Trans R Soc Trop Med Hyg
doi:10.1093/trstmh/trt081

ORIGINAL ARTICLE

West Nile virus surveillance, Brazil, 2008–2010

Tatiana Ometto[a,*], Edison Luiz Durigon[a], Jansen de Araujo[a], Rosalie Aprelon[b], Daniel Moura de Aguiar[c], Guacyara Tenorio Cavalcante[d], Rosane Marini Melo[e], José Eduardo Levi[f], Severino Mendes de Azevedo Júnior[g], Maria Virgínia Petry[h], Isaac Simão Neto[i], Patrícia Serafini[i], Eliana Villalobos[j], Elenice Maria Sequetin Cunha[j], Maria do Carmo Custódio S. H. Lara[j], Alessandra Ferreira Dales Nava[k], Marcello Schiavo Nardi[l], Renata Hurtado[a,m], Roberta Rodrigues[g], Angelo Luís Sherer[h], Janete de Fátima Martins Sherer[h], Marcelo Plaisant Geraldi[f], Marina Maria Moraes de Seixas[a], Cassio Peterka[l], Debora de Souza Bandeira[l], Jennifer Pradel[b], Nathalie Vachiery[b], Marcelo Bahia Labruna[m], Luiz Marcelo Aranha de Camargo[n], Robert Lanciotti[o] and Thierry Lefrançois[b]

[a]BSL3+ Laboratório de Virologia Clínica e Molecular Instituto de Ciências Biomédicas (ICB), Universidade de São Paulo (USP), 05508-900 São Paulo, Brasil; [b]CIRAD UMR CMAEE CIRAD-INRA, 97170 Petit Bourg, Guadeloupe, France; [c]Departamento de Clínica Médica Veterinária, Universidade Federal do Mato Grosso (UFMT), 78060-900 Cuiabá, Brasil; [d]Instituto de Física, Universidade de São Paulo, 05508-090 São Paulo, Brasil; [e]Instituto de Defesa Agropecuária do Estado do Mato Grosso (INDEA), 78050-970 Cuiabá, Brasil; [f]Laboratório de Virologia, Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, 05403-000 São Paulo, Brasil; [g]Universidade Federal Rural de Pernambuco (UFRPE), 52171-900 Recife, Brasil; [h]Universidade do Vale do Rio dos Sinos (UNISINOS), 93022-000 São Leopoldo, Brasil; [i]Centro Nacional de Pesquisa e Conservação de Aves Silvestres (CEMAVE), 88053-700 Florianópolis, Brasil; [j]Centro de Pesquisa e Desenvolvimento de Sanidade Animal, Instituto Biológico, 04014-002 São Paulo, Brasil; [k]EcoHealth Alliance, 05508-270 São Paulo, Brasil; [l]Instituto de Pesquisas Ecológicas (IPÊ), 19280-000 Teodoro Sampaio, Brasil; [m]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 05508-270 São Paulo, Brasil; [n]Instituto de Ciências Biomédicas V, Universidade de São Paulo, 78965-300 Monte Negro, Brasil; [o]Centers for Disease Control & Prevention (CDC), 80521 Fort Collins, United States

*Corresponding author: Tel: +55 11 3091 7293; Fax: +55 11 3091 7354; E-mail: tatiometto@usp.br

Received 13 March 2013; revised 25 July 2013; accepted 31 July 2013

Background: West Nile virus (WNV) is an emergent pathogen that is widely distributed in North and Central America. The recent introduction in South America has focused attention on the spread of WNV across Southern American countries. The transmission network involves mosquitoes, birds, horses and humans.

Methods: The serological evaluation of sera from 678 equids and 478 birds was performed using a WNV-specific blocking ELISA, and only the positive results were confirmed by plaque reduction neutralisation tests (PRNTs). Molecular analysis was performed on sera from 992 healthy equids and on 63 macerates of brains from equids that died of encephalitis and had previously tested negative for other pathogens. We also tested swabs from 928 birds. The samples analysed were collected in different biomes of Brazil.

Results: We identified WNV antibodies by ELISA in thirteen equids and five birds, and $PRNT_{90}$ confirmed WNV positivity in four equid samples collected in 2009 in an area between the Amazon and the Pantanal. None of the ELISA positive bird samples were confirmed by $PRNT_{90}$, and all samples tested by RT-PCR were negative.

Conclusion: WNV circulation is confirmed by this large scale survey even in the absence of detection of clinical cases.

