Title: *Culex saltanensis* and *Culex interfor* (Diptera: Culicidae) are susceptible and competent to transmit St. Louis encephalitis virus (Flavivirus: Flaviviridae) in central Argentina.

Short title: *Culex* competent vectors for the SLEV

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Abstract

Infectious diseases caused by mosquito-borne viruses constitute health and economic problems worldwide. St. Louis encephalitis virus (SLEV) is endemic and autochthonous in the American continent. *Culex pipiens quinquefasciatus* is the primary urban vector of SLEV; however, *Culex interfor* and *Culex saltanensis* have also been found naturally infected with the virus, suggesting their potential role as vectors.

OBJETIVE The aim of this study was to determine the vector competence of *Cx. interfor* and *Cx. saltanensis* for SLEV from central Argentina in comparison to a *Cx. p. quinquefasciatus*.

METHODS Adult female mosquitoes of the three *Culex* species were orally infected by feeding on viremic chicks that had been inoculated with SLEV CbaAr-4005, it is the same strain detected in the US and Argentina, and responsible for human disease. Then, abdomens, legs and saliva blood-fed mosquitoes were analyzed by viral plaque assay and the presence of cytopathic effect on the cell culture monolayer.

RESULTS Mosquitoes were permissive to orally acquire infections, to virus dissemination, and transmission of SLEV in the saliva. *Culex saltanensis* and *Cx. interfor* are potential vectors of SLEV.

CONCLUSIONS Our results demonstrate that in Argentina both *Culex saltanensis* and *Culex interfor* are susceptible to SLEV and competent for its transmission. Moreover they are abundant during SLEV epidemic period in urban area, positive for this virus in nature, and found to feed on natural hosts.

Key words

Arbovirus, Argentina, Culicidae, *Flavivirus*, Infectious diseases, Vector competence.

Introduction

Infectious diseases caused by vector-borne pathogens constitute health and economic problems worldwide [1]. Mosquitoes are an important group of arthropod vectors. Due to the hematophagous habit of females, mosquito species are competent vectors of infectious agents, including viruses (arthropod-borne viruses; 'arboviruses') [2]. Arbovirus is maintained by biologic transmission among vectors and hosts. Sometimes this biological transmission is specific and includes one vector and host species such as Chikungunya (CHIKV), Dengue (DENV), urban Yellow Fever (YFV) and Zika viruses (ZIKV). However, most of the arbovirus is generalist and they use many vectors and hosts species such as St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) [3]. The emergence and reemergence of diseases caused by arbovirus are a global phenomenon, in particular, those caused by CHIKV, DENV, WNV and ZIKV [1].

SLEV is an endemic neurothropic flavivirus in temperate and subtropical areas of the New World that is maintained between multiple avian hosts and *Culex* vectors, although incidental infections are possible in humans and other mammals, which are typically dead-end hosts [4]. In 2002, SLEV reemerged in the central area of Argentina and southern Brazil causing neurological diseases in humans. In 2005, the first outbreak occurred in Córdoba City with 47 confirmed cases and nine fatalities. After the 2005 outbreak, additional SLEV outbreaks in Argentina occurred in Parana (2006), Buenos Aires (2010), and San Juan (2011) [5]. Factors that promoted this emergence in Argentina include the introduction of a more virulent SLEV strain into a highly susceptible avian hosts community along with possible land use changes (urbanization, agriculture) [5]. Phylogenetic analyses indicate that the emerging SLEV in US (2015) is related to the

epidemic strains isolated during a human encephalitis outbreak in Córdoba, Argentina, in 2005 [5].

SLEV, as similar to other arboviruses transmitted by mosquitoes, is dependent upon a complex interaction with the tissues of mosquito vectors. The virus-vector interaction is essential for its effective transmission and is influenced by both viral and vector components [6]. A prerequisite for the systemic and persistent infection of the vector is the pathogen being able to overcome all tissue barriers [6, 7].There are barriers in mosquitoes that affect the likelihood for viral transmission after ingesting an infectious bloodmeal [7]. Viruses enter the midgut epithelial cells (midgut infection barrier-MIB), replicate, exit the cells (midgut escape barrier-MEB), and travel through the hemolymph-filled hemocoel to the salivary glands (salivary gland infection barrier-SGIB), where they again replicate and reside followed by the subsequent transmission through the saliva (salivary gland escape barrier-SGEB). Mosquito vector competence [susceptibility, extrinsic incubation period (EIP), transmission proportions], longevity, and host bloodmeal preferences are included among intrinsic factors. Each of these factors is affected by extrinsic factors such as larval density and nutrition, temperature, rainfall, avian host availability and avian host immunity [7].

