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Evaluation of Argentinean Bird Species as Amplifying Hosts for St. Louis Encephalitis Virus (Flavivirus, Flaviviridae)

Adrián Díaz,[1,2]* Fernando S. Flores,[1] Agustín I. Quaglia,[1] and Marta 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]*Instituto de Investigaciones Biológicas y Tecnológicas, CONICET—Universidad Nacional de Córdoba, Córdoba, Argentina*

Abstract. St.Louis encephalitis virus (SLEV) is an emerging human pathogen flavivirus in Argentina. Recently, it has reemerged in the United States. We evaluated the role as amplifying host of six resident bird species and analyzed their capacity as host during the 2005 encephalitis outbreak of SLEV in Córdoba. Eared Dove, Picui Ground Dove, and House Sparrow were the three species with highest host competence index. At a city level, Eared Dove and Picui Ground Dove were the most important amplifying hosts during the 2005 SLEV human outbreak in Córdoba city. This finding highlighted important differences in the SLEV ecology between Argentina and the United States. Characterizing and evaluating the SLEV hosts contribute to our knowledge about its ecology and could help us to understand the causes that promote its emergence as a human pathogen in South America.

INTRODUCTION

St. Louis encephalitis (SLE), caused by the homonymous virus (St. Louis encephalitis virus [SLEV], genus *Flavivirus*, family *Flaviviridae*), is a complex zoonosis in the New World.[1] It is an emerging/reemerging arbovirosis in South America. Febrile illness and encephalitis cases were reported in Argentina and Brazil.[2,3] In the central region of Argentina, SLEV emerged as a human pathogen during 2002 and, since then, outbreaks have been reported in Córdoba (2005), Entre Rios (2006), Buenos Aires (2010), and San Juan (2011) provinces.[2,4–7] In the southern area of Brazil, SLEV was identified as the etiologic agent of a meningoencephalitis outbreak and hemorrhagic cases among humans.[3,8,9] St. Louis encephalitis virus has been associated with neurological diseases in equines from Minas Gerais state, Brazil.[10] Recently, it reemerged as a neurological pathogen in Arizona and California states (US).[11,12]

St. Louis encephalitis virus is widely distributed in tropical, subtropical, and temperate-tropical areas of the American continent, and therefore, in most of the populated land masses of North and South America.[1] In the United States, this virus is known to be naturally maintained by transmission cycles between several *Culex* (*Cx*.) mosquito species and a variety of bird species, including the House Sparrow (*Passer domesticus*), House Finch (*Haemorhous mexicanus*), and Mourning Dove (*Zenaida macroura*).[1]

The ecological characteristics of SLEV in South America are practically unknown, but in Argentina the cycle involved *Culex quinquefasciatus* and *Culex interfor* mosquitoes as vectors.[13,14] In temperate and subtropical areas of Argentina, neutralizing antibodies (NTAb) against SLEV have been found in bird species belonging to the families Accipitridae, Ardeidae, Columbidae, Fringillidae, Furnariidae, Icteridae, Phytotomidae, and Tyrannidae.[15,16] The serological survey carried out during the 2005 SLE outbreak in Córdoba showed that species belonging to families Columbidae (14.0%), Tyrannidae (10.1%), Furnariidae (6.3%), Thraupidae (5.5%), Turdidae (4.5%), Passeridae (3.9%), and Icteridae (1.7%) were the most exposed.[17]

In the present work, we evaluated the role of six resident bird species as amplifying hosts and analyzed their capacity as host during the 2005 encephalitis outbreak of SLEV in Córdoba city, central Argentina.

MATERIALS AND METHODS

Bird collection and husbandry. Avian species selected for study were based on previous evidence of SLEV infection in nature, abundance in urban/periurban areas, and ease of maintenance in captivity.[17] Bay-winged Cowbird (*Agelaioides badius*, family Icteridae, order Passeriformes), Eared Dove (*Zenaida auriculata*, family Columbidae, order Columbiformes), House Sparrow (*P. domesticus*, family Passeridae, order Passeriformes), Picui Ground-Dove (*Columbina picui*, family Columbidae, order Columbiformes), Shiny Cowbird (*Molothrus bonariensis*, family Icteridae, order Passeriformes), and Spot-winged Pigeon (*Patagioenas maculosa*, family Columbidae, order Columbiformes) were collected using mist nets and in-house baited floor-traps for eared doves. Birds were kept at the Virology Institute biosafety facilities under seminatural conditions (photoperiod and temperature depended on environmental conditions) and were fed mixed grains ad libitum. Birds were handled according to the guidelines for the use of wild birds in research elaborated by the Ornithological Council (https://www.aaalac.org/accreditation/RefResources/SS_WildBirds.pdf). After collection, birds were bled and banded; 200 µL of blood was collected from each bird, stored at room temperature for 30 minutes to coagulate, and then centrifuged for separation of serum from clot. Sera diluted 1/10 were analyzed by plaque reduction neutralization test (PRNT) for antibodies against West Nile virus (WNV) (E/7229/06 strain) and SLEV (CbaAR-4005 strain). Only seronegative birds were used for the experiment (positive criteria: 80% of neutralization, > 1:10).