Keywords: West Nile virus, Serology, Molecular biology, Brazil, Equids, Migratory birds

Introduction

West Nile virus (WNV) is a mosquito-borne virus that belongs to the family *Flaviviridae* and the genus *Flavivirus*. WNV is maintained in nature by a mosquito–bird–mosquito cycle, and humans and other mammals act as dead-end hosts.[1] Human and animal outbreaks of WNV with neurological disorders have provided warnings of the changing impact of WNV fever on animal and public health.[2] WNV has emerged in the last three decades as a significant burden to public health and a major veterinary concern in Europe and the Americas.[3] The emergence of WNV, particularly the invasion into North America in 1999 and its subsequent spread throughout the Western Hemisphere,[3] corroborates the view that the virus is moving southward, placing millions of

individuals in South America and the Caribbean at risk for infection.[1] WNV is now recognised as the most widespread of the flaviviruses,[4] and the appearance in South American countries has stimulated intense interest. The first evidence of WNV activity in birds in South America was reported in Trinidad in 2004[5] and in Argentina in 2005.[6] Efforts to detect WNV-specific antibodies in resident and migrant birds in Brazil in 2002, 2003 and 2004 were unsuccessful.[7–9] WNV seropositivity in horses in South America was first reported in Colombia in 2004[10] and was subsequently reported in Venezuela,[11] Argentina[6] and Brazil.[2,12] The first report of WNV activity in South America surfaced in April 2006, when three horses died in Argentina.[13] However, the established transmission foci in Argentina are unknown[5] and have not yet been reported in Brazil.[14] A study in Brazil published in 2011 analysed 8703 human samples from blood banks, none of which were positive for the presence of the virus.[15] However, Brazil is a large tropical country with major ecological reserves that provide ideal conditions for many arboviruses, including WNV,[16] and it would not be surprising for WNV to circulate in several areas and biomes. Brazil has six different biomes with some particularities. The Amazon is the largest biodiversity reserve in the world and is dominated by a hot and humid climate and forests. In the Cerrado, there is a predominance of savannah formations and a hot sub-humid tropical climate, a dry season and a rainy season. The Atlantic Forest is a complex that includes mountain ranges, valleys and plateaus, with tropical rain forest vegetation and a hot and humid climate. The Caatinga is defined by the drought, heat and light, resulting in a steppe-like, thorny and deciduous savannah vegetation. The Pampa is marked by rainy weather, with freezing temperatures in the winter and vegetation consisting of pampa grass and shrubs. The Pantanal is characterised by long-term flooding; the predominant vegetation is savannah, but there are small areas of semi-deciduous and deciduous forests. Almost all of the Brazilian fauna are represented in the Brazilian Pantanal (Figure 1).[17]

Brazil has the largest herd of horses in Latin America and the third largest in the world. Including mules and donkeys, the total population is approximately 8 million head, equalling US$3.2 billion including herd management costs. Export expansion reached 524% between 1997 and 2009, from US$702 800 to US$4.4 million. Brazil is the eighth largest exporter of equine meat.[18]

Many questions regarding the potential for WNV spread in Brazil remain. The objective of this study was to address these questions by searching for evidence of WNV circulation in Brazil in resident equids and migratory and resident birds and by evaluating the importance of vaccinating horses before a possible Brazilian WNV outbreak.

Materials and methods

Study design

A serological survey was conducted of 678 equids, which were sampled between 2002 and 2009, and 478 birds, which were sampled between 2008 and 2010, in different Brazilian regions characterised by a variety of biomes (Figure 1).

The molecular survey was conducted on 928 samples (oral/cloacal swabs) from birds (2008 to 2010) and 992 serum samples from equids (2002 to 2009). We also performed molecular tests on the macerates of brains collected between 2008 and 2010 from 63 equids that died of encephalitis and had previously tested negative for other pathogens.

Equid study area

The sampled equids were privately owned and used primarily for cattle transport and handling. The equids were all asymptomatic at the time of sampling and had no history of vaccination against WNV or movement outside the area where they were sampled. For the retrospective study in 2002, we collected 99 samples, all tested by serological assay, in Monte Negro (Rondônia state) in the western Amazon.[19] The State Park Morro do Diabo, which is located in southwestern São Paulo state,[20] was the second retrospective study area from which 144 samples were collected in 2002 (86 equids) and 2006 (58 equids); all samples were tested by serological assay. The 2009 samples were collected in Mato Grosso state. There were a total of 570 samples of which, 217 were collected in Nova Brasilândia in the Cerrado region, and 218 collected from Juruena in northern Mato Grosso state. The remaining 135 (of the 570) samples came from 10 different cities in the same areas characterised by similar ecosystems and with increased farm activity, including Planalto da Serra, Arenápolis, Santo Antônio Leverger, Nova Bandeirantes, Jangada, Nova Mutum, Diamantino, Lucas do Rio Verde, Campo Verde and Poconé.

In addition, we performed molecular tests on the macerates of brains from 63 equids that died of encephalitis and had previously tested negative for herpes, rabies, western and eastern equine encephalitis, toxoplasma, *Neospora* and *Sarcocystis*. These samples were collected between 2008 and 2010 from the Southeast, Northeast and Central-West.