In Argentina, the Eared Dove (*Zenaida auriculata*) and Picui Ground-Dove (*Columbina picui*) are natural hosts of SLEV, while *Culex pipiens quinquefasciatus* is an efficient vector for the virus [3, 8]. For example, SLEV was detected and isolated in *Cx. p. quinquefasciatus* mosquitoes during an outbreak of encephalitis in humans in Córdoba City [9]. *Culex p. quinquefasciatus* have been detected during periods without reports of clinical disease symptoms in Santa Fe province between 1978-1983 [10] and Córdoba between 2001-2004 [11]. In addition viral isolations from field collected mosquitoes, and the

population abundance throughout the Córdoba City [9], laboratory studies have confirmed horizontal transmission [8, 12], and the infrequent vertical transmission of SLEV in *Cx. p. quinquefasciatus* [13]. The presence of naturally SLEV-infected *Culex interfor* and *Culex saltanensis*, dominant species in urban-vegetated sub-assemblage that frequently feed on competent hosts, suggest they could participate as vectors in the transmission network of SLEV [3, 8, 9, 14]. Based on these evidences we argue that *Cx. saltanensis* and *Cx. interfor* could be playing a role as vectors of SLEV in central Argentina; yet vector competence experiments have not been conducted on these species. Therefore, we evaluated the vector competence of *Cx. interfor* and *Cx. saltanensis* against SLEV from central Argentina compared to the primary urban vector, *Cx. p. quinquefasciatus*.

Materials and Methods

Capture and maintenance of mosquito colonies

Egg rafts of *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis* were collected during 2015 at the Bajo Grande sewage treatment plant (31°24'13"S 64°06'08"W) located in east of Córdoba City. The site is surrounded by aquatic vegetation, reservoirs, low income human settlements and crop lands (vegetables and fruits). Authorization for mosquito field collections was obtained from the Municipality of Córdoba.

Egg rafts were collected with a dipper (350 ml) and transferred to polypropylene bottles with a fine brush and transported to the Instituto de Virología "Dr. *J. M. Vanella*" Facultad de Medicina, Universidad Nacional de Córdoba (FM-UNC). Mosquitoes were maintained 27ºC, 70% humidity, and 12:12 h light: dark (L: D) photoperiodic. Adult F1 females from the field collected rafts were used in the vector competence assay (Table 1).

Egg rafts were placed in a plastic container with distilled water and hatched larvae were fed with suspension of liver powder (0.25 mg/0.5 ml distilled water) every 48 h. After identification, larvae were grouped by species, pupae of the same species were placed inside screened cages (26 x 22 cm) until emergence, and adults provided a 10% sugar solution by soaked cotton balls. Identification of adult females and 4th instars were based on morphological keys [15]. Adult males were also collected to confirm the taxonomic identification based on the genitalia morphology [16, 17]. The small number of adult females used in this study, was caused by low feeding success and high mortality, which limited the sample size (Table 1).

Viral stock

Adult female mosquitoes were orally infected by feeding on viremic chicks that had been inoculated with SLEV CbaAr-4005 [9]. Viral stock was obtained from collections stored at the Instituto de Virología "Dr. *J. M. Vanella*" (FM-UNC). Viral stock was prepared from brain of an infected Swiss albino suckling mice homogenized in 10% P/V solution in Eagle's minimal essential medium (MEM) (Gibco, Ireland), supplemented with 10% fetal bovine serum (FBS) (Natocor, Argentina) and 1% of gentamycin (Klonal, Argentina).

Viral titration was carried out by viral plaque assay in VERO cell monolayer (African green monkey kidney, *Cercophitecus aethiops*). We inoculated 0.1 ml of the samples onto VERO cell monolayer on a 12-well plate and incubated the plates for 60 min at 37°C with 5% CO_2 and a humid atmosphere to favor the adsorption of the virus to the cell. After incubation 0.5 ml of MEM 2x was mixed with 1% methylcellulose and added to plate followed by incubation at 37°C for 7 days, until the formation of lytic plaques. The plates

were then fixed with 10% formalin solution for 2 h and stained with crystal violet for the observation and plaque forming units (PFU) counts. Viral concentrations were then expressed as $\log_{10}$ PFU per milliliter (PFU/ml).

Vector competence assay

Twenty-four hour-old chicks (*Gallus gallus*) were inoculated intraperitoneally with 0.1 ml of a viral suspension containing approximately 400 PFU of SLEV. In accordance with viremia kinetic [8], 48 hr post-inoculation starved female mosquitoes were fed on chicks (Table 1). Prior to and following blood feeding by mosquitoes, 0.1 ml of blood was taken from the chick jugular vein to determine the viremia titer (potential viral load ingested by mosquitoes). The chick blood was diluted in 0.45 ml of MEM supplemented with 10% FBS and 1% gentamycin, followed by centrifugation at 4°C for 20 min at 2,300 g before supernatant stored at -80°C. Titers were determined by plaque assay and expressed as the mean viremia load before and after feeding ($\log_{10}$ PFU/ml). Mosquitoes were anesthetized by cold and fully engorged females were maintained in a screened cage at 27°C, H° 70%, 12:12 h L: D photoperiod and provided a 10% sugar solution. After 14 days EIP, the females were aspirated and anesthetized for 2 min at 4°C, placed on a refrigerated plate where the legs and wings were gently removed. Saliva samples were recovered after live females were placed on a flat surface with adhesive tape and the proboscis inserted for 30 min into a capillary tube with 0.001 ml glycerin (Todo Droga, Argentina) [18]. The abdomen was then removed, and saliva, legs and abdomen samples individually stored at -80°C with 1 ml of MEM supplemented with 10% FBS and 1% gentamycin. Legs and abdomen were individually homogenized by agitation with glass beads for 4 min and centrifuged at 11,200 g for 30 min. Subsequently, 0.1 ml of the sample was inoculated with

two cellular and viral controls and infective virus particles detected by plaque assay as described above.