Viral strain. Birds were experimentally infected with a sympatric SLEV CbaAr-4005 strain isolated from *Cx. quinquefasciatus* mosquitoes in the city of Córdoba (Argentina) during the 2005 human encephalitis outbreak. The viral strain was passaged three times in VERO cell monolayers. The viral suspension was prepared from infected suckling-mouse brain diluted 10% in minimum essential medium (MEM) with Earle's salts and L-glutamine, 10% fetal calf sera (FCS), and 1%

*Address correspondence to Adrián Díaz, Arbovirus Laboratory, Institute of Virology "Dr. J. M. Vanella," School of Medicine, National University of Córdoba, Enfermera Gordillo Gómez s/n, CPX5016GCA, Córdoba, Argentina. E-mail: adrian.diaz@conicet.gov.ar

gentamicin centrifuged at 11,400 × *g* at 4°C for 30 minutes. The viral suspension was tittered by VERO cell plaque assay and 100 µL aliquots was stored at –80°C. The viral titer was expressed as plaque-forming units per milliliter of serum (PFU/mL).

Viremia assays. Birds were subcutaneously inoculated in the abdominal region with approximately 300 PFU SLEV diluted in 100 µL. This viral load was chosen in accordance with values observed in field-collected mosquitoes.[18] Following inoculation, birds were observed every 12 hours to detect any clinical signs of illness. Birds were bled (100 µL) daily for 10 days via jugular or brachial venipuncture. Whole blood was diluted in 0.45 mL of refrigerated MEM with 10% FCS and 1% gentamicin to avoid bacterial contamination, centrifuged at 1,500 × *g* for 15 minutes, and the supernatant stored at –80°C. Viremia titers were determined by plaque assay on VERO cells and expressed as PFU/mL[19] (detection threshold: 2 $\log_{10}$ PFU/mL). Mean daily viremia was calculated using detectable and no-detectable viremia values. In those individuals with no-detectable viremia, the limit of detection value (2 $\log_{10}$ PFU/mL) was used. Viremia values were not $\log_{10}$-transformed in calculations and only expressed as $\log_{10}$-transformed values in the text.

Serology. To verify seroconversion to SLEV in inoculated birds, all survivors were bled on the 14th day postinoculation (dpi). Blood was allowed to coagulate at room temperature for 30 minutes, followed by centrifugation to separate the serum. The samples were stored at –20°C and heat inactivated at 56°C for 30 minutes before testing. For PRNT, sera were diluted 1:10 in MEM, and endpoint antibody titers were determined using serial 2-fold dilutions.[19]

Host competence and capacity indexes. Host competence index (C_i) is a term that describes the infectiousness of an infected host and provides an estimate of the number of infectious mosquitoes generated by each individual of a given species.[20] Values for C_i were calculated according to the formula ($C_i = i \times d \times s$), where "*s*" is the susceptibility to infection (a proportion of viremic birds), "*i*" is the extrapolated mean daily infectiousness (the proportion of feeding *Cx. quinquefasciatus* that are expected to become infected after a viremic blood meal and survive the extrinsic incubation period), and "*d*" is the mean duration of infectious viremia (in days).[20] Infectiousness was extrapolated using data published by Mitchell et al.[21] on oral infectivity of *Cx. quinquefasciatus* for 78V-6507 SLEV Argentinean strain. These data indicated an approximate minimum infection threshold (MIT) of $10^{2.9}$ PFU/mL for infectious viremia titers.[21] To compare C_i among species, we also calculated relative host competence index (C_r), which results in the division of two C_i values.