Bird study area

The sampled birds were migratory and resident, and asymptomatic or symptomatic at the time of sampling. The first sampling occurred in September 2008 in Glória city, Bahia state, in the Caatinga biome, with 71 samples collected and all tested by molecular assay. Between October 2008 and August 2009, we sampled 245 birds in the metropolitan region of Porto Alegre in the state of Rio Grande do Sul in four different city sites. All of these samples were tested by molecular assay, and 69 samples were tested by serology. In 2009, 400 bird samples were collected at Canela's Island in Pará state in the Amazon region, which is an important stopover site for migratory birds. All of these samples were tested by molecular assay, and 116 samples were tested by serology. From 2009 to 2010, we sampled 212 birds in the Lagoa do Peixe National Park in Rio Grande do Sul state. This national park is recognised as the largest migration point for birds in Brazil.[8] All of these samples were tested by molecular assay, and 136 samples were tested by serology. In 2010, we collected samples from 142 birds in the Reentrâncias Maranhenses environmental protection area, Maranhão state, which has been designated by the Western Hemispheric Shorebird Reserve Network as an internationally important site that receives large populations of migratory birds.[21] All of these samples were tested by serological assay. The last sampling was conducted in 19 birds in Fernando de Noronha Island, Pernambuco state, which is an oceanic island

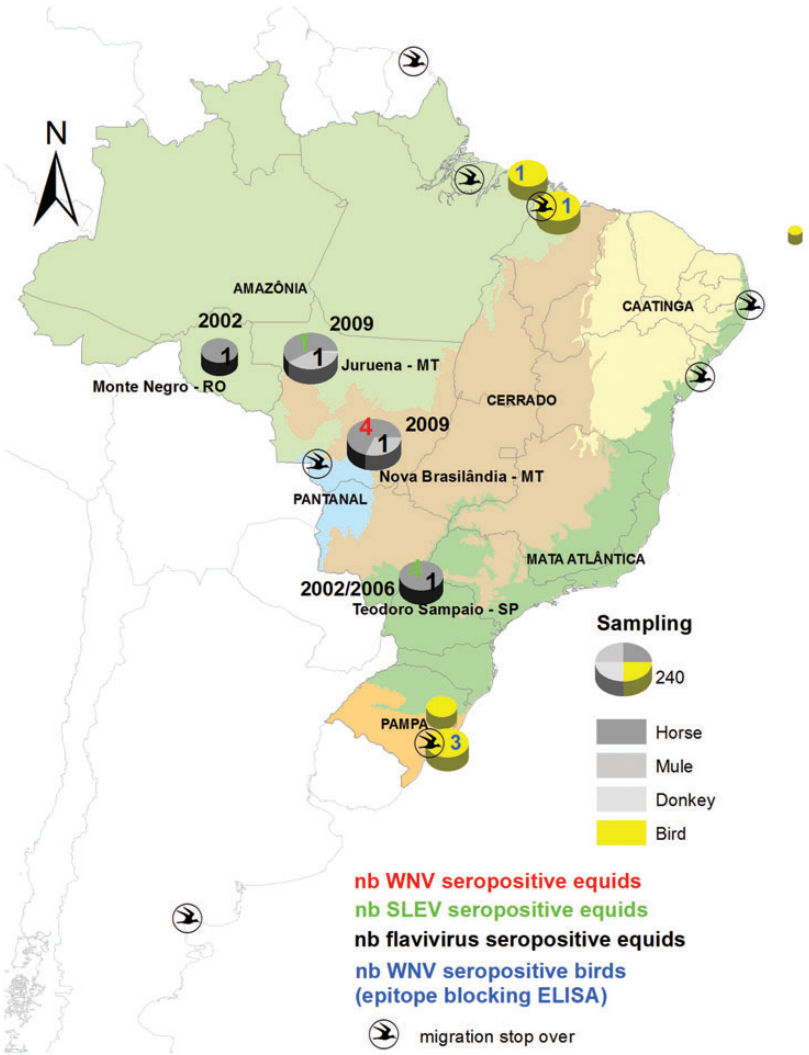

Figure 1. Site and year of sampling, major Brazilian biomes and serological results. The major biomes of Brazil are indicated by different colours as follows: Amazonia (light green), Cerrado (light brown), Pantanal (blue), Mata Atlantica (dark green), Caatinga (yellow) and Pampa (orange). The grey lines represent the borders of the Brazilian states. MT: Mato Grosso state; nb: number of; RO: Rondônia state; SLEV: St Louis encephalitis virus; SP: São Paulo state; WNV: West Nile virus.

located on the Brazilian coast. All samples were tested by molecular assay, and 15 were analysed by serological assay.

