



Recent infection of West Nile Virus in horses from eastern Yucatán, Mexico

José E. Ortíz-Esquivel¹ ; José Alberto Rosado-Aguilar¹ ; Roger Iván Rodríguez-Vivas^{1*} ; Marco Torres-Castro² ; Edwin José Gutiérrez-Ruiz¹ ; Arturo Bates-Acosta³ ; Fernando I. Puerto² .

¹Universidad Autónoma de Yucatán, Campus de Ciencias Biológicas y Agropecuarias, Facultad de Medicina Veterinaria y Zootecnia, Cuerpo Académico de Salud Animal, km. 15.5 carretera Mérida-X'matkuil, Mérida, México.

²Universidad Autónoma de Yucatán, Campus de Ciencias de la Salud, Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Laboratorio de Enfermedades Emergentes y Reemergentes, Avenida Itzáes x 59, No. 490, Mérida, México.

³Centro de Equinoterapia del Oriente de Yucatán, km. 2 carretera Tizimín-Xkekén, Tizimín, México.

*CorrespondenceE: rvivas@correo.uady.mx

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ABSTRACT

Objective. To determine the circulation of West Nile virus (WNV) in horses from the eastern Yucatán, Mexico. **Materials and Methods.** For convenience, 184 horses from 23 production units in the municipalities of Tizimín and Panabá, Yucatán, were studied. A blood serum sample was obtained from each studied horse and evaluated with an immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) to detect immunoglobulin M (IgM) against WNV. Additionally, positive reactor and suspect serum samples were analyzed by reverse transcription polymerase chain reaction (RT-PCR) to detect viral RNA. **Results.** Eight studied horses were seropositive (4.3%, 8/184) to WNV and two were suspects (1.1%, 2/184). All serum samples were negative by RT-PCR. **Conclusions.** The detection of IgM specific against WNV in the studied horses shows recent infections with the virus and indicates its circulation in eastern Yucatán, Mexico.

Keywords: Antibodies; MAC-ELISA; *Flavivirus*; IgM; serology (*Source: DeSC*).

RESUMEN

Objetivo. Determinar la circulación del virus del Oeste del Nilo (VON) en caballos del oriente de Yucatán, México. **Materiales y métodos.** Por conveniencia se estudiaron 184 caballos localizados en 23 unidades de producción de los municipios de Tizimín y Panabá, Yucatán. De cada caballo se obtuvo una muestra de suero sanguíneo, la cual fue evaluada con un ensayo de inmunoabsorción enzimática de captura (MAC-ELISA) para detectar inmunoglobulinas de clase M (IgM) específicas contra VON. Adicionalmente, los sueros seroreactores y los sospechosos fueron analizados con una reacción en cadena de la polimerasa por retrotranscripción (RT-PCR) para detectar ARN viral. **Resultados.** Ocho caballos estudiados fueron seroreactores en la ELISA-MAC (4.3%, 8/184) y dos fueron sospechosos (1.1%, 2/184). Todos estos sueros fueron negativos a la RT-PCR. **Conclusiones.** La detección de IgM específicas contra VON en los caballos estudiados, demuestra infecciones recientes e indica la circulación del virus en el oriente de Yucatán.

Palabras clave: Anticuerpos; MAC-ELISA; *Flavivirus*; IgM; serología (*Fuentes: DeSC*).

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INTRODUCTION

West Nile virus (WNV), classified as a re-emerging pathogen in the American continent due to an increase in its distribution and in the number of infections (1,2), is a positive-polarity RNA virus belonging to the genus *Flavivirus* and represents a public health problem worldwide (2,3). In the transmission cycle, mosquitoes of the genus *Culex* are the biological vectors and wild birds are the amplifying hosts (2,4).

Humans and equines, including domestic horses, are final and accidental hosts; the manifestations of infection range from asymptomatic (mild) to severe encephalitis (2,4).

The detection of viral RNA or class M immunoglobulins (IgM) specific against the virus in the blood serum of affected hosts is used to determine the circulation or recent infection with WNV (5,6), a strategy that helps in the prevention of outbreaks and prevents the spread of endemic areas (7). In this sense, in regions with tropical climates and populations of *Culex* mosquitoes, domestic horses with recent infections are relevant because in the absence of epidemiological surveillance programs, they act as sentinels in monitoring the virus and the subsequent risk of transmission to human beings (7,8).

In Mexico, the first reports of WNV infections were in wild birds and domestic horses with encephalitis from Coahuila, Tamaulipas and Chihuahua during the summer of 2002 (1,9); however, a low number of clinical cases in humans and domestic horses throughout the national territory have been reported (1). The detection and isolation of the virus has also been achieved in crows and American sparrows captured in areas near the border with the USA and southeastern Mexico (1,9).