SLEV-negative saliva samples from females with positive abdomen samples were re-analyzed to reduce false negatives due to dilution. Thus, 0.2 ml of each sample was inoculated in two cell monolayer plates (‘A’ and ‘B’ plates) using the same controls as mentioned above. ‘A’ plates followed the same culture protocol described above. For ‘B’ plates, MEM, FBS (2%) and gentamycin (1%) were added, and after 96 h the cytopathic effect (cell detachment, rounding and non confluent monolayer) was determined under an inverted microscope. To amplify viral particles not detected in the first passage, monolayer from the ‘B’ plates were harvested on the fourth day post inoculation, by collecting 0.75 ml from each of the duplicates wells followed by centrifugation at 9,300 g for 30 min (passage 1). Finally, another plate was inoculated (duplicate, same ‘B’ plates protocol) with 0.2 ml of the samples without cytopathic effect in the ‘B’ plates. Therefore, PFU/ml was recorded in ‘A’ plates and the cytopathic effect presence in ‘B’ plates.

Ethical Guidelines

The protocol used was approved by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Instituto de Virología "Dr. *J. M. Vanella*" (FM-UNC). The chicks were maintained in cages with a substrate of wood shavings and with a 12:12 h L: D photoperiod, 40-80% humidity and 20-26°C temperature. Food and water were available *ad libitum* (balanced concentrate feed, Gepsa Feeds, Argentina). The experimental procedures used were to minimize or eliminate pain and distress. Awareness were taken to avoid chicks suffering, as anesthetic protocols were not used. Euthanasia was carried out with neck dislocation by a trained laboratory technician.

Statistics analysis

Abdomens, legs, and saliva were considered positive when they showed at least one PFU or the presence of cytopathic effect on the cell culture monolayer. Infection rates were defined as the numbers of positive mosquito abdomens of the total number of blood feed mosquitoes. Dissemination rates were calculated as the number of mosquitoes with positive legs out of the number of mosquitoes fed analyzed. Transmission rates were determined by the number of mosquitoes with positive saliva of the total number of mosquitoes that blood fed. Variations in the infection, dissemination and transmission rates between the three *Culex* spp., were analyzed using a generalized linear model (GLM) with binomial distributions of errors and logit link function. *Culex p. quinquefasciatus* was a reference species as the assayed population was susceptible to SLEV [8]. Differences in viremia levels (range: 2.4-3.4 $\log_{10}$ PFU/ml) between feeding trials could be a source of variation confounding the outcomes analyzed, therefore viremia level was added as a co-variable. Significance at infections, dissemination, and transmission models were evaluated by Likelihood Ratio test (α=0.05, type II error) [19]. Contrast between species using the Tukey test, where the odds ratio (OR) informed the magnitude for the differences between species for each significant rate. Normal distribution and homogeneity of the variance of the residuals for each model were graphically explored [20]. All analyses and graphical presentations were made in the R [21] with car [22], factorplot [23] and ggplot2 [24] packages.

Results

The numbers of eggs collected *Cx. p. quinquefasciatus*, *Cx. interfor*, *Cx. saltanensis* and females that fed and were positive for SLEV are shown in Table 1. There was a narrow range of viremia during blood feeding (*Cx. p. quinquefasciatus*=2.9 $\log_{10}$ PFU/ml, *Cx. interfor*=3.5 $\log_{10}$ PFU/ml and *Cx. saltanensis*=3.2 $\log_{10}$ PFU/ml, σ^2=0.22) not contribute to the variation in infection (LR X^2=1.01, df=2, n=76, p=0.604), dissemination (LR X^2=4.94, df=2, n=76, p=0.084) and transmission (LR X^2=1.20, df=2, n=76, p=0.548) (Table 2).

Infection rates were not significantly different among the three species (LR X^2=3.42, df=2, n=76, p=0.181, σ^2=0.09), although infections rates of *Cx. saltanensis* being slightly higher (8/12, 67%), followed by *Cx. interfor* (14/25, 56%), and lastly *Cx. p. quinquefasciatus* (13/39, 33%) (Figure 1). Viral loads (range=1.3-5.3 $\log_{10}$ PFU/ml, σ^2=16.3) were evaluated in 89% (31/35) of the three species mosquitoes.

Dissemination rates were significantly higher in *Cx. saltanensis* (8/12, 67%; p=0.01; OR=8.9, 95% CI [2.12-44.10], σ^2=45.6) than *Cx. p. quinquefasciatus* (7/39, 18%) and *Cx. interfor* (10/25, 40%; p=0.09, σ^2=24.6). Both, *Cx. p. quinquefasciatus* and *Cx. interfor* showed similar dissemination rates (p=0.813, σ^2=0.2). Among infected mosquitoes, dissemination was achieved in 100% of those individuals tested (8/8) for *Cx. saltanensis*, while are 71% (10/14) of *Cx. interfor* and 54% (7/13) of *Cx. p. quinquefasciatus* demonstrated disseminated infections. These results suggest the possible existence of a midgut barrier to SLEV in *Cx. p. quinquefasciatus* (Figure 1). Viral load ranged from 1.0-5.4 $\log_{10}$ PFU/ml (σ^2= 17.04) for 76% (19/25) of the three species mosquitoes with viral dissemination.

