological criteria; for example, they must show competence to transmit the virus, they must show population abundance during virus activity, and they must demonstrate a preference for vertebrate hosts, among other criteria.

Ecological studies carried out in Córdoba city have helped us to understand the population dynamics of mosquito communities and thus to infer their environmental interactions with arboviruses. Studies by Diaz (2009) and Batallán (2013) conducted in Córdoba city determined that *Oc. albifasciatus* and *Cx. quinquefasciatus* were the most abundant species among the culicidae fauna and were widely distributed in the city. Moreover, climatologic variables were highly influential on their abundance. *Ochlerotatus* species are more abundant during the autumn, whereas *Culex* species frequently peak in late summer. In addition, *Ochlerotatus* species are particularly abundant after periods of heavy rain (Forattini *et al*., 2000; Gleiser *et al*., 2002). Unfortunately, it was not possible to sequence and characterize all RT-PCR positive pools and therefore specific identification was missed. However, viral activity recorded by the detection of infected mosquitoes coincided with the peak in abundance of infected species. In 2009, *Oc. scapularis*, which in 2003 was the least representative species and which had not been recorded as infected, turned out to be the second most abundant species (Batallán, 2013) and had the largest number of positive pools. Most of the infected mosquitoes (five of six) were collected during spring (October and November), a period that coincided with maximum abundance as a result of explosive population growth (Batallán, 2013).

In the region of the Mar Chiquita lagoon, the most abundant species during the study period were *Oc. scapularis* and *Oc. albifasciatus* (Visintin, 2012; Berrón, 2014). Only individuals of *Oc. scapularis* collected near the town of La Para in November 2008 were infected with BUNV. Bloodmeal identification studies showed that *Oc. scapularis* females collected at this site during the same period fed mainly on cattle (Berrón, 2014). Furthermore, high prevalences of BUNV infection were detected in the cattle of this area in recent years (Tauro & Contigiani, 2005).

The new BUNV amplicons circulating in the centre and northeast of Córdoba province represent the first record of infection by BUNV in *Oc. scapularis* mosquitoes in Argentina. The isolation of BUNV from *Oc. albifasciatus* (Bianchini *et al*.,