To evaluate the role of these inoculated bird species as host during the SLEV outbreak in Córdoba city, we calculated the host capacity index (M_i) according to the formula ($M_i = C_i \times Ir \times Ab$) developed by Komar et al.[22] This index represents the number of infectious mosquitoes produced by the population of a specific bird in a certain epidemiological scenario. To calculate the M_i, we used the host competence values obtained for each bird species during our experimental assays (C_i), seroprevalence obtained during the SLEV human encephalitis outbreak (Ir),[17] and abundance data (Ab) for the city of Córdoba. Relative host capacity index (M_r) was calculated in the same manner as C_r values were obtained.

Bird community assemblage. Estimates of abundance in bird urban assemblage were obtained by an observational and acoustic sampling in four sites in the city of Córdoba (Botanical Garden, Camino San Carlos, Bajo Grande, and Villa Parque). For a detailed description of these sites, consult Diaz et al.[17] We estimated bird abundance using the point and line transect methods.[23] Ten transect points spaced 200 m apart were established within a 1-km radius of each study site. All transect points were surveyed once by a single observer. Surveys took place during March 14–21, 2005, between 6:00 and 10:00 AM Point counts lasted for 10 minutes during which all bird species seen and heard were recorded.

RESULTS

A total of eight bay-winged cowbirds were collected, out of which one individual cowbird had SLEV NTAb (12.5%). One of seven inoculated individuals developed detectable viremia on day 3 postinoculation. The viremia titer was 3.1 $\log_{10}$ PFU/mL (Figure 1A). The other icterid species analyzed was Shiny Cowbird. These individuals developed a viremia profile similar to the one detected in Bay-winged Cowbird. All the inoculated individuals (N = 3) developed viremia on the third dpi, ranging between 3.5 and 4.3 $\log_{10}$ PFU/mL (average daily viremia = 3.9 $\log_{10}$ PFU/mL) (Figure 1C). Only one of nine collected house sparrows was positive by PRNT against SLEV (11.1%). Seven of eight (87.5%) inoculated individuals developed detectable viremia. Viremia titers were only detectable on the third dpi, ranging between 2 and 4.5 $\log_{10}$ PFU/mL (average daily viremia = 3.9 $\log_{10}$ PFU/mL) (Figure 1B).

Three columbid species were tested in this assay. Four picui ground-doves resulted positive for SLEV PRNT (26.7%). Eight of 11 inoculated individuals developed detectable viremia (average daily viremia = 4.3 $\log_{10}$ PFU/mL) ranging between 2 and 5.8 $\log_{10}$ PFU/mL (Figure 1A). The highest percentages of viremic birds were detected on days 2 and 3 pi (64% and 55%, respectively). The viremia was observed between the second and fifth dpi (mean duration = 2.25 ± 1.2 days). Twelve spot-winged pigeons were collected and four resulted positive for PRNT against SLEV (33.3%). Seven of eight inoculated individuals showed detectable viremia (87.5%). The average daily viremia was 2.9 $\log_{10}$ PFU/mL (range: 2–3.7 $\log_{10}$ PFU/mL) (Figure 1C). Detectable viremias were observed between the second and fifth dpi. Days 3 and 4 pi registered the highest viremic bird percentage (50% and 38%, respectively). Ten eared doves were collected and 50% of those individuals had SLEV NTAb. All inoculated individuals developed detectable viremia. The average daily viremia observed was 4 $\log_{10}$ PFU/mL (2–5.3 $\log_{10}$ PFU/mL). Detectable viremias were registered on days 2 and 7 pi with a mean duration of 4 days (Figure 1B). On day 3 pi, all inoculated individuals proved to be viremic. With the exception of one Picui Ground-Dove and one Bay-winged Cowbird that did not develop viremia, all inoculated individuals showed seroconversion at day 14 pi. None of the birds used in the host competence assays were positive for WNV.

The calculated C_i values are shown in Table 1. Eared Dove and Picui Ground-Dove obtained the highest values. The C_i value obtained for Eared Dove is explained by its having the highest susceptibility (100% viremic individuals), highest titers, and longest lasting viremia. According to the relative host competence index (C_r), an Eared Dove and a Picui