Transmission rates between *Cx. interfor* (20%, 5/25), *Cx. p. quinquefasciatus* (18%, 7/39) and *Cx. saltanensis* (17%, 2/12); (LR X^2=2.82, df=2, n=76, p=0.093, σ^2=0.4) were not significantly different (Figure 1). All *Cx. p. quinquefasciatus* females with disseminated infections demonstrated SLEV in their saliva (7/7); this rate was only 50% (5/10) in *Cx. interfor* and 25% (2/8) in *Cx. saltanensis*, indicating a potential salivary gland barrier in both *Cx. interfor* and *Cx. saltanensis* (Figure 1). The saliva viral load range was 1.1-2.3 $\log_{10}$ PFU/ml (σ^2= 2.5) in *Cx. saltanensis* and *Cx. interfor* (5/7, 71%). Because lysis plaques in *Cx. p. quinquefasciatus* were not confluent the SLEV load in saliva was not able to measure.

Discussion

Culex p. quinquefasciatus, *Cx. interfor* and *Cx. saltanensis* were competent vectors for SLEV based on 1) acquired infections, 2) disseminated virus, and 3) transmission of SLEV in the saliva. The three *Culex* species displayed similar infection susceptibility and comparable transmission in the saliva, whereas dissemination patterns appeared to be species specific. While, *Cx. saltanensis* had a greater chance of becoming infected and disseminating (67%) SLEV than *Cx. interfor* (infection=56%; dissemination=40%), and both with respect to *Cx. p. quinquefasciatus* (infection=33%; dissemination=18%) they were not observed statistical differences, however, the number of tested mosquitoes was low, thus our results are not conclusive.

To be considered a vector, a mosquito species must fulfill several biological and ecological characteristics [25, 26]. The coevolution between pathogen and arthropods determine the vector competence, and thus the ability to acquire, maintain and eventually

transmit it [25]. Variation in vector competence has been documented with all of the major disease agents they transmit (i.e. malaria and filarial parasites, and arboviruses) [6, 27, 28]. Although the life cycle of each pathogen is distinct, they all face the common events of being ingested, exposed to the midgut environment, and traversing the hemolymph-filled hemocoel to reach their tissue site of development and/or suitable site for transmission back to a new vertebrate host. The pathogen must cope with innate immune responses and overcome tissue barriers associated with the midgut and the salivary glands. Each of these migratory steps presents potential barriers that might be manipulated to interfere with normal pathogen migration and/or development [26]. There is considerable specificity in the vector-arbovirus relationship, and some of this specificity comes from the ability of a particular arbovirus to overcome tissue barriers in the vector to establish a persistent infection. Factors that strongly affect VC of a mosquito for a particular arbovirus include MIB, MEB, SGIB, and SGEB. These barriers can be virus dose-dependent and/or -independent [6]. The tested species showed similar infection and transmission rates, but *Cx. p. quinquefasciatus* and *Cx. saltanensis* demonstrated differences in dissemination rates that could be related to a species-specific tissue barriers affecting the infection-transmission kinetics [29-31]. In our study, *Cx. p. quinquefasciatus* presented possible existence of a midgut barrier (MIB/MEB) and likely partially limited the infection/dissemination of SLEV (54%, 7/13) for others specie dissemination was achieved in (100%, 8/8) for *Cx. saltanensis*, while are (71%, 10/14) of *Cx. interfor* of demonstrated disseminated infections. Kramer and colleagues were the first to demonstrate that the inability of infected *Cx. tarsalis* mosquitoes to transmit WEEV was associated with a MEB [32] and Richards et al (2012) detected *Cx. p. quinquefasciatus* with low or zero WNV titer in their thoraces with high abdomen titers was consistent with the possibility of a barrier to infection and/or virus

replication to the thorax tissues in these mosquitoes [31]. Moreover, in our study, a salivary gland barrier of *Cx. p. quinquefasciatus* was not observed since 100% those disseminated with to SLEV transmitted the virus in saliva, while *Cx. interfor* was intermediate vector and *Cx. saltanensis* the SGIB/SGEB likely partially limited the transmission of SLEV since (100%, 8/8) of the infected mosquitoes demonstrated disseminated virus, while only 25% (2/8) transmitted it. Similar observations indicating a significant barrier in the salivary glands were detected in *Cx. p. molesus* was less efficient for the Japanese encephalitis virus [33], in *Cx. tarsalis* infected with the western equine encephalitis virus, where the effectiveness of this barrier decreased with the extrinsic incubation period but was still evident after 21 days [32], and *Psorophora cingulata* and *Coquillettidia venezuelensis* were susceptible to infection by Venezuelan equine encephalitis virus; however they had low transmission rates, even after intrathoracic inoculation [34]. In addition, Reisen et al. quantified the viral particles of SLEV expectorated in the saliva of *Cx. tarsalis* (1.1-2.2 $\log_{10}$ PFU) [35] and we obtained similar data for *Cx. interfor* (range=1.1-2.3 $\log_{10}$ PFU) and *Cx. saltanensis* (1.8 $\log_{10}$ PFU). Further studies are needed to have deeper understanding regarding the SLEV infection and dissemination barriers in these three *Culex* species.