In 2003, IgG seroreactor horses against WNV were reported for the first time in the state of Yucatán (10). Furthermore, Loroño-Pino et al (11) described that 15% of a studied population of horses from the east of this same state presented IgG against WNV. Additionally, recently, Chaves et al (12) using a reverse transcription polymerase chain reaction (RT-PCR) test, estimated a WNV frequency of 6–27% in different families of Yucatán resident birds, indicating active transmission of the virus. Despite these studies, no IgM seroreactor horses against WNV have been detected in the region, which would indicate a recent infection in these animals (12).

The objective of this work was to determine the WNV circulation in horses from eastern Yucatán, Mexico.

MATERIALS AND METHODS

Place of study. The study was carried out in the municipalities of Tizimín and Panabá, located in the eastern part of the state of Yucatán, Mexico. Both municipalities have agricultural holdings of variable extents that cover almost half their area (49%), so that areas of grasslands, introduced crops and semi-extensive livestock production predominate. Its predominant climate is warm sub-humid with rains in summer, an annual average temperature of 26°C and annual rainfall of 1.228 mm (13).

Study type and studied animals. A descriptive, cross-sectional observational study applying a non-random sampling was carried out. One hundred and eighty-four horses from 23 production units (7–10 animals per unit) were chosen for convenience. Horses were considered regardless of sex, breed or zootechnical function. The inclusion criteria were: animals older than one year, good body condition (according to the Henneke scale) and no history of immunization against WNV, which was verified with the veterinarian in charge of the production unit or with the animal's owner.

During the sampling period, all selected horses were evaluated physically by the responsible veterinarian to identify clinical signs associated with WNV infection, such as fever, loss of appetite, stupor, muscular weakness in the hind limbs, muscle tremor, irritability, postural instability and movement disorders (4,14). In addition, the clinical history was obtained for each horse, provided by the veterinarian.

Taking and processing of biological samples. From each horse, up to 5 ml of whole blood was collected by puncture of the jugular vein with a 21G x 1.5" needle (0.8 mm x 38 mm) and deposited in a Vacutainer® tube without anticoagulant (Becton, Dickinson and Company®, Franklin Lakes, NJ, USA), which were kept in plastic coolers with refrigerants during their stay in the production unit and until their arrival at the laboratory for further processing.

Each blood sample was identified with a number corresponding to the sampling order. Likewise, names, ages, sexes and geographic coordinates

were recorded in a digital database as part of the internal data control.

The blood samples were transferred to the Laboratory of Emerging and Re-emerging Diseases (LEER for its acronym in Spanish) of the Regional Research Center "Dr. Hideyo Noguchi" (CIR for its acronym in Spanish) of the Autonomous University of Yucatán (UADY for its acronym in Spanish). All samples were centrifuged at 1500 *g* for 5 min to separate the serum, which was collected and deposited in 1.5-ml cryopreservation vials with the information corresponding to the animal. Subsequently, they were frozen at -79°C until their use in testing for WNV infection.

Detection of class M immunoglobulins (IgM) specific against WNV by IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA). After dilution to a concentration of 1:400, as recommended by Long et al (15), the blood sera of all the studied horses were analyzed with the MAC-ELISA IDEXX IgM WNV Capture ELISA Test® (IDEXX®, Westbrook, ME, USA), which is based on the qualitative detection of IgM-class antibodies against WNV in the serum of infected equines. This test has reported a sensitivity of 91.7% and a specificity of 99.2% (15).

Four wells in each microplate were used for the positive and negative controls included in the kit, and in the remaining 92, 46 diluted sera with a nonspecific antigen against WNV (in the upper row of the microplate) and with a recombinant antigen against WNV (in the bottom row) were dispensed in duplicate.

All microplates were observed at wavelength 450 nm. In reactive sera, IgM against WNV is bound to the anti-equine IgM coating (layer) placed in the wells of the microplate. The color intensity is proportional to the number of antibodies present in the tested serum. To determine reactive or suspicious sera, the immune response index (ISR) was calculated, which is the interpretation coefficient between optical densities. It was considered negative at <2.0, suspicious at ≥2.0–3.0 and positive at >3.0, according to scales established by the manufacturer.

Viral RNA detection by reverse transcription polymerase chain reaction (RT-PCR). All positive or suspect sera were evaluated by RT-PCR. The viral RNA extraction in these was carried out according to the described protocol in the QIAamp Viral RNA Mini Kit® (QIAGEN®, Barcelona, Spain).

The reverse transcriptase (RT) reaction was carried out with the protocol of the RevertAid H Minus First Strand cDNA Synthesis kit® (Thermo Fisher Scientific®, Waltham, MA, USA), using 5 µl of RNA. The conditions in the thermal cycler were alignment at 42°C for 2 min, amplification at 42°C for 50 min, and an additional cycle at 70°C for 15 min to inactivate the enzyme.