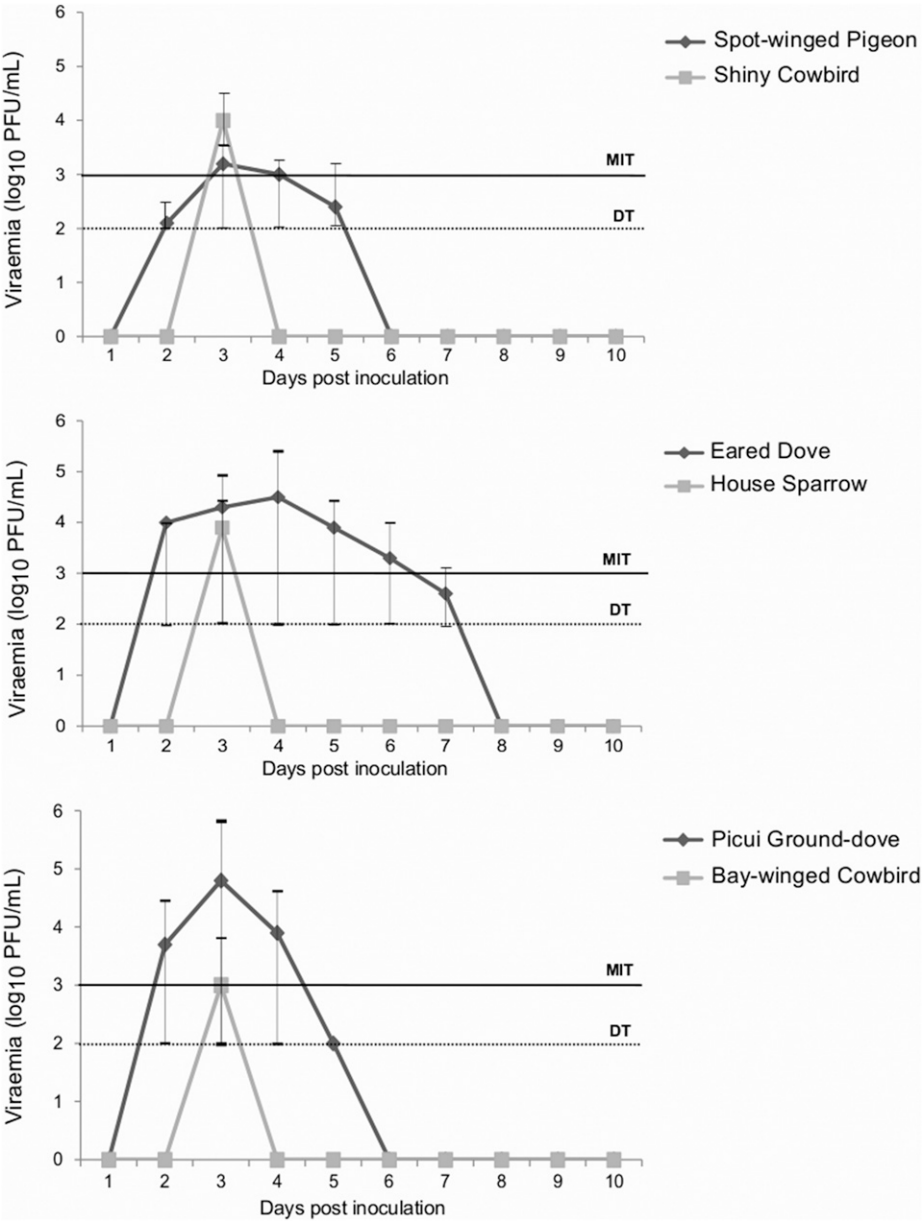

FIGURE 1. Mean, minimum, and maximum daily viremia developed in subcutaneously inoculated birds with CbaAr-4005 St. Louis encephalitis virus (SLEV) strain. MIT = minimum infection threshold of SLEV in *Cx. quinquefasciatus* from Argentina; DT = detection threshold.

Ground-Dove produce, respectively, 485 and 83 times more infectious mosquitoes compared with the Bay-winged Cowbird (Table 1). At a city level, Eared Dove and Picui Ground-Dove showed the highest M_i values during the 2005 SLEV human outbreak in Córdoba city (Table 2). However, differentiating by neighborhood, Eared Dove amplified the virus solely in Bajo Grande (Supplemental Figure 1). On the other hand, Picui Ground-Dove contributed to the viral amplification in all analyzed sites. Species such as Spot-winged Pigeon and Shiny Cowbird were not exposed to the virus during the outbreak (M_i = 0). According to the relative host capacity index (M_{ir}), the Eared Dove and Picui Ground-Dove populations produced 1,283 and 183 times more infectious *Cx. quinquefasciatus* mosquitoes during the outbreak than the Bay-winged Cowbird (Table 2).