Serologic tests

Blocking ELISAs

ELISAs were performed using the WNV-specific monoclonal antibody 3.1112G (Chemicon, Temecula, CA, USA) as previously described.[22,23] The capacity of the test sera to prevent the binding of the WNV-specific monoclonal antibodies to the WNV antigen was compared to the blocking capacity of horse serum without antibodies to WNV. The sera were considered to be positive when the inhibition value was greater than 30% and to be ambiguous when the value was between 25% and 30%. This assay which has previously been used for epidemiological studies of horses and birds[24,25] has high specificity and sensitivity for WNV and has the advantage of being rapid, reproducible and less expensive than other methods.[4]

Plaque reduction neutralisation tests (PRNTs)

Positive and ambiguous samples were further evaluated using a $PRNT_{90}$, as previously described.[26,27] $PRNT_{90}$s were performed with WNV and St. Louis encephalitis virus (SLEV). A serum sample was considered to contain antibodies to WNV if it significantly inhibited the binding of monoclonal antibody 3.1112G by ELISA and had a 90% $PRNT_{90}$ titre to WNV that was at least 4-fold greater than the corresponding SLEV $PRNT_{90}$ titre. A serum sample was considered to contain antibodies to SLEV if the $PRNT_{90}$ titre to SLEV was at least 4-fold greater than the corresponding WNV titre. A serum sample was considered to have antibodies to a flavivirus of undetermined origin if it contained

ELISA-detected or neutralising antibodies but did not meet the criteria for WNV or SLEV infection.

Molecular tests

In house one-step real-time RT-PCR

One-step real-time RT–PCR was performed using specific primers and a probe designed according to the sequence of gene E (viral envelope) deposited in the GenBank database (Accession number: AY660002.1 - SEQ1F 5'-GCGATCTCTCCACCAAAGCT-3'; SEQ1R 5'-TGGGTCAGCACGTTTGTCAT-3'; SEQ1M1 FAM- CCATGGG AGAAGCTCACA 5 NFQ). For RNA extraction, a 5x MagMax™ - 96 Viral Isolation Kit and an AgPath-ID™ One-Step RT-PCR Kit were used according to the manufacturer's instructions (Ambion, Inc., Austin, TX, USA). Real-time RT-PCR was performed in an ABI 7300 PCR System (Applied Biosystems, Foster City, CA, USA). All procedures were conducted in a Biosafety Level 3^+ Laboratory ($BSL3^+$) at the Biomedical Institute at the University of São Paulo. The positive controls used were inactivated human plasma samples from blood donors, which were kindly provided by Dr Susan Stramer of the American Red Cross, NY, USA.

Cobas® TaqScreen West Nile Virus Test

An automated real-time RT-PCR method (Cobas® TaqScreen West Nile Virus Test, Roche, Pleasanton, CA, USA) that is routinely used in the United States and Canada to screen blood donors was adopted to evaluate the serum and brain macerate samples. The analytical sensitivity of this method is reported to be 40 copies/mL (95% CI: 35.1–47.8 copies/mL) for WNV lineage 1.[28]

Results

Of the 478 birds analysed, 5 (1.05%) tested positive based on the WNV-specific blocking ELISA. Of the 678 equids analysed (517 horses, 156 mules and 5 donkeys), 13 (1.9%) tested positive based on the WNV- specific blocking ELISA (Table 1). The results of the $PRNT_{90}$ did not confirm the flavivirus seropositivity for the 5 positive bird samples. Among the equid samples, the results of the $PRNT_{90}$ confirmed the flavivirus seropositivity for all 13 of the positive samples by specific blocking ELISA (WNV in 4 samples, SLEV in 5 samples and undifferentiated *Flavivirus* in 4 samples). The WNV and SLEV $PRNT_{90}$ titres ranged from 40 to 2560 (Table 1). Antibodies to SLEV were identified in horses in Teodoro Sampaio in 2002 and 2006 and in Juruena in Mato Grosso in 2009 (Figure 1). Equids with antibodies only to WNV were identified in Nova Brasilândia in Mato Grosso state in 2009 (1.8%, 3 horses and 1 mule of 217 samples). The WNV-seropositive horses were between 5 and 8 years old.

All of the bird and equid samples (928 birds and 570 equids) tested by one-step real-time RT-PCR and the equid samples (422 serum and 63 brain) tested by automated real-time RT-PCR were negative for the presence of WNV viral RNA.

Discussion

This is the largest molecular and serological screening of WNV in migratory birds and equines in Brazil. The spread of WNV throughout North America and periodic outbreaks of this virus in Eastern and Western Europe have increased worldwide interest in understanding the viral, host and ecological factors that result in WNV outbreaks.[3] Although WNV is considered a public health problem, there have been no reports of human cases in Brazil. A recent molecular study analysed 8703 blood bank sera samples in 2011 in Amazon, São Paulo and Mato Grosso do Sul states,[15] in locations close to those where we confirmed positivity for WNV antibodies in four equids. No positive results were obtained for the presence of WNV in the sera of healthy donors,[15] despite the established prevalence of 1.49 (2003) and 0.44 (2004) out of 10 000 donors in blood banks of the United States, a country where WNV is established.[29]