Our results corroborate the findings reported by Diaz et al. on the susceptibility of *Cx. p. quinquefasciatus* for SLEV infection [8]. These authors observed an infection rate of 70% using the same viral strain CbaAr-4005 and feeding viremia level of 5.2 $\log_{10}$ PFU/ml. In our study only one third of the *Cx. p. quinquefasciatus* females became infected. This difference could be related to the lower viremia level that the mosquitoes were exposed to in this assay (2.9 $\log_{10}$ PFU/ml). Mitchell et al. obtained a transmission rate of (90.5%, 19/21) with strain 78V-6507 for *Cx. p. quinquefasciatus* from Santa Fe Province [12]. In our study, the transmission rate was (18%, 7/39). Dissemination and transmission rate

formulation in Mitchell and herein are different. Even through dissemination rates are higher with a higher infectious dose (Mitchell, 4.1-4.8 $\log_{10}$ PFU/ml vs our study, 2.9 $\log_{10}$ PFU/ml), adjusting the formulation for rates the dissemination between Mitchell and the current study would be similar. For the species used in our study regardless of the host's viremia, we did not find differences between infection, dissemination and transmission rates. The effect of viremia could be affected by the small number of individuals used in this study. Future studies to determine the Minimum Infection Threshold (MIT) and the EIP are needed.

The maintenance of SLEV in nature is complex and requires the coexistence in time and space of mosquito vectors and avian hosts. The detection of naturally infected mosquitoes does not represent, by itself, a reliable proof of their role as a competent vector. In the case of *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis*, there is evidence supporting their intervention as vectors in SLEV transmission. *Culex p. quinquefasciatus* is considered the primary vector, because it was abundant and *pools* assayed were positive for SLEV [9-11, 14]. *Culex saltanensis* and *Cx. interfor* participate in the maintenance of SLEV and could assist in the spillover of SLEV to humans. In 2004, prior to the outbreak of encephalitis in Córdoba City, SLEV infected *Cx. interfor* were detected [11]. In 2010, there were small outbreaks of SLEV in provinces, e.g., Buenos Aires, Córdoba and San Juan [37]. Not long thereafter, SLEV infected *Cx. saltanensis* were detected for first time in Córdoba City [14]. *Culex interfor* and *Cx. saltanensis* are mainly ornithophiles, and bloodmeal from Columbiformes and Passeriformes have been also detected, although the pattern of host preference and its drivers have not been established yet [14, 38, 39]. In Argentina, *Z. auriculata* and *C. picui* are amplifier hosts of SLEV and have been recorded in engorged *Cx. saltanensis*, *Cx. interfor* and *Cx. p. quinquefasciatus* sustaining that SLEV

maintenance could relied on multiple vectors [3, 36]. However, the transmission load in SLEV episystem could be unequal between the three *Culex*, despite they showed similar transmission rate experimentally (ranged 17-20%). For instance, among other traits, lifespan difference among mosquito species is expected to impact vector capacity as longest lifespan increase the odds of extrinsic incubation completeness and delivering infectious bites [7, 25]. Here, *Cx. p. quinquefasciatus* was less able to survive after a viremic bloodmeal than *Cx. saltanensis* and *Cx. interfor* suggesting that the role of the last species has been neglected. In addition, it has been proposed that *Cx. interfor* could transmit SLEV from birds to mammals and thus fulfill a role of "bridge vector" [14, 38, 40] as *Cx. interfor* was recorded in human baited barley traps [40] and *Cx. interfor* and *Cx. saltanensis* switch between bird feeding profile in spring-summer to bird-mammals in autumn in a rural environment [41]. The local populations of *Culex* spp. increase in abundance with peaks in summer, with are temporal distribution of *Culex* spp. coinciding with the activity peaks of SLEV in human infection [3, 42]. Adult mosquitoes belonging to the species *Cx. saltanensis* and *Cx. interfor* have been found in increasing numbers in Córdoba City, with a higher abundance in urban and periurban areas where vegetation is more robust. This differs from *Cx. p. quinquefasciatus*, which is predominant throughout a vast range of city-type environments [42]. Our results support the hypothesis that SLEV is transmitted by multiple sympatric *Culex* spp., and that both *Cx. saltanensis* and *Cx. interfor* can be considered potential vectors of SLEV. In the United States, this has been observed as well; however, different mosquito species serve as the primary vector transmitting SLEV in different geographical areas. *Culex quinquefasciatus* and *Cx. nigripalpus* are vectors for the virus in Florida, *Cx. tarsalis* in the western and *Cx. pipiens* in the northern United States [4].

SLEV is a multi-host and multi-vector flavivirus in the process of an ongoing reemergence in Argentina. This is the first completed vector competence evaluation of *Cx. interfor* and *Cx. saltanensis* against SLEV. Further studies are required to understand the spatial compartmentalization of these mosquito species in the transmission network of SLEV by performing vector capacity studies. By having insights into its ecoepidemiology, we will have a better understanding of which factors are causing this reemergence and how biological and environmental factors interact with and affect its activity. In addition, knowledge of the potential mosquito species vectors of SLEV will provide information to be used by different public agencies related to human health for the control of vector mosquito populations and improve efficiency in SLEV prevention programs.