TABLE 1

Host competence index for St. Louis encephalitis virus derived from six resident bird species

Species	*s*	*i*	*d*	C_i	C_r
Bay-winged Cowbird	0.14	0.12	1	0.02	1
Spot-winged Pigeon	0.87	0.23	1.4	0.28	14
House Sparrow	0.87	0.65	1	0.57	29
Shiny Cowbird	1	0.98	1	0.98	49
Picui Ground-Dove	0.73	1.03	2.2	1.65	83
Eared Dove	1	2.94	3.3	9.70	485

C_i = host competence index, C_r = relative host competence index; *d* = viremia mean duration; *i* = infectiousness; *s* = susceptibility.

DISCUSSION

In Argentina, the avian hosts for SLEV are poorly characterized because serological prevalence studies in free-ranging

TABLE 2
Host capacity index calculated for six resident bird species during the human encephalitis outbreak by St. Louis encephalitis virus in Córdoba (2005)

Species	C_i	Estimated abundance	Infection rate*	M_i	M_{ir}
Shiny Cowbird	0.98	40	0	0	0
Spot-winged Pigeon	0.28	47	0	0	0
Bay-winged Cowbird	0.02	99	0.03	0.06	1
House Sparrow	0.57	493	0.04	11	183
Picui Ground-Dove	1.65	301	0.15	74	1233
Eared Dove	9.70	303	0.12	352	5867

C_i = host competence index; M_i = host capacity index; M_{ir} = relative host capacity index.
* Seroprevalence data obtained from Diaz et al.[17]

bird populations are the only studies that have been carried out.[15,17] In this study, we tested the host competence and host capacity of six bird species for SLEV. Viremias developed by Columbiformes species (Eared Dove, Picui Ground-Dove, and Spot-winged Pigeon) were higher and longer than those registered for Passeriformes species (House Sparrow, Bay-winged Cowbird, and Shiny Cowbird). All evaluated species developed mean viremias higher than MIT for *Cx. quinquefasciatus* mosquitoes (2.9 $\log_{10}$ PFU/mL); this lasted only one day in Bay-winged Cowbird, House Sparrow, and Shiny Cowbird. Although the Spot-winged Pigeon developed viremias lasting 4 days, its host competence index was low (C_i = 0.32). This finding indicates the high influence of viremia titer over the generation of infectious *Cx. quinquefasciatus* mosquitoes. Besides the titer and duration of viremia, susceptibility to viral infection was an important factor influencing the competence indexes.[24–26] For example, all inoculated individuals of the Eared Dove developed detectable viremias (100%), whereas only 14% of inoculated Bay-winged Cowbird did. This finding opens a new question about the relationship between the role as amplifying host and intrinsic features such as resistance (neither viremia nor antibodies develop) and tolerance (development of viremia but no antibodies). Previous host competence assays remarked the infection resistance of icterid species stating that life history traits such as mating system, geographic range, and breeding system and phylogenetic factors influence the outcome of viral infection.[27] Further studies that focus on evaluating these attributes are needed.

House Sparrow represents one of the most important avian hosts for SLEV in the United States.[1] Current reported evidence indicates that this species has a poor role as amplifying host for SLEV in the central area of Argentina. Monath et al.[15] analyzed 230 sera samples of House Sparrow with no positive samples. During the 2005 outbreak in Córdoba city, Diaz et al.[17] detected seropositive House Sparrows at low levels (3.9%). Based on our host competence assays (C_i = 0.65; M_i = 0.10), we can confirm that House Sparrows can amplify SLEV, but its contribution might be a small fraction to the total viral flow in an ecosystem. It is interesting to point out the differential viral response detected between house sparrows from Córdoba (Argentina) and Colorado (United States) inoculated with the same viral strain (CbaAr-4005). Diaz et al.[28] inoculated adult house sparrows collected in Ft. Collins subcutaneously. Inoculated sparrows developed higher (2.3–5.9 $\log_{10}$ PFU/mL) and longer lasting viremias (mean = 2.5 days) than those observed in the present assay (mean = 1 day; 3.3–4.5 $\log_{10}$ PFU/mL). Recently, evidence for co-evolution of WNV in House Sparrow was reported in the United States.[29] According to this study, actual House Sparrow populations are more resistant to infection by modern WNV strains. Host competence differences observed among Argentinean and U.S. House Sparrow populations[28] and variation detected among inoculated bird species could reflect some degree of co-evolution for SLEV strains. Further assays should test adaptation of SLEV in native bird species.