For the viral RNA detection, an endpoint PCR was performed in which the oligonucleotides described by Lanciotti et al. (16) were used: 5'-TTGTGTTGCTCTCTCTTGGCGTTCTT-3' sense and 5'-CAGCCGACAGCACTGGACATTCATA-3' anti-sense, which amplify a conserved fragment of 408 base pairs (bp) located between the WNV genes of the nucleocapsid protein and the pre-membrane.

The reagents used in the reaction had the following final concentrations for a volume of 25 µl: 1X buffer, 0.2 mM dNTPs, 2.5 mM MgCl₂, 0.4 µM of each primer, 1 U of Taq polymerase (Promega®, Madison, WI, USA), 3 µl of complementary DNA (cDNA) and the corresponding volume of ultrapure water. The thermocycler program was an initial denaturation at 95°C for 5 min, followed by 40 cycles (denaturation at 95°C for 1 min, alignment at 60°C for 1 min, and amplification at 72°C for 2 min), and final elongation at 72°C for 5 min.

In all reactions, cDNA synthesized from inactivated WNV RNA (contained in a commercial vaccine and obtained with the previously described RT protocol) was used as a positive control. The negative control was a mixture of all the reagents used in the reaction but without cDNA. The electrophoresis of the PCR products was carried out in 1.2% agarose gels stained with 5% ethidium bromide. A photodocumentation system (Bio-Rad®, Hercules, CA, USA) was used for the recording of results.

RESULTS

One hundred and eighty-four horses were sampled, of which 123 were from the Tizimín municipality and 63 from the Panabá municipality.

The MAC-ELISA showed eight (three from Panabá and five from Tizimín) positive reactor horses (4.3%, 8/184) and two (one per municipality) as suspects (1.1%, 2/184; Table 1). All these sera were negative by RT-PCR.

In the clinical history provided by the veterinarian and in the physical evaluation carried out during the sampling, no clinical signs associated with infection with WNV were detected in any studied horse.

Table 1. List of positive and suspects results obtained by the MAC-ELISA performed on the blood sera of studied horses from the Tizimín and Panabá municipalities, Yucatán, Mexico.

| ID | Municipality of the unit production | MAC-ELISA results | | |
|-----|-------------------------------------|----------------------------------|-----------|------------|
| | | Absorbance degrees at OD 450 nm* | ELISA ISR | Diagnostic |
| 95 | Panabá | WNRA: 0.68 NCA: 0.053 | 12.83 | Positive |
| 147 | Panabá | WNRA: 1.055 NCA: 0.123 | 8.58 | Positive |
| 90 | Panabá | WNRA: 0.281 NCA: 0.069 | 4.07 | Positive |
| 49 | Tizimín | WNRA: 0.562 NCA: 0.077 | 7.30 | Positive |
| 65 | Tizimín | WNRA: 0.424 NCA: 0.068 | 6.24 | Positive |
| 58 | Tizimín | WNRA: 0.374 NCA: 0.066 | 5.67 | Positive |
| 168 | Tizimín | WNRA: 0.259 NCA: 0.059 | 4.39 | Positive |
| 51 | Tizimín | WNRA: 0.176 NCA: 0.053 | 3.32 | Positive |
| 97 | Panabá | WNRA: 0.283 NCA: 0.119 | 2.38 | Suspect |
| 57 | Tizimín | WNRA: 0.149 NCA: 0.06 | 2.48 | Suspect |

ID: horse identification, ISR: immune response index, *optical densities (OD) read at 450 nm for reagents with WNRA (WNV recombinant antigen) and with NCA (nonspecific antigen).

DISCUSSION

The eight horses seroreactive to IgM against WNV in this work represent a frequency of 4.3%. Previously, Loroño-Pino et al (10) reported IgG

antibodies against WNV at a frequency (obtained by neutralization test) of 1.1% (3/252) in horses from the coastal zone and central and eastern Yucatán, those from Tizimín making up more than half the positive cases. In another study, Loroño-Pino et al (11) obtained (through a blocking ELISA) an IgG antibody frequency of 15% (28/186) in horses from municipalities in eastern Yucatán, including Tizimín and Panabá. The difference between the frequencies of horses seroreactive against WNV identified in different years may be due to the population density (lower or higher) of susceptible or non-immunized horses at the study sites, as well as an increase or decrease in the introduction rate of the virus by the migration of wild birds in southeastern Mexico (11).

In the works by Loroño-Pino et al (10,11), WNV infection was demonstrated in domestic horses from Yucatán; however, this did not indicate recent infections in the affected animals. In this context, Joó et al (14) point out that infected horses can present high IgG titers against WNV up to three years post-infection.