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Conflicts of Interest

The authors of this preprint declare that they have no financial conflict of interest with the content of this article.

Adrian Diaz is one of the PCI Entomology recommenders.

References

1. Gould E, Pettersson J, Higgse S *et al*. Emerging arboviruses: Why today? *One Health* 2017: 4: 1-13.
2. Agarwal A, Parida M, Dash PK. Impact of transmission cycles and vector competence on global expansion and emergence of arboviruses. *Rev Med Virol* 2017: e1941.
3. Diaz LA, Flores FS, Quaglia A *et al*. Intertwined arbovirus transmission activity: Reassessing the transmission cycle paradigm. *Front Physiol* 2013: 3: 493.
4. Reisen WK. Epidemiology of St. Louis encephalitis virus. *Adv Virus Res* 2003: 61: 139-184.
5. Diaz A, Coffey LL, Burket-Cadena N *et al*. Reemergence of St. Louis encephalitis virus in the Americas. *Emerg Infect Dis* 2018: 24: 2150-2157.
6. Franz AWE, Kantor AM, Passarelli AL *et al*. Tissue barriers to arbovirus infection in mosquitoes. *Viruses* 2015: 7: 3741-3767.
7. Kramer LD & Ebel GD. Dynamics of *Flavivirus* infection in mosquitoes. *Adv Virus Res* 2003: 60: 187-232.
8. Diaz LA, Flores FS, Beranek M *et al*. Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013: 107: 332-334.
9. Diaz LA, Ré V, Almirón W *et al*. Genotype III Saint Louis encephalitis virus outbreak, Argentina, 2005. *Emerg Infect Dis* 2006: 128: 1752-1754.

10. Sabattini MS. Historical, epidemiological and ecological aspects of arboviruses in Argentina: Flaviviridae, Bunyaviridae and Rhabdoviridae. An overview of Arbovirology in Brazil and neighbouring countries (ed. by Travassos da Rosa APA, Vasconcelos PFC & Travassos da Rosa JFS). Brasil: Instituto Evandro Chagas; 1998. 113-134.
11. Diaz LA, Albrieu Llinás G, Vázquez A *et al*. Silent circulation of St Louis encephalitis virus prior to an encephalitis outbreak in Córdoba, Argentina (2005). *PLoS Negl Trop Dis* 2012: 6: 1489.
12. Mitchell CJ, Monath TP & Sabattini MS. Transmission of St. Louis encephalitis virus from Argentina by mosquitoes of the *Culex pipiens* (Diptera: Culicidae) Complex. *J Med Entomol* 1980: 17: 282-285.
13. Flores FS, Diaz LA, Batallán GP *et* al. Vertical transmission of St. Louis encephalitis virus in *Culex quinquefasciatus* (Diptera: Culicidae) in Córdoba, Argentina. *Vector Borne Zoonotic Dis* 2010: 10: 999-1002.
14. Batallán GP. Binomía de *Culex interfor* Dyar en Córdoba, Argentina. [theses]. Córdoba: Universidad Nacional de Córdoba; 2013. 197 p.
15. Darsie RF. Mosquitoes of Argentina. Part I. Keys for identification of adult females and fourth stages larvae in English and Spanish (Diptera: Culicidae). *Mosquito Systematics* 1985: 17: 153-253.
16. Harbach RE, Jakov WL & Peyton EL. Recognition of *Culex bidens* Dyar and *Culex interfor* Dyar (Diptera: Culicidae) as separate species. *Mosquito Systematics* 1986: 18: 139-144.
17. Laurito M, Visintin AM & Almirón WR. *Culex saltanensis* morphological redescription of the inmature and adult stage. *J Am Mosq Control Assoc* 2008: 24: 203-210.

18. Anderson SL, Richards SL & Smartt CT. A Simple method for determining arbovirus transmission in mosquitoes. *J Am Mosq Control Assoc* 2010: 26: 108-111.

19. Zuur AF. GLM and GAM for Absence–Presence and Proportional Data. New York: Springer; 2009. Chapter 19, Mixed Effects Models and Extensions in Ecology with R. *Statistics for Biology and Health*. 245-259.

20. Zuur AF, Ieno EN & Elphick CS. A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution* 2010: 1: 3-14.

21. R Core Team. R: A language and environment for statistical computing. [cited 2015]. https://www.R-project.org.

22. Fox J & Weisberg S. An R Companion to Applied Regression. 2nd Ed. Thousand Oaks CA: Sage Publications, 2011. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

23. Armstrong D. factorplot: factorplot. R package version 1.1-1. 2014. https://CRAN.R-project.org/package=factorplot.

24. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, 2009.

25. Gubler DJ. Vector-borne diseases. *Rev sci tech* 2009: 28: 583-588.

26. Beerntsen BT, James AA & Christensen. Genetics of Mosquito Vector Competence. *Microbiology and Molecular Biology Reviews* 2000: 64: 115-137.

27. Cohuet A, Harris C, Vincent R *et al*. Evolutionary forces on *Anopheles*: what makes a malaria vector? *Trends Parasitol* 2010: 26: 130-136.

28. Ahid SMM, da Silva Vasconcelos PS & Lourenço-de-Oliveira R. Vector Competence of *Culex quinquefasciatus* Say from Different Regions of Brazil to *Dirofilaria immitis*. *Mem Inst Oswaldo Cruz* 2000: 95: 769-775.

