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Alphaviruses: Serological Evidence of Human Infection in Paraguay (2012–2013)

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Abstract

Introduction: Alphaviruses can produce febrile illness and encephalitis in dead-end hosts such as horses and humans. Within this genus, the Venezuelan Equine Encephalitis virus (VEEV) complex includes pathogenic epizootic subtypes and enzootic subtypes that are not pathogenic in horses (except subtype IE, Mexican strains), although they can cause febrile symptoms in humans. The Rio Negro virus (RNV—VEEV subtype VI) circulates in Argentina, where it was associated with undifferentiated febrile illness. Mayaro (MAYV) and Una (UNAV) viruses belong to a different group, the Semliki Forest virus complex, with confirmed circulation.
Objective: The present study aimed to determine RNV, MAYV, and UNAV seroprevalences by plaque reduction neutralization test in 652 samples of Paraguayan individuals mainly from the Central Department, between years 2012 and 2013.
Methods: Samples with antibodies titer >1:20 against RNV were also tested for Mosso das Pedras—subtype IF, subtype IAB, and Pixuna (PIXV)—subtype IV viruses that belongs to VEEV antigenic complex.
Results: The overall seroprevalence of RNV was 3.83%, and for UNAV it was 0.46%, and no neutralizing antibodies were detected against MAYV in the studied population. Two of the twenty-seven heterotypic samples were positive for PIXV. The 50.1% of neutralizing antibody titers against RNV were high (equal to or greater than 1/640), suggesting recent infections. The effect of age on the prevalence of RNV was negligible.
Conclusions: These results bring new information about neglected alphaviruses in South America, and these data will serve as the basis for future studies of seroprevalence of other VEEV, and studies to search potential hosts and vectors of these viruses in the region.

Keywords: alphaviruses, Paraguayan human population, serological evidence, Una virus, Venezuelan Equine Encephalitis virus

Introduction

THE *ALPHAVIRUS* GENUS (family Togaviridae) includes 31 viral species (International Committee on Taxonomy of Viruses 2015; https://data.ictvonline.org/taxonomy-search.asp?msl_id=30), which are clustered into two groups according to their geographical location: Old World viruses, associated with febrile illness and arthralgia in humans, such as Chikungunya virus (CHIKV), and New World viruses that cause encephalitis in humans and horses, such as Venezuelan Equine Encephalitis virus (VEEV) (Strauss and Strauss 1994, De Figueiredo and Figueiredo 2014).

The VEEV complex comprises six antigenic subtypes that are divided into epizootic (IAB and IC) and enzootic subtypes. Epizootic subtypes are highly pathogenic to equines and humans, and emerge periodically causing outbreaks with variable

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morbidity and mortality. Enzootic subtypes are also important to human health because they can cause febrile illnesses with similar clinical symptoms to Dengue virus (DENV) (Weaver et al. 2004, Taylor and Paessler 2013). The Rio Negro virus (RNV, VEEV subtype VI) is an enzootic subtype that was first detected in Argentina between 1978 and 1980 (Mitchell et al. 1985). It was associated with an outbreak of DENV-like febrile illness (Contigiani et al. 1993). Molecular and serological studies in recent years show an expansion of its circulation into new regions of Argentina, probably due to climate and environmental changes that influence the ecology of its vectors and hosts (Pisano et al. 2013).

The Mayaro virus (MAYV) is closely related to CHIKV, and both produce similar symptoms that make them clinically indistinguishable (Strauss and Strauss 1994, Tesh et al. 1999, Coimbra et al. 2007, Griffin 2007, De Figueiredo and Figueiredo 2014). MAYV was first isolated in 1954 in Trinidad from an ill human (Anderson et al. 1957). Its circulation was detected later in Bolivia, Brazil, Colombia, Venezuela, Peru, and Central American countries (Powers et al. 2006). In addition, there have been outbreaks of this virus in rural and urban areas of Brazil and Peru (Azevedo et al. 2009, Mourão et al. 2012). In fact, MAYV, Oropouche virus, and DENV are considered the most common cause of febrile syndrome in Brazil. Therefore, MAYV was included as a notifiable disease in that country since 2011 (Azevedo et al. 2009, Pego et al. 2014). The Una virus (UNAV), phylogenetically related to MAYV, has not been associated with specific human diseases and it is widely distributed, with low seroprevalences in tropical and subtropical regions of South America (Walder et al. 1984, Karabatsos 1985).

