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Do saint Louis Encephalitis and West Nile Viruses Fly Together?

Maritza Pupo[1*], Vazquez Y[2], Andonova M[3], Alvarez M[4], Vazquez S[5], Bell R[6], Morier L[7] and Guzman MG[8]

[1]Maritza Pupo, Department of Microbiology, University of Havana, Cuba

[2]Vazquez Y, Center for Research and Rehabilitation of Ataxia, Cuba

[3]Andonova M, National Microbiology Laboratory, Public Health Agency of Canada, Canada

[4]Alvarez M, Department of Virology, Institute of Tropical Medicine Pedro Kouri, Cuba

[5]Vazquez S, Department of Virology, Institute of Tropical Medicine Pedro Kouri, Cuba

[6]Bell R, Department of Physiology, Animal Science Institute, Cuba

[7]Morier L, Department of Microbiology, University of Havana, Cuba

[8]Guzman MG, Director of Researches, Institute of Tropical Medicine Pedro Kouri, Cuba

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Corresponding author:
Maritza Pupo,
Department of Microbiology, University of Havana, Cuba, Tel: +53-7-832 92 52; Fax: +53-7-832;
Email: mpupo@fbio.uh.cu

ABSTRACT

St Louis Encephalitis Virus (SLEV) and West Nile Virus (WNV) co-circulated in humans and horses in Cuba, from 2004 to 2006, based on serological testing using neutralization assays. During this time frame, serum samples from 659 humans suspected to have been exposed to flaviviruses were collected and tested for WNV IgM and IgG by ELISA and plaque reduction neutralization test. Avidity of IgG antibodies against WNV was determined on a subset of confirmed human serum samples. A total of 8 paired serum samples (1.2%) and 23 monosera had plaque reduction neutralization test antibodies against SLEV while 16 paired sera samples (2,42%) were positive for WNV neutralizing antibodies. Antibodies to both viruses were detected in 23 of 210 horses and 7 of them were specific to WNV. Antibodies to SLEV in birds were detected before to 2004. PCR testing of tissues from 1479 birds collected in 2004 and 2005 were negative to both viruses. This research confirmed the co-circulation of SLEV and WNV, also the possibility for using an alternative serological test to differentiate, in absence of IgM, SLEV or WNV infections. This study provided direct evidence that SLEV and WNV have circulated in common hosts throughout Cuba.

INTRODUCTION

Saint Louis Encephalitis Virus (SLEV) and West Nile Virus (WNV) are closely related mosquito-borne flaviviruses which are widely distributed throughout the Western Hemisphere. Both viruses are maintained in a mosquito-bird-mosquito cycle and their amplification occurs in peridomestic birds and *Culex* spp. mosquitoes [1]. SLEV activity is restricted to the Western Hemisphere and outbreaks have occurred in North America since 1933 [2]. In South America, SLEV has been known to occur in Argentina and Brazil since the 1960s [3]. However, SLEV have re-emerged in the central region of Argentina during 2002 and, since then, several outbreaks have been reported in Argentina and also in Brazil [4-7]. On the other hand, in 1999 WNV

appeared in USA and since then it spread southward into the Caribbean area basin and Latin America [8]. Both viruses, as members of the same family, genera and antigenic complex share similar composition, clinical symptoms and also transmission cycle, hosts and vectors [9,10]. All of this made possible the simultaneous transmission of both viruses in the same area [11,12]. As a consequence, the diagnosis of patients inside geographic areas where multiple flaviviruses are circulating is extremely difficult [13]. Therefore, several diagnostic algorithms have been alternatively used to differentiate between SLEV and WNV human infections. One of them has been avidity assay, which differentiates between acute or primary infection and persistent or recurrent infection, or reactivated disease [14].

In Cuba, the presence of antibodies related with SLEV and WNV in animals and humans has been reported. SLEV antibodies were detected in humans from the beginning of national serological surveys in 1972 [15]. Meanwhile, such antibodies were identified in sentinel birds from 1988, 1989 and 1994 [16]. Further evidence of SLEV circulation was observed after the establishment of a WNV surveillance system in 2002 [17]. This surveillance system was developed through molecular, virological and serological methods. In a first stage, samples from birds and horses, which died or showed clinical symptoms of flavivirus infections, were studied by virological and molecular methods. In parallel, human samples from suspected cases were studied by the methods depending on the time course of the illness. Here, we present data to confirm SLEV and WNV co-circulation in Cuba from 2004 to 2006. Also, we studied if an IgG avidity test is useful to differentiate previous infections between SLEV and WNV in our conditions.

MATERIALS AND METHODS

During 2004-2006 period, serum samples from 659 humans suspected to have been exposed to flaviviruses were collected across Cuba and tested for WNV IgM and IgG antibodies by ELISA. In parallel, more than 200 samples of equine sera were studied and 1479 birds were tested for WNV by molecular methods during 2004-2005.