29. Weaver SC, Scott TW & Lorenz LH. Patterns of eastern equine encephalomyelitis virus infection in *Culiseta melanura* (Diptera: Culicidae). *J Med Entomol* 1990: 27: 878-891.

30. Reisen WK, Barker CM, Fang Y *et al*. Does variation in *Culex* (Diptera: Culicidae) vector competence enable outbreaks of West Nile virus in California? *J Med Entomol* 2008: 45: 1126-1138.

31. Richards SL, Anderson SL, Lord CC *et al*. Relationships between infection, dissemination, and transmission of West Nile virus RNA in *Culex pipiens quinquefasciatus* (Diptera: Culicidae). *J Med Entomol* 2012: 49: 132-142.

32. Kramer LD, Hardy JL, Presser SB *et al*. Dissemination barriers for western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low viral doses. *Am J Trop Med Hyg* 1981: 30: 190-197.

33. Turell MJ, Mores CN, Dohm DJ *et al*. Laboratory transmission of Japanese encephalitis and West Nile viruses by molestus form of *Culex pipiens* (Diptera: Culicidae) collected in Uzbekistan in 2004. *J Med Entomol* 2006: 43: 296-300.

34. Turell MJ, Jones JW, Sardelis MR *et al*. Vector competence of Peruvian mosquitoes (Diptera: Culicidae) for epizootic and enzootic strains of Venezuelan equine encephalomyelitis virus. *J Med Entomol* 2000: 37: 835-839.

35. Reisen WK, Chiles RE, Kramer LD *et al*. Method of infection does not alter response of chicks and house finches to western equine encephalomyelitis and St. Louis encephalitis viruses. *J Med Entomol* 2000: 37: 250-258.

36. Diaz LA, Flores FS, Quaglia IA *et al*. Evaluation of Argentinean birds species as amplifying hosts for St. Louis encephalitis virus (Flavivirus, Flaviviridae). *Am J Trop Med Hyg* 2018: 99: 216-221.

37. Vergara Cid C, Spinsanti L, Rivarola M *et al*. Detección de infecciones humanas por *Flavivirus* en la ciudad de Córdoba durante el año 2010. *Rev Argent Microbiol* 2011: 43: 37.

38. Beranek MD. Mosquitos del género *Culex* (Diptera: Culicidae) como vectores del virus Saint Louis encephalitis (Flavivirus) en ambientes urbanos de la ciudad de Córdoba. [theses]. Córdoba: Universidad Nacional de Córdoba; 2019. 188 p.

39. Berrón CI. Preferencia de hospedadores aviares en especies de mosquitos Género *Culex* asociadas a la transmisión de Flavivirus (Flaviviridae) en el arco sur de la Laguna Mar Chiquita. [theses]. Córdoba: Universidad Nacional de Córdoba; 2014. 155 p.

40. Stein M, Zalazar L, Willener JA *et al*. Culicidae (Diptera) selection of humans, chickens and rabbits in three different environments in the province of Chaco, Argentina. *Mem Inst Oswaldo Cruz* 2013: 108: 563-571.

41. Quaglia AIE. Comunidades de Mosquitos Vectores y Hospedadores Aviares y su Asociación en el Mantenimiento del virus St. Louis encephalitis (Flavivirus). [theses]. Córdoba: Universidad Nacional de Córdoba; 2017. 217 p.

42. Batallán GP, Estallo EL, Flores FS *et al*. St. Louis encephalitis virus mosquito vectors dynamics in three different environments in relation to remotely sensed environmental conditions. *Acta Trop* 2015: 146: 53-59.

Table 1: The egg collections and number females of *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis* in Córdoba City.

Species	Nº of egg rafts per species*	Total Nº of females fed on chicks†	Nº of engorged females‡	Nº of SLEV positive females§
Culex p. quinquefasciatus	12	64	39	13
Culex interfor	15	32	25	14
Culex saltanensis	9	18	12	8

***Number of egg rafts collected for each mosquito species, assuming that each raft was from a different female. Number of eggs not determined. Male/female ratio of hatched individuals was ~50%.**

† Number of females provided blood meals on viremic chicks.

‡Number of engorged females after feeding on viremic chicks.

§Number of females positives for SLEV.

Table 2: Vector competence of *Culex p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis* for SLEV measured as infection, dissemination and transmission.