Due the reports of circulation of alphavirus in neighboring countries, as previously mentioned, the presence of a great variety of mosquito species (Belkin et al. 1968) in the territory that can act as vectors of alphavirus, in addition to climatic conditions, deforestation, and other favorable factors, it is expected that alphaviruses circulate in Paraguay. Therefore, we aimed to evaluate for the first time the degree of exposition to alphavirus infection (RNV, MAYV, and UNAV) in Paraguayan human population during 2012 and 2013.

Materials and Methods

Design and study population

The study area is located in the Eastern region of Paraguay, including the capital city Asunción (between 25° 00′–26° 00′ South and 57° 11′–57° 50′ West) and the neighboring departments shown in Figure 1. The type of climate varies from tropical to subtropical, and it is governed by tropical and polar air masses with extremely hot and rainy summers and dry winters characterized by lower temperatures. The average annual temperature is 24°C and a maximum annual average is 29°C. The departments included in the study have similar characteristics of temperature, rainfall, as well as circulation of mosquitoes, and 99% of the population comes from nearby departments that are a maximum of 229 kilometers (Misiones) of Asuncion (Barrios Kuck et al. 2012).

A cross-sectional study was carried out in 652 blood samples taken from human participants ranging between 1 and 80 years of age. From the total of human participants, 131 (20.1%) were enrolled during 2012, and 521 (79.9%) participants were enrolled during 2013 in reference health centers (Clinical

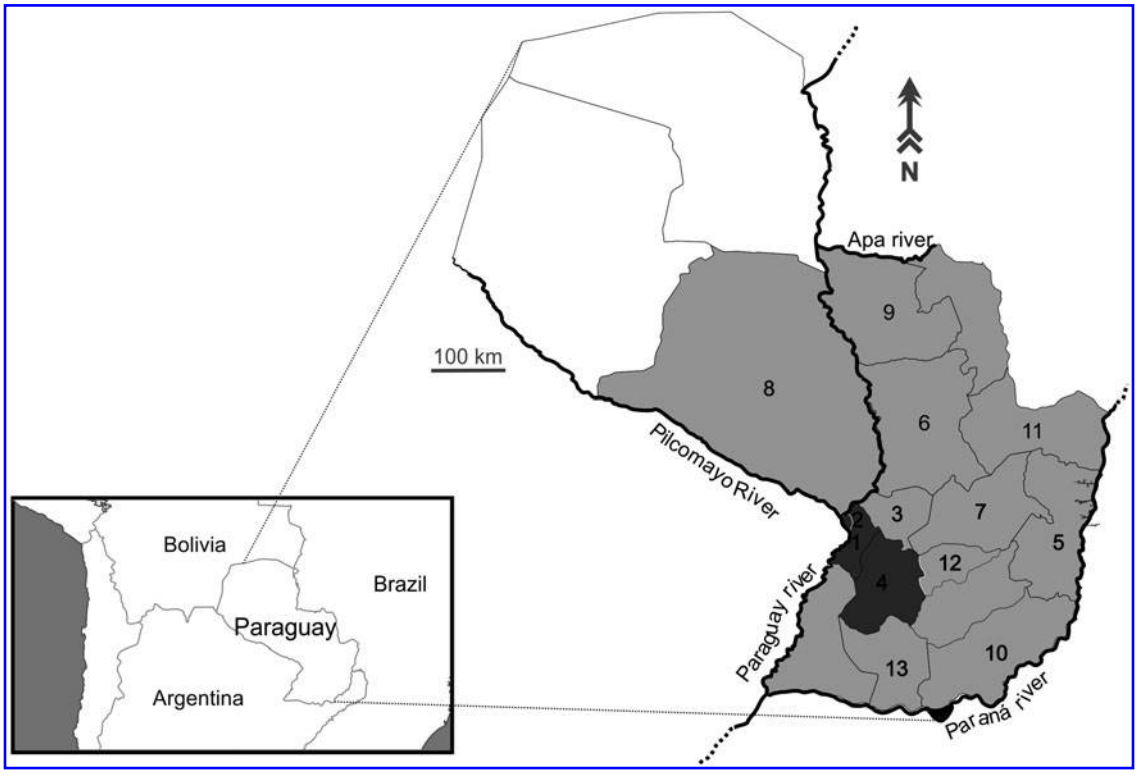

FIG. 1. Geographical distribution of the sampled departments and those that were positive for at least one RNV case in *dark gray*. Each department is listed as follows; 1. Central. 2. Capital district. 3. Cordillera. 4. Paraguarí. 5. Alto Paraná. 6. San Pedro. 7. Caaguazú. 8. Presidente Hayes. 9. Concepción. 10. Itapúa. 11. Canideyú. 12. Guará. 13. Misiones. RNV, Rio Negro virus.