Human samples

The distribution of human serum samples across the years of study was as follows: 110 from 2004, 399 from 2005 and 150 from 2006. Most cases suffered fever with clinical symptoms of both viral infections such as arthralgia, cephalous, and vomits with 5 cases of viral meningoencephalitis in this period. All samples were negative to Dengue Virus (DV) diagnosis.

Samples were screened for WNV IgM and IgG by using commercial IgM and IgG ELISA kits (Focus Technologies, Cypress, CA, USA) according to manufacturer's instructions.

All reactive serum samples were further tested using a plaque reduction neutralization test (PRNT) with the following strains: WNV (NY99, Ontario, Canada, 2001 isolate), SLEV (Parton strain, American Type Culture Collection catalog No. VR-1265), and dengue virus (dengue 2, NG-C strain). PRNT endpoint titrations were defined as the highest dilution of serum that reduced plaque formation by >90% [17].

Avidity of IgG antibodies against WNV (EUROIMMUN, Luebeck, Germany) was determined on a subset of human serum samples confirmed by PRNT. Sera were tested using an ELISA (EuroimmunAG, Luebeck, Germany). Briefly, 100 µl of serum diluted 1:100 in buffer phosphate-buffered saline–Tween, was added to each of two wells coated with WNV. After incubation for 60 min at 37°C, wells were exposed to either 6 M urea solution or to phosphate buffer for 10 min. After washing, wells were incubated with peroxidase-labeled anti-human IgG for 60 min at 37°C, substrate was added, and the reactions were stopped after 15 min by the addition of 100 µl stop solution per well. The reactions were read immediately at a wavelength of 450 nm using a reference wavelength of 650 nm. A Relative Avidity Index (RAI) was calculated to classify antibodies as low (<40%) or high avidities (60%) antibodies for each specimen and was expressed as the percentage of reactivity remaining in the urea-treated well [18].

Calculations

OD samples urea treated / OD samples without urea treatment X 100 = RAI%

RAI <40% low avidity, 40% - 60% equivocal, >60% high avidity

RAI determination is not useful in samples with OD < 0.14 after urea treatment.

Horse samples

The distribution of 278 equine serum samples across the years of study was as follows: 210 from 2004, 73 from 2005 and 5

from 2006. They came mainly from Sancti Spiritus and Camagüey provinces.

Samples were tested by an ELISA containing a recombinant WNV enveloped protein antigen for detection of IgG antibodies to flavivirus and WNV using monoclonal antibodies 6B6/C (Chemicon®, Catalogue NumberMAB8744) and 3.91Chemicon®, Catalogue NumberMAB8151, respectively. An inhibition value higher of 30% was considered indicative of the presence of viral antibodies [19]. In addition, samples were assayed by Hemagglutination Inhibition (HI) test for antibodies SLEV and WNV [20]. To confirm the specificity of the results, samples were tested by $PRNT_{50}$ for antibody against SLEV and $PRNT_{90}$ against WNV [17].

Bird samples

Throughout 2004-2005, samples from 1479 birds previously identified by ornithologist experts were processed at the Tropical Medicine Institute "Pedro Kourí". Brain, heart, and kidneys were removed and tested for WNV by using Reverse Transcription–Polymerase Chain Reaction (RT-PCR) [21]. When the firsts human WNV infections were confirmed the study on birds was only done in cases of great bird populations were ill or dead.

RESULTS

Human samples

Of 110 human samples collected in 2004, just one (0.9%) was positive to WNV specific antibodies, meanwhile 5 (4.5%) were positive to SLEV infections. During 2005, from a total of 399 samples 2 cases (0.005%) were confirmed to WNV infections and 8 (2%) to SLEV. Most of the samples tested were pair sera. However, 13 monosera had neutralizing antibodies to SLEV (data not shown). Until May 2006, of 150 samples, 7 cases (4. 6%) were confirmed to WNV infections and 5 monosera (3.3%) to SLEV. There were not evidences of IgM antibodies for both viruses (Table 1).

A subset of 20 cases was studied for IgG WNV affinity and the general behavior of the samples is shown in (Table 2). All samples after the convalescent phase were between 80 to 100 % of affinity to IgG WNV antibodies, whereas samples from the acute phase showed low or intermediate affinity values. However, the samples of negative cases to WNV, with a SLEV diagnosis and in the convalescent phase showed high affinity to IgG antibodies, which demonstrate the presence of cross reactivity antibodies.

Table 1: Human confirmed cases to SLEV and WNV by PRNT from 2004, 2005.