In contrast to findings observed in the United States, where Passeriformes are the main SLEV hosts, our results pointed out the importance of Columbiformes (Eared Dove and Picui Ground-Dove) as amplifying hosts for SLEV in Argentina. This ecological finding could explain the differences observed in the epidemiological behavior of SLEV between Argentina and the United States. In Argentina, SLEV was not considered a public health concern until its emergence in 2002 when outbreaks were reported mainly in the central area (Córdoba, Entre Ríos, and Buenos Aires).[2,4,7] In the last decade, the central area of Argentina has seen intensive land-use changes, basically transforming autochthonous vegetation (grasslands and thorn forest) into croplands.[30] As a result of this environmental disturbance, Eared Dove and Picui Ground-Dove populations have been increasing in the agricultural area of Argentina (Córdoba, Entre Ríos, and Santa Fe) during the last 10 years.[31] Most of the outbreaks due to SLEV took place in cities (Córdoba, Paraná, Santa Fe, and Buenos Aires) where the Eared Dove constitutes an abundant species in urban bird assemblages (A. Diaz, personal communication). Eared Dove flocks usually feed on agricultural areas during the day and use cities as roosting places, thereby generating a synchronization with the feeding time period of *Cx. quinquefasciatus*, one of the SLEV mosquito vectors. These movements could allow for the exchange of SLEV strains through different environments and also permit the persistence of the virus in urban areas. Both competent dove species (Eared Dove and Picui Ground-Dove) are natives of South America and can be found in a variety of ecosystems (grasslands, croplands, steppes, savannas, and disturbed forest). They are well adapted to urban areas where they reproduce year round, with the peak of chick production during spring and summer.

All evaluated avian species developed infectious viremia ($C_i > 0$); however, only Eared Dove, Picui Ground-Dove, and House Sparrow contributed to the viral flow during the 2005 SLEV outbreak in Córdoba city (Table 2). Spatial analysis of the host capacity index during the SLEV encephalitis outbreak (Supplemental Material) allowed us to identify variations in the host amplification role among neighborhoods in Córdoba city. Bay-winged Cowbird, Shiny Cowbird, and Spot-winged Pigeon did not represent a source of viral amplification for the vector because apparently they were not exposed to infectious mosquitoes' bites. Only the Picui Ground-Dove played a role as host in all analyzed sites and the Eared Dove did so only in Bajo Grande (Supplemental Figure 1). This fact is related to the absence of seropositive Eared Dove individuals found during the outbreak and essentially because only a few sera samples were analyzed per site (Botanic Garden = 10, Camino San Carlos = 6, and Villa Parque = 2). Eared Dove is quite difficult to collect with mist nets; thus, it is not well represented in these collections. Therefore, our estimation for the host capacity index by site could be biased. However, previous research indicate that the eastern area of Córdoba city (Bajo Grande) has landscape determinants that allow high infected human

populations, promote *Cx. quinquefasciatus* mosquito vector populations, and potentially serve as roosting site for Eared Dove flocks.[32,33] In line with these findings, the highest seroprevalence values in birds were detected in the same area during the outbreak.[17]

Considering the extreme intrinsic aptitude to amplify SLEV and its abundance in urban habitats, Eared Dove could be considered as a superspreader host.[34] Although the abundance of evaluated bird species represent up to 70% of the total, a study must still be carried out to analyze the amplifying role of other avian species such as Great Kisadee (*Pitangus sulphuratus*), Rufous Hornero (*Furnarius rufus*), Brown Cachalote (*Pseudoseisura lophotes*), Rufous-collared Sparrow (*Zonotrichia capensis*), and Creamy-billed Thrush (*Turdus amaurochalinus*), all of which are often found infected by SLEV and abundant in urban avian communities.[17] Characterizing and evaluating bird species' role as hosts for SLEV give us the foundation necessary to know its neglected ecology and to understand the causes that promote its emergence as a human pathogen in South America.

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Authors' addresses: Adrián Díaz, Fernando S. Flores, Agustín I. Quaglia, and Marta S. Contigiani, Laboratorio de Arbovirus, Instituto de Virología "Dr. J. M. Vanella," Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina, E-mails: adrian.diaz@conicet.gov.ar, fernandosflores@gmail.com, quaglia.agu@gmail.com, and martascontigia@hotmail.com.

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