Hospital, National University of Asunción, and in the ''Central Hospital of the Social Security Institute'' (HC-IPS).

The participants did not present symptoms of febrile disease at the time of sampling. The date of collection, age of individuals (years), sex, and location were registered. Sera were provided by the mentioned health centers as an opportunity sampling. The study was confidential and anonymous, and it was approved by the Scientific and Ethics Committees of the ''Research Institute in Health Sciences, National University of Asunción'' (Project code: P34/2012). The date of collection, age of individuals (years), sex, and location were registered.

Viral strains

Viruses used in this study were: RNV (VEEV subtype VI) strain AG80-663 (Mitchell et al. 1985), VEEV IAB strain TC83 (Berge et al. 1961), Mosso das Pedras virus (MDPV, VEEV subtype IF) strain 78V3531 first isolated in 1978 in Brazil (Powers et al. 1997), Pixuna virus (PIXV; VEEV subtype IV) strain BeAr35645 (Shope et al. 1964), UNAV AN979 (Sabattini et al. 1998), and MAYV AR20290 (Espósito and da Fonseca 2015). All these viral strains are available in the Arbovirus laboratory, at the Virology Institute ''Dr. Vanella''—National University of Córdoba, where the serological tests were performed. Viral stocks were prepared as a 10% dilution of infected suckling mice brain in Minimum Essential Medium with 10% fetal bovine serum and 1% gentamicin, and then centrifuged at $11.400 \times g$ for 30 min so as to decontaminate.

Serological tests

Detection of neutralizing antibodies was performed using plaque reduction neutralization tests in African green monkey kidney cells (*Cercopithecus aethiops*, Vero 76 ATCC® CRL-587) as previously described (Earley et al. 1967).

Sera (1:10) were incubated with 100 plaque-forming units (PFU) of each virus. Those that neutralized at least 80% of inoculated PFU were considered positive and they were further titrated with the same technique, using two-fold serial dilutions, to determine the endpoint titer.

Previous studies had shown cross reactions among some subtypes of VEEV in one or both directions, hence they were considered for a correct interpretation of the present results. The samples that were positive for RNV were submitted to the detection of antibodies against other VEEV subtypes with reported crossed reactions and recent circulation in South America (Pisano et al. 2013): MDPV-subtype IF, subtype IAB, and PIXV-subtype IV.

According to the guidelines of the Centers for Disease Control and Prevention (CDC, www.cdc.gov), a titer difference of four-fold or greater is used to confirm exposition; when a four-fold difference is not observed, the specific exposition to the agent cannot be defined. Therefore, in heterotypic patterns, only samples with a virus with neutralization titers at least four-fold greater than the other viruses, were considered positive. Those samples with titer difference lower than four-fold were considered Alphavirus antibody positive with no specific virus identified and labeled as ''Indeterminate.''

Statistical analysis

The influence of year of sampling, gender, and age (as discrete predictor) on the exposure to RNV was analyzed by generalized linear model (GLM) with binomial error distribution and log link function. Also, the percentile 2.5% and 97.5% of the prevalence ratio (PR) was estimated after 1000 bootstrap replicates using the packages prLogistic (Ospina and Amorim 2013). All the analytical and graphical procedures were made with the statistical R platform (R Core Team 2016), packages visreg (Breheny and Burchett 2017), and ggplot2 (Wickham 2009).

Table 1. Rio Negro Virus Human Seroprevalence Distribution by Department

Variables	N	*No. positive*	*% Positive [$CI_{95\%}$]*[a]
Department			
Central	436	19	4.36 [2.64–6.72]
Capital	115	5	4.35 [1.43–9.85]
Cordillera	38	0	0 [0–9.25]
Paraguarí	18	1	5.56 [0.14–27.29]
Alto Paraná	9	0	0 [0–33.63]
San Pedro	8	0	0 [0–36.94]
Caaguazú	7	0	0 [0–40.96]
Presidente Hayes	6	0	0 [0–45.93]
Concepción	4	0	0 [0–60.24]
Itapúa	3	0	0 [0–70.76]
Canindeyú	2	0	0 [0–84.19]
Guairá	1	0	0 [0–97.5]
Misiones	1	0	0 [0–97.5]
NA[b]	4	0	
Total	652	25	3.83 [2.5–5.61]

[a]Percentage and 95% CI.
[b]NA, missing department record.
CI, confidence interval.