2004		2005		2006	
Code	Nt/Result	Code	Nt/Result	Code	Nt/Result
1	40/WNV	1	80/WNV	1	>80/WNV
2	640/SLEV	2	80/WNV	2	>80/WNV
3	320/SLEV	3	160/SLEV	3	>80/WNV
4	80/SLEV	4	320/SLEV	4	80/WNV
5	40/SLEV	5	>320/SLEV	5	320/WNV
6	80/SLEV	6	160/SLEV	6	>80/WNV
		7	160/SLEV	7	>80/WNV
		8	>320/SLEV	8	>320/SLEV
		9	80/SLEV	9	>320/SLEV
		10	160/SLEV	10	160/SLEV
				11	>80/SLEV
				12	80/SLEV

Nt neutralization; West Nile virus (WNV); St. Loius encephalitis virus (SLEV).

Table 2: Summary of human sera tested for WNV and SLE infections by PRNT and affinity EUROIMMUN ELISA.

Code	WNV Nt	Daysafteronset	IgG WNV Avidity RAI%	Diagnosis (PRNT)
1	<20	>15 convalescent	70.6	SLEV
2	<20	>30 convalescent	71.3	SLEV
3	320	>30 convalescent	100	WNV
4	>80	>30 convalescent	100	WNV
5	>80	>30 convalescent	89.5	WNV
6	80	>30 convalescent	87.7	WNV
7	80	2 (acute)	73.6	WNV
8	80	2 (acute)	59	WNV

Neutralization (Nt). Rate avidity index (RAI).

Horse samples

From the horse WNV surveillance, in 2004, 4 and 8 animals had WNV- and SLEV-specific antibodies respectively (data not shown) [17]. The 80% of the samples from 2005 had antibodies capable to block the binding of the flavivirus-specific monoclonal antibody (6B6C-1) and 7 of them to a WNV-specific epitope on the NS1 protein. In addition, 23 samples were positive to SLEV according to a neutralization test (Table 3).

Table 3: Horse sera samples positive to flavivirus and specific WNV antibodies, 2004-2006.					
Locality/Samples	% inhibition by anti-flavi ELISA	% inhibition by anti-WNV ELISA	SLEV $PRNT_{50}$ titer	WNV $PRNT_{90}$ titer	Interpretation
Nu1	75	57.3	10	>40	WNV
Nu2	98.4	44	74	>160	WNV
Na13	93.4	43.4	10	>40	WNV
Ca6	96.7	54.4	10	>40	WNV
J10	85.7	68	40	>80	WNV
J12	88.5	52.5	10	>40	WNV
J16	65.8	40	14	>40	WNV

Bird samples

During 2004-2006 period, significant bird mortality was not observed. The birds studied mainly belonged to *Caradriformes, Passeriformes, Anatidae, Columbiformes, Gruiformes, Strigiformes, Ardeidae, Pelecaniformes, Ciconiformes* orders with more than 200 species among them. No one was positive to WNV by isolation or molecular biology techniques. Up to 2006, no great bird's population was ill or dead by a cause probably related to a flavivirus infections.

(Table 4) summarizes the total number of samples studied and the kind of assays used.

Table 4: Summary of samples tested.				
Samples/No	ELISA IgG WNV/ SLEV Positive Negative	PRNT WNV/SLEV Positive Negative	WNV IgG avidity HiL/Eq	RT-PCR WNV Positive Negative
H/659	659/659 0/0	10/18 649/641		
H/20			7* 12/1	NT
	ELISA WNV/ SLEV Positive Negative	PRNT WNV/SLEV Positive Negative		
E/278	11/31 267/247	7/23 271/247	NT	NT
B/1479	NT	NT	NT	0 1479

H: human, E: equines, B: birds, Hi: high, L: low Eq: equivocal, *2 samples were SLEV infections and NT: no tested.

DISCUSSION

In the tropical countries of Americas the co-circulation of Multiple flaviviruses is common and in most of them some of these flavivirus are endemic [11,21]. Concurrent circulation of different arboviruses has been recently reported in Latin America [22-25].

Several studies have been conducted to understand the epidemiological patterns of WNV and SLEV, regarding to the more limited nature of SLEV activity in relation to the widespread dissemination of WNV [10]. Several hypotheses have been proposed to explain this difference. First, competitive pressures might alter the transmission cycle of WNV, SLEV, or both. Also the neutralizing cross-protective effect of immune responses to heterologous flaviviruses and the different virulence between WNV and SLEV in avian host could have an impact in the different dissemination of both viruses [26,27]. However, both viruses have recently been proposed as etiological agents of concurrent human encephalitis outbreaks in Arizona, US showing a high SLEV activity from 2015 [28]. Recent reports revealed reemergence of SLEV in California and Arizona resulted from introduction of a South American strain of SLEV [29].