Following the introduction of WNV vaccinations for horses in the United States, the incidence of neuroinvasive disease in horses decreased from more than 14 000 cases in 2002 to approximately 5100 in 2003, suggesting that the WNV vaccination had a substantial impact on equine health.[1]

As Brazil has no confirmed clinical cases of WNV, it is considered an exotic disease, and the Ministry of Agriculture and public health policies do not permit import or vaccine production for these types of diseases. Our work indicates a low prevalence of WNV antibodies in equids in Brazil, therefore, the vaccination of horses should be discussed from an economic standpoint with a model of free demand by the owners, as the cost-benefit of the vaccination may be greater than that of treating the animals, considering that Brazil has the largest herd of horses in South America. Based on serological evidence of WNV in birds and equines in Colombia, Venezuela and Argentina,[6,10,11] similar findings were anticipated in Brazil, which shares borders with these three countries.

Brazil has abundant circulating birds and mammals and also hosts a large and diverse population of mosquitoes, including *Culex* sp. and *Aedes* sp., which carry several flaviviruses and can produce important outbreaks, as has been observed with yellow fever and dengue. A large serological survey in Brazil between 2003 and 2004 did not demonstrate the presence of WNV antibodies in wild and domestic birds,[7–9] although recent studies in the Pantanal area identified antibodies to WNV in five out of 168 equids in 2009[2] and in three out of 38 equids in 2010.[12] However, these recent studies included small sample numbers, lacked a wide range of collection sites and sampled fewer species of animals than the current study; these study design differences have allowed us to better visualise the circulation of WNV in the country.

In our study, WNV seropositivity was identified in 2009 in equids ranging from 5 to 8 years old, providing a broad indication of the period of circulation of WNV (2004–2009) in this area. Positive WNV serology was not detected before 2009 (samples from 2002 and 2006 were negative), which could be due to the small number of samples or a reflection of the history of the dispersion of WNV from North to South America. The WNV equine seroprevalence in Nova Brasilândia was 1.8%, which can be compared to the first identification of WNV in Yucatán, Mexico (1.2%),[24] and to the seroprevalence determined in Pantanal (3%).[2] However, this seroprevalence is lower than that observed in Guadeloupe after the establishment of the WNV cycle there (19.3%)[30] or in Mexico in areas with a history of clinical encephalitis (22%).[31]

In this study, we analysed two areas of Mato Grosso that were sampled during the same period with similar population sizes (217 and 218 equids), WNV circulation was only identified in Nova Brasilândia. This finding may be related to the specific

Table 1. West Nile Virus (WNV) seroprevalence in equids and wild birds in Brazil, 2002–2010

Location Geographical coordinates	Date of sampling	Species	Inhibition ELISA 3.112G	PRNT WNV titre	PRNT SLEV titre	PRNT diagnosis
Monte Negro, Rondônia state S 10°17′13″ W 63°14′27″	6/15/2002	*Equus caballus* (horse)	43%	80	160	Flavivirus
Teodoro Sampaio, São Paulo state S 22°22′70″ W 52°25′66″	3/17/2002	*Equus caballus* (horse)	49%	20	160	SLEV
	3/19/2002	*Equus caballus* (horse)	35%	40	160	SLEV
	4/12/2002	*Equus caballus* (horse)	39%	10	20	Flavivirus
	1/18/2006	*Equus caballus* (horse)	44%	320	1280	SLEV
	2/6/2006	*Equus caballus* (horse)	64%	160	2560	SLEV
Nova Brasilândia, Mato Grosso state S 14°57′25″ W 54°57′56″	4/27/2009	*Equus caballus* (horse)	51%	80	20	WNV
	4/27/2009	*Equus caballus* (horse)	60%	160	320	Flavivirus
	7/6/2009	*Equus caballus* (horse)	32%	80	20	WNV
	9/21/2009	*Equus caballus* (horse)	48%	80	10	WNV
	10/16/2009	*Equus sp.* (mule)	42%	40	10	WNV
Juruena, Mato Grosso state S 10°19′05″ W 58°21′32″	11/6/2009	*Equus caballus* (horse)	30%	20	160	SLEV
	11/6/2009	*Equus caballus* (horse)	26%[a]	1280	640	Flavivirus
Pinheiro, Maranhão state S 2°31′22,64″ W 45°05′33,23″	5/14/2010	*Dendrocygna autumnalis* (bird)	53%	<20	<20	negative
Ilha de Canela, Pará state S 00°46′54,77″ W 46°43′44,90″	11/25/2008	*Arenaria interpres* (bird)	37%	<20	<20	negative
Parque Nacional da Lagoa do Peixe, Rio Grande do Sul state S 31°21′17,94″ W 51°02′52,37″	11/19/2009	*Sterna hirundo* (bird)	41%	<20	<20	negative
	11/20/2009	*Sterna hirundo* (bird)	37%	<20	<20	negative
	3/26/2010	*Sterna hirundo* (bird)	34%	<20	<20	negative