Results

The study covered 13 out of 17 departments of Paraguay, and 93.10% (607/652) of the participants was distributed in four of them. Only three of these departments had at least one RNV-positive participant (Fig. 1), and they showed the same seroprevalence (range: 4.35–5.56; p-value = 0.759) (Table 1).

Table 2. Rio Negro Virus Human Seroprevalence, Grouped by Age Range, Gender, and Year of Sampling

	Total		
	N	*No. positive*	*% Positive [$CI_{95\%}$]*[a]
Age class[b]			
1–22	146	2	1.37 [0.17–4.86]
23–32	189	4	2.12 [0.58–5.33]
33–42	157	9	5.73 [2.65–10.6]
43–80	153	10	6.54 [3.18–11.69]
Gender			
Female	256	8	3.12 [1.36–6.06]
Male	394	17	4.31 [2.53–6.82]
Year			
2012	131	5	3.82 [1.25–8.68]
2013	521	20	3.84 [2.36–5.87]
Total	652	25	3.83 [2.5–5.61]

[a]Percentage and 95% CI.
[b]Interquartile age class.

Table 3. Generalized Linear Model Output

	Prevalence ratio (PR)	*PR* $[P_{2.5}–P_{97.5}]$[a]	*Pr(>|z|)*
Intercept	0.004		<0.001
Age (discrete)	1.049	[1.020–1.085]	0.001
Gender[b]	0.730	[0.244–1.826]	0.427
Year[c]	1.625	[0.654–7.817]	0.420

[a]2.5th and 97.5th percentile of the prevalence ratio after 1000 bootstrap replicates.
[b]Gender reference: female.
[c]Year reference = 2012.

The ages of the screened participants ranged from 1 to 80 years of age, with median value of 31 years (interquartile range = 19). Out of the total number of participants (652), 394 (60.43%) were male, and 256 (39.26%) were female; no gender data were available for the remaining 2 (0.3%). The characteristics of the analyzed population and the RNV seroprevalence are summarized in Table 2. After a first screening, from the total set of samples, 3.83% (25/652; confidence interval [CI]: 2.50–5.61%) were positive for RNV, whereas 0.44% (3/376; CI: 0.09–1.30%) were positive for UNAV with low titers (between 1/20 and 1/40). No neutralizing antibodies were detected against MAYV in the analyzed population. The annual prevalence was 3.82% (5/131; CI: 1.25–8.68%) in 2012, and 3.84% (20/521; CI: 2.36–5.87%) in 2013. The seroprevalence of RNV in females were 3.12% (8/256; CI: 1.36–6.06%) and 4.31% (17/394; CI: 2.53–6.82%) in males. Likewise, the exposure to RNV across gender (PR: 0.73; CI: 0.24–1.82; *p*-value: 0.427) and year of sampling (PR: 1.62; CI: 0.65–7.82; *p*-value: 0.420) were similar (Tables 2 and 3).

The age of the youngest RNV-seropositive participant was 15, and the count of seropositive cases increased slightly with age (Table 2). Furthermore, participants showed a slight increase in the risk of exposure to RNV even when the age was included as a quantitative discrete predictive variable in the fitted GLM (PR: 1.05; CI: 1.02–1.08; *p*-value: 0.001) (Table 3; Fig. 2).

Finally, serological crossreactions between RNV and the other members of the VEEV complex were tested, and these results are summarized in Table 4.

Following the criterion suggested by the CDC, 8 out of 35 samples did not show a four-fold titer difference and they were considered ''indeterminate.'' In three cases, the sample volume was not enough for testing. Out of the remaining 27 samples, 4 were monotypic (only positive for RNV) and 23 heterotypic (23/27; 85.18%; CI: 66.27–95.81%), and none of them was positive for IAB. Two of these heterotypic samples were positive for PIXV (2/27; 7.41%; CI: 0.91–24.23%).

Discussion

Based on previous studies, which have reported circulation of RNV in Argentina (Mitchell et al. 1985, Pisano et al. 2010, 2013), as well as the circulation of UNAV and MAYV in South America (Tesh et al. 1999, Díaz et al. 2007, De Figueiredo and Figueiredo 2014), in the present study the seroprevalence of RNV, UNAV, and MAYV was analyzed for the first time in human sera of Paraguayan individuals.