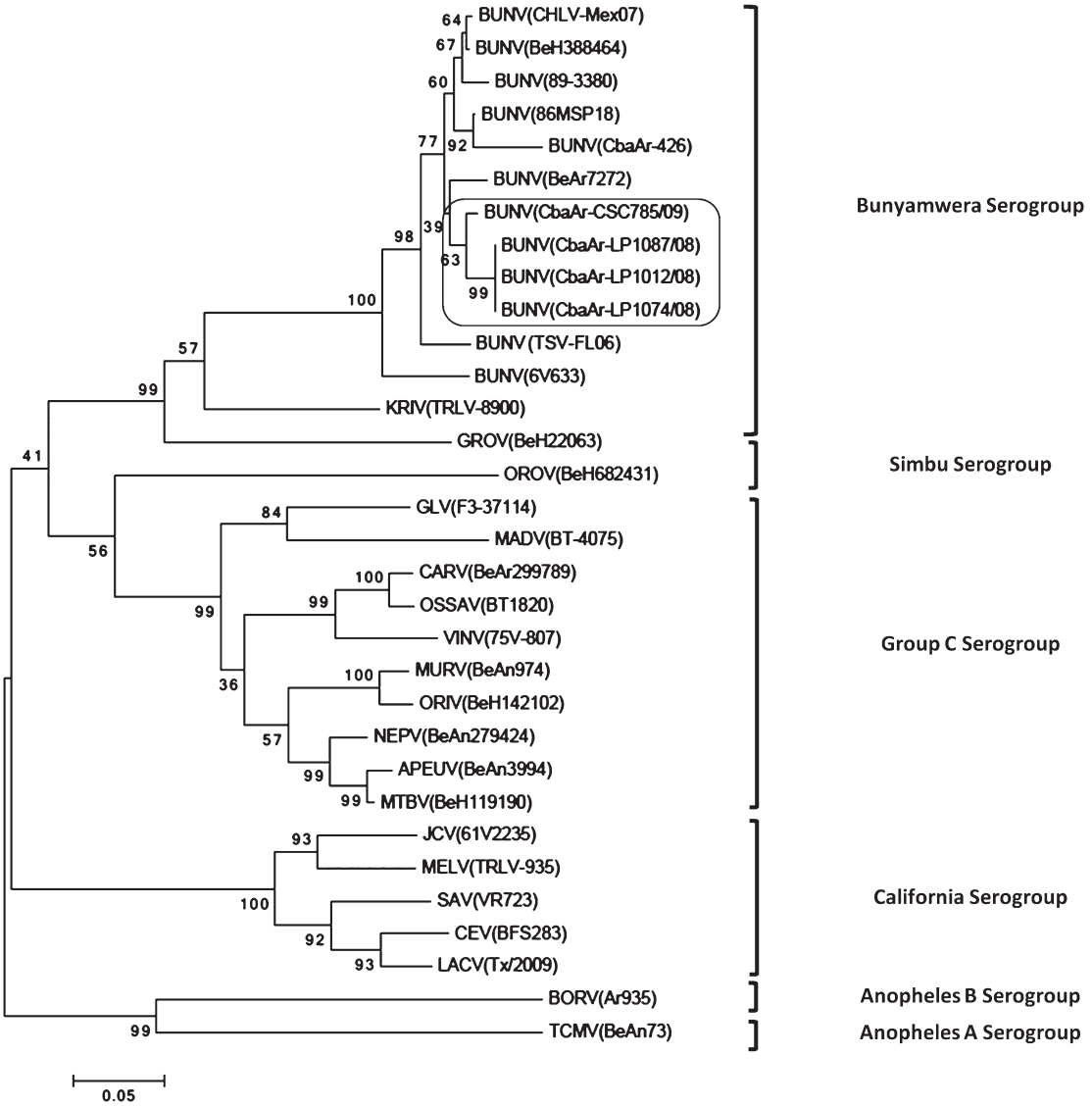

Fig. 2. Consensus tree generated from nucleotide sequences of a fragment of 239 bp of the S segment, using neighbour joining and the p-distance parameter. Virus of *Orthobunyavirus* genus detected in America belonging to serogroups Anopheles A, Anopheles B, California, Simbu, Group C and Bunyamwera were included. GenBank accession numbers for sequences of BUNV used in the phylogenetic analysis are as follows: CbaAr-LP1074/08 (KM234270); CbaAr-LP1087/08 (KM234271); CbaAr-LP1012/08 (Km234269); CbaAr-CSC785/09 (KM234272); 86MSP18 (EU564829); CbaAr-426 (KM205207); BeAr7272 (M28380); CHLV-Mex07 (EU879062); 89-3380 (AY72965.1); BeH388464 (EU5648430); 6V633 (X73465); TSV-FL06 (FJ943507), and 89-3380 (AY7296521). APEUV, Apeu virus; BORV, Boraceira virus; CARV, Caraparu virus; CEV, California Encephalitis virus; GROV, Guaroa virus; GLV, Gumbo Limbo virus; JCV, Jamestown Canyon virus; KRIV, Kairi virus; LACV, La Crosse virus; MADV, Madrid virus; MTBV, Marituba virus; MELV, Melao virus; MURV, Murucutu virus; NEPV, Nepuyo virus; ORIV, Oriboca virus; OROV, Oropouche virus; OSSAV, Ossa virus; SAV, San Angelo virus; TCMV, Tacaiuma virus; VINV, Vinces virus.

1968), the abundance of this species and *Oc. scapularis* in Córdoba province, and the molecular detection of virus infection in both species in the present study suggest that these mosquito species may act as vectors of these viruses in nature. Future studies of vector competence will be performed to elucidate the real roles of these mosquito species as vectors of BUNV.

These results, in addition to the serological evidence, demonstrate the need for constant virological and clinical surveillance in Córdoba.

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