[a]Doubtful between 25% and 30%. Inhibition values of ≥30% are considered significant. A serum sample was considered to contain antibodies to WNV if the PRNT90 titre to WNV was at least 4-fold greater than the corresponding SLEV titre. A serum sample was considered to contain antibodies to SLEV if the PRNT90 titre to SLEV was at least 4-fold greater than the corresponding WNV titre. PRNT: plaque reduction neutralization test; SLEV: St Louis encephalitis virus; WNV: West Nile virus.

environmental and ecological characteristics of this area, such as the predominance of migratory birds from North America in the Pantanal region.[32] This region is characterised by strong anthropogenic disturbance, including the recent expansion of farming, which involves human activities that are linked to deforestation. These factors can interfere with the natural cycle of the virus and could increase the risk of WNV transmission to humans.

Although SLEV was not the focus of this study, the circulation of SLEV was detected in Teodoro Sampaio (São Paulo state) and Juruena (Mato Grosso state). The seroprevalence of SLEV was not assessed in this study because only samples that tested positive based on the results of WNV-specific blocking ELISA were further tested with the $PRNT_{90}$. Some of the samples that were seropositive by the WNV-specific blocking ELISA were positive by $PRNT_{90}$ for SLEV at a higher titer than for WNV. If the PRNT results (gold standard) suggest that these samples are negative for WNV, considering the expected high specificity of the WNV-specific blocking ELISA, an alternative explanation could be that these samples were positive for both SLEV and WNV but that the PRNT test was not sensitive enough to detect WNV antibodies. The same reason could also indicate that the flavivirus positive samples could be in fact a co-infection with both WNV and another flavivirus.

The specific blocking ELISA used in our work uses MAb 3.112G, which can potentially discriminate between WNV and SLEV infections.[23] Given the cross-reactivity among flaviviruses, particularly in areas such as South America, where multiple flaviviruses circulate, the specific blocking ELISA results are considered to be the more reliable ones even if $PRNT_{90}$ is still used as the reference assay for the specific diagnosis of WNV infection. Even though the diagnostic sensitivity of ELISA is 100% compared to $PRNT_{90}$, the specificity is only 79.5%.[4] Using $PRNT_{50}$s in samples that were positive by ELISA and negative by $PRNT_{90}$ could have improved the sensitivity of the results. It is likely that we have underestimated the WNV circulation in Brazilian birds by using $PRNT_{90}$ because it has low sensitivity for the detection of antibodies in animals that have not been clinically infected, even though they may have been sub-clinically infected.

The limitations of this study include the failure to undertake the sampling of birds and equids in the same regions and at the same times, which makes it difficult to derive any solid ecological picture of what might have been occurring. Another limitation is related to the application of the $PRNT_{90}$, which used only WNV and SLEV antigens. This methodology could lead to cross-reaction with other flaviviruses present in Brazil that are not commonly found in North America.

The direct molecular detection of WNV in equid and bird samples did not confirm the presence of WNV in the studied areas during any of the years of the study (2008, 2009 and 2010) but this is not surprising considering the short viraemia. WNV is therefore probably circulating in equids and birds, but as a sub-clinical infection due to the influence of other flaviviruses circulating in Brazil. It is likely that the WNV epidemic in Brazil behaves differently from that observed in North America: in Brazil, there is a greater diversity of flaviviruses, which increases the level of genetic resistance in South America. WNV could be circulating with the same pattern observed with other flaviviruses.[2,12] These other flaviviruses infect equids and birds but do not cause clinical symptoms that are relevant to Brazilian health surveillance. Similar to other South American countries, Brazil appears to have established a form of co-existence with WNV in which the virus can circulate, albeit at a low level in terms of pathogenicity, without causing any real problems.

The lack of evidence for WNV in birds in Brazil or the very low prevalence if the results are based on the WNV-specific blocking ELISA remains puzzling because several migratory species were evaluated, and seropositive birds have been prevalent for the last 5 years in countries neighbouring Brazil. Experimental infections in several bird species have indicated that viraemic levels remain high only for a short duration (a maximum of 7 days), making the introduction of WNV by long-distance migrators an unlikely event[33] and corroborating the idea that the virus has spread through resident or short-distance migrant birds, thereby explaining the spread of the virus sequentially southward through the Americas and not directly by migrating birds from North America.

Our work used a large number of migratory bird samples from different stop-over points, and given our results, we believe that the input of WNV in Brazil is most likely due to birds resting at convenient 'staging posts' on their north-to-south journey. Those birds probably have gradually moved the virus to the south in stages, rather than clinically infected birds travelling the full distance. These data have been reinforced by the identification of WNV antibodies in equids only since 2009, along with other Brazilian studies that have also reported positive serology during the same period.[2,12] The introduction of WNV in Argentina occurred early via birds in January 2005.[6] This information reinforces the idea of staging posts, as there are bird displacements between Brazil and Argentina (Figure 1), and quite intense contact occurs among these posts.