The seroprevalence of RNV in the studied population was 3.83% (25/652), and it represents the first report of its circulation within humans in Paraguay. Various studies show RNV circulation in a wide region of northern Argentina, including cities close to Paraguay in the province of Formosa, located by the Paraguay river, which represents the limit between the countries (Mitchell et al. 1985, Contigiani et al. 1993, Cámara et al. 2003, Pisano et al. 2013). Therefore, Paraguay is the second country that reports serological evidence of circulation of RNV (VEEV subtype VI). Paraguay shares environmental conditions with the Northeast and Northwest of Argentina, where endemic circulation of this virus was also recorded.

In our study, no crossreactions were observed among the tested alphaviruses (RNV, MAYV, UNAV). However,

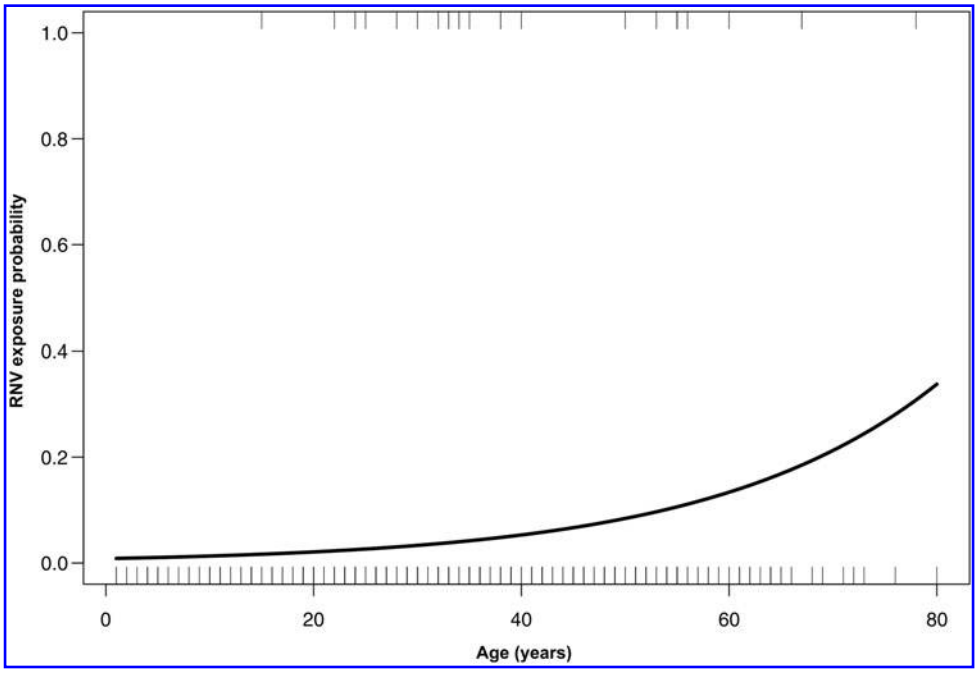

FIG. 2. RNV exposure probability by age (years).

Table 4. Titer Values of Neutralizing Antibodies Against Rio Negro Virus, Mosso Das Pedras Virus, Pixuna Virus, and Venezuelan Equine Encephalitis Virus IAB by Means of Plaque Reduction Neutralization Test, in Human Population of Paraguay (2012–2013)

Virus				
RNV (VEEV subtype VI)	*MDPV (VEEV subtype IF)*	*VEEV IAB*	*PIXV (VEEV subtype IV)*	*Result interpretation*
≥1280	NA	NA	NA	—
≥320	NA	NA	NA	—
≥1280	<20[a]	<20	320	RNV
40	<20	<20	160	PIXV
160	<20	<20	<20	RNV
≥1280	80	-<20	≥1/640	Indeterminate[b]
≥1280	<20	<20	160	RNV
1280	<20	<20	160	RNV
40	<20	<20	<20	RNV
≥1280	<20	<20	320	RNV
1280	<20	<20	80	RNV
1280	<20	<20	80	RNV
≥1280	<20	<20	80	RNV
640	<20	<20	40	RNV
80	<20	<20	40	Indeterminate
≥1280	<20	<20	80	RNV
1280	<20	<20	20	RNV
1280	<20	<20	80	RNV
640	<20	<20	160	RNV
80	<20	<20	160	Indeterminate
≥5120	20	<20	320	RNV
≥320	<20	<20	320	Indeterminate
40	<20	<20	160	PIXV
1280	<20	<20	320	RNV
≥80	<20	<20	80	Indeterminate
≥80	<20	<20	20	RNV
≥1280	NA	NA	NA	—
160	<20	<20	<20	RNV
40	<20	<20	40	Indeterminate
80	<20	<20	20	RNV
1280	<20	<20	80	RNV
640	<20	<20	80	RNV
40	<20	<20	20	Indeterminate
≥ 5120	<20	<20	160	RNV
≥1280	<20	<20	80	RNV
160	<20	<20	<20	RNV
40	<20	<20	20	Indeterminate
80	<20	<20	20	RNV