In the period of this study (2004-2006) several reports of SLEV and WNV co-circulation in birds, equines and humans in the region were published [11,30- 35]. However, SLEV infections were the most reported in the region, with a significant human outbreak in Argentina in 2004 and 2005 [35]. In Brazil, 2004, after more than two decades of absence a clinical case of infection by SLEV was detected in Sao Paulo [36]. Then, in 2006, the first community outbreak of SLEV in Brazil was detected in São José do Rio Preto. This outbreak was concurrent with a large outbreak of dengue serotype 3 [37].

In general, our study revealed a major SLEV activity such as in horses and humans which was higher in 2005. At the same time, WNV was lightly increased from 2005 to 2006. Taken together, these reports indicate an elevated activity of SLEV in the region and both viruses were co-circulating.

The broad range of co-infecting viruses and the similar signs and symptoms at the early stages of infection have complicated their diagnosis. Therefore alternative diagnostic tools are needed to discriminate them. Common laboratory diagnosis of these viruses is primarily based on sero-diagnosis. However, the differential diagnosis of them may be complicated due to the high degree of cross-reactivity. Also the confirmation of each virus is based on a PRNT assay with several flaviviruses which are cumbersome and time-consuming [13]. Despite the modern, faster and more sensitive molecular techniques, serological assays continue to be the first line of diagnosis in countries with limited resources and technology [38,39].

WNV and SLEV diagnosis are usually based on the demonstration of Immunoglobulin M (IgM) in serum or in the cerebrospinal fluid by capture ELISA [40]; although other classes of immunoglobulins have been detected [41,42]. For serological diagnosis it is essential to have paired sera samples collected in the adequate time (acute- and convalescent-phase) which is frequently difficult. Sometimes, samples collected too late provoke the lost or decrease of IgM and Immunoglobulin G (IgG) antibodies titer respectively, leading to false negative results.

Avidity testing is based on IgG avidity which is low after primary antigenic challenge but increases progressively during subsequent weeks and months due to affinity maturation and antigen-driven B-cell selection [18]. Such test has been useful to differentiate between recent and past exposure to WNV and other flaviviruses [43]. However, there are not enough evidence to confirm that cross-reacting flavivirus antibodies will cause a high-avidity result, and its implication in WNV infection classification [18].

Ours result indicate that, in our context with absence of IgM antibodies, the affinity test was not useful complement and confirm the serological diagnosis. Previous studies have reported that low-avidity IgG together with a positive IgM ELISA is a convincing evidence of an acute or recent WNV infection [44] or just high IgM levels are indicative of WNV infection, making unnecessary IgG avidity determination [42]. Apparently, IgG SLEV cross-reacted antibodies have a high avidity for WNV, in spite of reduced number of samples assayed. Recently, studies in an immunocompetent mouse model have been conducted to assess the influence of preexisting cross-reactive immunity on the development of immunity to a subsequent flavivirus challenge. A group of these mice was infected with viruses from independent sero-complexes Japan Encephalitis virus (JEV), Yellow Fever virus (YFV), (live-attenuated vaccine strains) and DENV1 (clinical isolate) to quantify the generation of specific and sero-complex cross-reactive antibody titers. The avidity to the virus structural antigens for each homologous or heterologous antibody was measured. The DENV1 clinical isolate induced specific high-avidity but low-avidity cross-reactive antibodies against YFV and JEV. However, for JEV and YFV vaccine strains, both specific and cross-reactive antibodies generated were low avidity. These results showed that mice evoke similar immune responses to flaviviruses as humans in terms of generating immunity consistent with human sero-complexes [43]. So it is expected that SLEV elicit cross-reacting antibodies against WNV with high affinity. The serological cross-reactivity between WNV and SLEV has been complicated. Then, differentiation among flaviviruses in human cases is needed. A good example is MAC-ELISA, one of the reasons why the 1999 outbreak of WNV in New York City was confused with SLEV; leading to the implementation of a new algorithm for the identification of both viruses [44].

Despite the several commercially available serological assays to identify different flavivirus sometimes they lack many others [45]. Moreover, the vast flavivirus circulation in the region makes difficult to include all of them. Therefore it is fundamental to deep inside accurate diagnosis methods capable of differentiating these related viruses which seemingly many times fly together.

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ETHICS STATEMENT

This research was approved by the "Pedro Kouri" Tropical Medicine Institute's Ethical and Scientific Committees, National Direction of Veterinary and Direction of Flora and Fauna. The animal capture and identification techniques were the less invasive to preserve the welfare of the animals and avoid potential stress.

INFORMED CONSENT

The content of the consent will be verbally explained to the individuals by qualified personnel with a clear and simple language to facilitate their understanding. After knowing their consent to participate, written consent (Annex 1) will be requested and the demographic and clinical history data will be collected using the national database of the WNV Surveillance Laboratory. A copy of the informed consent will be given to each participant for future reference.

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