In relation to humans, there is a strong vaccination program for yellow fever in Brazil. Furthermore, the dengue virus is endemic in Brazil. Both the yellow fever virus and the four subtypes of the dengue virus can confer cross-immunity to other flaviviruses, resulting in WNV producing only sub-clinical infections. Similar correlations can be observed with equids with immunity to different flaviviruses due to constant exposure to these different viruses.

Considering the large population of reservoir birds and the abundance and diversity of mosquito species in Brazil, WNV may have become endemic in some states. The continuous evolution of WNV in North America[34] and the possible consequences of WNV circulation in Brazil on public health, wild bird conservation and the economy emphasise the need to establish WNV surveillance and Brazilian WNV research programs to minimise possible damage to Brazilian society from the introduction of another lethal arbovirus in addition to those already established in the country.

Authors' contributions: TO, TL, ELD and JA conceived the study and designed the study protocol; TO, ELD, JA, DMA, GTC, RMM, SMAJ, MVP, ISN, PS, EV, EMSC, MCCSHL, AFDN, MSN, RFH, RR, AS, JS, MMMS, CP, DSB, MBL and LMAC carried out the sampling and critically revised the manuscript for intellectual content; TO, JA, JEL, RFH, MPG, MMMS, RA, JP, NV, TL and RL conducted the laboratory work, analysis and interpretation of the data. TO, TL, ELD, MMMS and JA drafted the manuscript; TO, TL, ELD, JA, DMA, JEL, MMMS and RL critically revised the manuscript for

intellectual content. All authors read and approved the final manuscript. TO, ELD and TL are the guarantors of the paper.

Acknowledgments: We thank Nicolas Gaidet for mediating communication between CIRAD and the University of São Paulo.

Funding: This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2009/05994-9); Interreg IV Caraïbe 'CaribVET' (Europe Project) and FEDER 2007-2013 'Risque en santé animale et végétale'.

Competing interests: None declared.

Ethical approval: This study was conducted in strict accordance with the recommendations of the Ethical Principles of Animal Experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL) and the animal protocol was approved by the Ethics Committee on Animal Experiments (EAEC) (Permit Number: 105, page 74, book 2). The equid sampling was approved by the Institutional Committee for Ethics in Animal Research of the Federal University of Mato Grosso N°23108.017192/10-6 and the sampled birds were licensed by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/SISBIO).

References

1 Martina BEE, Koraka P, Osterhaus ADME. West Nile virus: is a vaccine needed? Curr Opin Investig Drugs 2010;11:139–46.

2 Pauvolid-Corrêa A, Morales MA, Levis S et al. Neutralising antibodies for West Nile virus in horses from Brazilian Pantanal. Mem Inst Oswaldo Cruz 2011;106:467–74.

3 Pesko KN, Ebel GD. West Nile virus population genetics and evolution. Infect Genet Evol 2012;12:181–90.

4 De Filette M, Ulbert S, Diamond M, Sanders NN. Recent progress in West Nile virus diagnosis and vaccination. Vet Res 2012;43:16.

5 Komar N, Clark GG. West Nile activity in Latin America and the Caribbean. Rev Panam Salud Publica 2006;19:112–7.

6 Adrián Diaz L, Komar N, Visintin A et al. West Nile virus in birds, Argentina. Emerg Infect Dis 2008;14:689–91.

7 Araujo FAA, Vianna RST, Andrade Filho GV et al. Primeiro inquérito sorológico em aves migratórias e nativas do Parque Nacional da Lagoa do Peixe/RS para detecção do vírus do Nilo Ocidental. Bol Eletron Epidemiol 2003;1:3–12.

8 Araujo FAA, Wada MY, Silva EV et al. Segundo inquérito sorológico em aves migratórias e residentes do Parque Nacional da Lagoa do Peixe/RS para detecção do vírus do Nilo Ocidental e outros vírus. Bol Eletron Epidemiol 2004;5:1–8.

9 Araujo FAA, Vianna RST, Andrade Filho GV et al. Inquérito sorológico em aves migratórias e residentes de Galinhos/RN para detecção do vírus da Febre do Nilo Ocidental e outros vírus. Bol Eletron Epidemiol 2004;2:1–12.

10 Mattar S, Edwards E, Laguado J et al. West Nile virus antibodies in Colombian Horses. Emerg Infect Dis 2005;11:1497–8.

11 Bosch I, Herrera F, Navarro JC et al. West Nile virus, Venezuela. Emerg Infect Dis 2007;13:651–3.

12 Melandri V, Guimarães AÉ, Komar N et al. Serological detection of West Nile virus in horses and chicken from Pantanal, Brazil. Mem Inst Oswaldo Cruz 2012;107:1073–5.