[a]<20: negative.
[b]Indeterminate: following the criteria of the Centers for Disease Control and Prevention, samples that did not have a difference of four times in the title.
RNV, Rio Negro virus; MDPV, Mosso das Pedras; PIXV, Pixuna; VEEV, Venezuelan Equine Encephalitis virus; NA, not analyzed.

heterotypic reactions were observed within VEEV subtypes, mostly between RNV and PIXV, which is consistent with previous results obtained by other authors (Cámara 1997, Pisano et al. 2013). The high titers observed for PIXV (320), and the fact that PIXV is not neutralized by antibodies against subtype IF, could be interpreted as specific infections by PIXV, whereas low titers are likely due to unspecific cross-reactions. Those undetermined samples that showed high titers for RNV and PIXV (≥320), might suggest sequential infections with both viruses.

A 50.1% of the titers found in RNV positive were equal or greater than 640 suggesting a recent infection or circulation in the studied population. However, no positive cases have been found in individuals under 15 years, possibly because this age range was not well represented in the sample (8.5%). Although the association between age and exposure rate to RNV was statistically relevant, the effect of age, in terms of effect size, showed a low signal. Our sample size provides sufficient statistical power to find differences in the studied population. However, the biological magnitude of the age–RNV association could be masked. Therefore, this scenario could be alternatively explained in terms of an apparent low circulation of RNV in the population, reducing the probability of observing the size of the effect imposed by age. For instance, Vittor et al. (2016) showed a low seroprevalence of VEEV in Darien province, Panama, and also found an increasing pattern of exposure among age that could be expected for an endemic virus. The authors also state that

antibody titers may wane over time, leading to underestimates of prior exposure in older age groups (Vittor et al. 2016). Finally, the age structure of the studied population might not be reflecting the total population, so the strong expected association between RNV and age could be underestimated by this fact.

Moreover, to confirm the recent infection, other tests would be necessary as virus isolation, IgM, and molecular detection.

Regarding UNAV and MAYV, Díaz et al. (2007) observed a high seroprevalence for UNAV (73%) and no positive sera for MAYV in black howler monkeys (*Aloutta caraya*) from subtropical regions of Argentina and Paraguay (Díaz et al. 2007). In our study also, no positive sera were detected for MAYV and the observed seroprevalence (0.44%) and titers were very low for UNAV. These differences should indicate that UNAV is circulating more intensely in sylvatic areas than in urban regions. Our observations highlight the need for more serosurvey studies and detailed eco-epidemiological studies to describe the circulation of UNAV and possible introductions of this virus to urban environments.

There are limitations in the design of our study. As a hospital-based study, there is a potential bias related to the selection of the population, where the participants included come mostly from the capital and the Central Department, and only 8% of the sampled population was younger than 15 years of age, which may lead to erroneous extrapolations to the general population of the country. However, our results are consistent with previous studies using neutralization assays, including cities close to Paraguay demonstrating a serological evidence of RNV circulation in the region (Mitchell et al. 1985, Contigiani et al. 1993, Cámara et al. 2003, Pisano et al. 2013).

In summary, this is the first serological evidence of circulation of *Alphavirus,* mainly VEEV (RNV and PIXV) in Paraguay. However, the impact of this virus on public health in this region is still unknown. It is known that RNV causes a febrile syndrome similar to DENV and previous reports in other cities in America, where DENV outbreaks occur, show that there may be an underestimation of the actual number of cases by VEEV (Aguilar et al. 2004). All of the above stresses the need to intensify the surveillance of this virus and include representatives of the genus *Alphavirus* in the diagnosis of febrile illness cases, and to perform new isolations and eco-epidemiological studies designed to identify potential hosts and vectors of these *Alphavirus* in Paraguay. Our results remark the importance of improving the specific and sustained arboviral epidemiological surveillance in our country.

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Author Disclosure Statement

No competing financial interests exist.

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