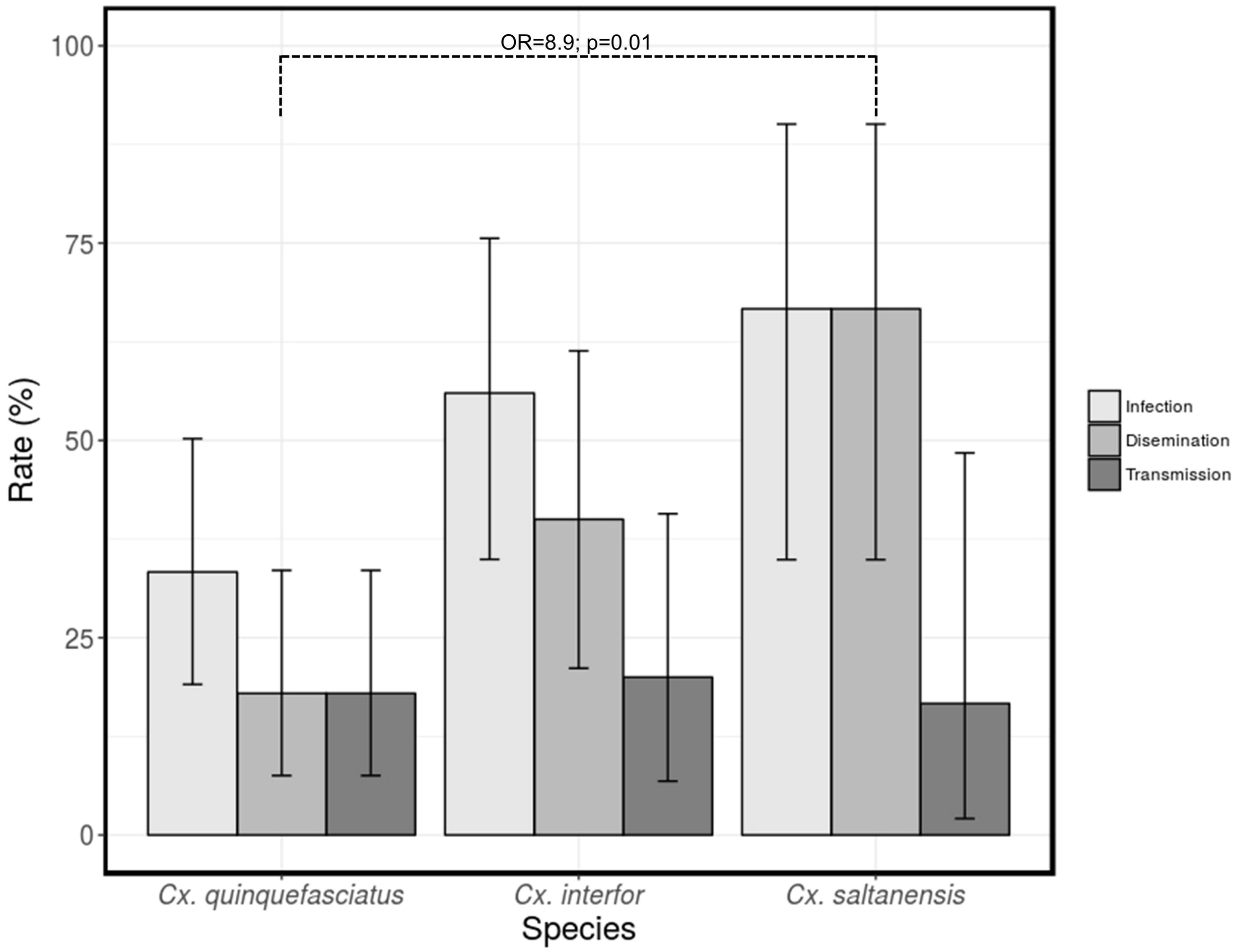
Species Vector competence	*Culex p. quinquefasciatus*			*Culex interfor*			*Culex saltanensis*		
	N*	Rates† (0.95%CIs)	Viral load‡ (0.95%CIs)	N*	Rates† (0.95%CIs)	Viral load‡ (0.95%CIs)	N*	Rates† (0.95%CIs)	Viral load‡ (0.95%CIs)
Infection	13/39	33 (19.1-50.2)	2.4 (1.3-2.9)	14/25	56 (34.9-75.6)	4.8 (2.4-5.3)	8/12	67 (34.9-90.1)	4.8 (3.8-5.1)
Dissemination	7/39	18 (7.5-33.5)	2.9	10/25	40 (21.1-61.3)	4.8 (1-5.4)	8/12	67 (34.9-90.1)	4.6 (1.6-5.1)
Transmission	7/39	18 (7.5-33.5)	No data	5/25	20 (6.8-40.7)	1.8 (1.1-2.3)	2/12	17 (2.1-48.4)	1.8

***Number of mosquitoes positive for SLEV/total number of mosquitoes assayed**

†Infection rate (number of positive mosquito abdomens/total number of mosquitoes fed); dissemination rate (number of mosquitoes with positive legs/total number of mosquitoes fed) and transmission rate (number of mosquitoes with positive saliva/total number of mosquitoes fed).

‡ Average SLEV titers (log_{10} PFU/ml) in abdomen, legs and saliva of each mosquito species.

Figure 1: Vector competence for SLEV of *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis*. Infection rate (number of positive mosquito abdomens/total number of mosquitoes fed) in light gray, dissemination rate (number of mosquitoes with positive legs/total number of mosquitoes fed) in gray and transmission rate (number of mosquitoes with positive saliva/total number of mosquitoes fed) in dark gray; with their 0.95% CIs. Dashed line indicates statistical difference on dissemination rate for *Cx. saltanensis* in relation to *Cx. p. quinquefasciatus* (OR = 8.9; p= 0.01; σ^2=45.6).


OR=8.9; p=0.01
100
75
50
25
0
Rate (%)
Infection
Disemination
Transmission
Cx. quinquefasciatus
Cx. interfor
Cx. saltanensis
Species