13 Morales MA, Barrandeuguy M, Fabbri C et al. West Nile virus isolation from equines in Argentina. Emerg Infect Dis 2006;12:1559–61.

14 Domingues RB, Teixeira AL. Management of acute viral encephalitis in Brazil. Braz J Infect Dis 2009;13:433–9.

15 Levi JE, Geraldi MP, Torres KL et al. Investigação molecular da presença do virus do Oeste do Nilo em doadores de sangue e pacientes de três regiões brasileiras. Revista Brasileira de Hematologia e Hemoterapia 2011;33:399.

16 Rosa JFST, Rosa APAT, Vasconcelos PFC et al. Arboviruses isolated in the Evandro Chagas Institute, including some described for the first time in the Brazilian Amazon region, their known hosts, and their pathology for man. In: Rosa APAT, Vasconcelos PFC, Rosa JFCT (Editors). An Overview of Arbovirology in Brazil and Neighbouring Countries. Belém, Brazil: Instituto Evandra Chagas 1998: p. 19–31.

17 Ministério do Meio Ambiente. Brazilian biomes. Brasília, Brazil. http://www.brasil.gov.br/sobre/environment/geography [accessed 15 May 2013].

18 Ministério do Agicultura, Pecuária e Abastecimento. Equídeos. Brasília, Brazil. http://www.agricultura.gov.br/animal/especies/equideos [accessed 20 May 2013].

19 Carmargo LMA, Moura MM, Engracia V et al. A rural community in Brazilian Western Amazonian region: Some demographic and epidemiological patterns. Mem Inst Oswaldo Cruz 2002;97:193–5.

20 Alessi CAC, Galati EAB, Alves JR, Corbett CEP. American cutaneous leishmaniasis in the Pontal of Paranapanema–SP, Brazil: ecological and entomological aspects. Rev Inst Med Trop 2009;51:277–82.

21 Rodrigues RC, Azevedo-Júnior SM, Larrazábal ME, Araujo HFP. Temporal variations of body mass and plumage in Arenaria interpres (Aves: Scolopacidae) along the Brazilian coast. Zoologia 2009;26:386–90.

22 Blitvich BJ, Bowen RA, Marlenee NL et al. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. J Clin Microbiol 2003;41:2676–9.

23 Blitvich BJ, Marlenee NL, Hall RA et al. Epitope-blocking enzyme-linked immunosorbent assays for the detection of serum antibodies to West Nile virus in multiple avian species. J Clin Microbiol 2003;41:1041–7.

24 Loroño-Pino MA, Blitvich BJ, Farfán-Ale JA et al. Serologic evidence of West Nile virus infection in horses, Yucatan State, Mexico. Emerg Infect Dis 2003;9: 857–9.

25 Blitvich BJ, Fernadez-Salas I, Contreras-Cordero JF et al. Serologic evidence of West Nile virus infection in horses, Coahuila State, Mexico. Emerg Infect Dis 2003;9:853–6.

26 Beaty B, Calisher CH, Shope RE. Arboviruses. In: Lennette EH, Lennette DA, Lennette ET (Editors). Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections. 7th ed. Washington, DC: American Public Health Association. 1995: p. 189–212.

27 Fernández-Salas I, Contreras-Cordero JF, Blitvich BJ et al. Serologic evidence of West Nile Virus infection in birds, Tamaulipas State, México. Vector Borne Zoonotic Dis. 2003;3:209–13.

28 Pai A, Kleinman S, Malhotra K et al. Performance characteristics of the Food and Drug Administration–licensed Roche Cobas TaqScreen West Nile virus assay. Transfusion 2008;48:2184–9.

29 Stramer SL, Fang CT, Foster GA et al. West Nile virus among blood donors in the United States, 2003 and 2004. N Engl J Med 2005;353:451–9.

30 Lefrançois T, Blitvich B, Pradel J et al. West Nile virus surveillance, Guadeloupe, 2003–2004. Emerg Infect Dis 2005;11:1100–3.

31 Estrada-Franco JG, Navarro-Lopez R, Beasley DW et al. West Nile virus in Mexico: evidence of widespread circulation since July 2002. Emerg Infect Dis 2003;9:1604–7.

32 Nunes AP, Tomas WM. Embrapa Pantanal. Aves migratórias e nômades ocorrentes no pantanal. http://www.cpap.embrapa.br/publicacoes/online/Livro033.pdf [accessed 10 June 2008].

33 Koopmans M, Martina B, Reusken C, Van Maanen K. West Nile virus in Europe: waiting for the start of epidemic? In: Taakken W, Knols BGJ (Editors). Emerging Pests and Vector-Borne Diseases in Europe. Volume 1. Wageningen, The Netherlands: Wageningen Academic Publishers. 2007: pp. 123–52.

34 McMullen AR, May FJ, Li L et al. Evolution of new genotype of West Nile virus in North America. Emerg Infect Dis 2011;17:785–93.