On the other hand, Mattar et al (17) described, using an ELISA test, IgM against WNV in two of 12 studied horses in Colombia, concluding that these infections probably had a course of no more than three months. This description reinforces the evidence that the seroreactor horses identified in the present work presented recent infections and that, in addition to past studies (10,11), the WNV circulation has an endemic tendency in these animals. In this sense, Pérez-Ruiz et al (5) mentioned that to establish WNV circulation in a particular region, detection of the viral agent and/or the demonstration of specific IgM against WNV in blood serum or cerebrospinal fluid is necessary. They also serve to detect the early circulation of the virus and avoid the generation of outbreaks in susceptible animals or humans.

In this study, two suspect seroreactor horses (1.1%) were also identified, which could be due to the fact that the IgM titers in these animals were not high enough to reach the cut-off point established in the MAC-ELISA and be considered as positives. This finding is common in hosts with subacute infections characterized by a low titer and by occurring 4–10 days after initial exposure to the virus (14,18). Similarly, it has been reported that IgM can persist in low levels for up to two months after the first exposure (14,19).

It is important to mention that the specificity of ELISA tests has improved with the implementation of WNV-specific protein fragments to differentiate it among flaviviruses; the domain (DIII) of the E protein that protrudes on the surface of the virus is the most used (18). In particular, MAC-ELISA has been validated to detect IgM against WNV in a study carried out on horses from Florida, USA (15), in which it was concluded that this test can identify recent exposure to the virus with a specificity of 91.7% and sensitivity of 99.2%. This type of test has also been used to detect seroreactor horses in endemic regions of other countries such as Croatia (20), Hungary and Italy (14).

The MAC-ELISA (IDEXX IgM WNV Capture ELISA Test®) used in the present investigation has been evaluated by Joó et al (14), establishing a sensitivity and specificity of 95% (taking the neutralization test as the gold-standard test) in a WNV-endemic region (Hungary) where other flaviviruses also circulate.

Both the positive and suspect seroreactor horses found by MAC-ELISA were negative by RT-PCR, which shows that they did not have a viremia detectable or active at the time of blood sampling. In this sense, the World Organization for Animal Health (OIE for its acronym in French), points out that viremia in affected horses is brief and occurs during the disease incubation period (3–15 days after WNV transmission by the bite of the vector mosquito). During this time, antibodies are scarce at the early stage of production, which precedes the appearance of clinical signs, if any (19).

Tizimín and Panabá municipalities have an annual mean temperature of 26°C and an annual rainfall of 1.228 mm (13). These environmental characteristics, as reported by the Environmental Protection Agency (EPA), are the ideal conditions for the maintenance of WNV vector mosquito populations, especially in the jungle and peridomestic areas (21), elements that influence the virus circulation (1). Another factor that influences this circulation is the population of susceptible hosts necessary for enzootic stability to occur in the wild birds principal responsible for transmission cycle maintenance (1,22).

Infection in horses and other susceptible hosts such as humans occurs by overflowing of the

enzootic cycle, typical of jungle areas in tropical regions (7). This overflow is favored by the proximity to the vector mosquitoes and the reservoir bird species (7, 21). In this context, in Yucatán, a wide distribution (in the jungle and peridomestic areas) of mosquitoes of the genus *Culex* (23) that could be participating in the WNV transmission has been described.

On the other hand, according to Kilpatrick (24), in some regions of North America the incidence of WNV in susceptible animals such as domestic horses will increase due to urbanization and the destruction of natural habitats, since it has been shown that distribution of the virus is associated with the anthropogenic use of the land and the increase in the populations of domestic animals and wild bird species that tolerate humans. These characteristics may influence the infection with WNV of the horses studied, since Tizimín and Panabá municipalities have almost 49% of their surface destined for livestock or agriculture (13).

In the physical evaluation and the clinical history of the seroreactive or suspect horses, no clinical signs related to WNV disease were identified, which indicates the presence of asymptomatic infections. In this sense, the OIE mentions that worldwide most infections in domestic horses are asymptomatic (19). Because of this, some hypotheses have been formulated about asymptomatic infections in susceptible hosts from countries with tropical climates (including Mexico) as opposed to North American countries (where most of the infections are symptomatic), the most accepted being the cross-protection granted by infection with other endemic flaviviruses (25) and the circulation of WNV strains of attenuated virulence in Mexico (25,26).

The research results provide recent antecedents of the WNV epidemiology in horses from eastern Yucatán. More research is needed to isolate and characterize the viral strains that infect other susceptible hosts and the biological vectors from the region (27) to describe the risk of transmission, especially to the inhabitants of the eastern part of the state.

It is concluded that the detection of IgM seroreactor horses against WNV indicates recent infections and circulation of the virus in the Tizimín and Panabá municipalities, Yucatán, Mexico.

Conflict of interests

The authors declare no conflicts of interest.

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