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Epidemiological surveillance of West Nile virus in the Eastern Plains of Colombia

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ABSTRACT

Objective. Identify the presence of West Nile virus in horses and mosquitoes in eight municipalities of the Department of Meta. **Materials and methods.** The research was supported by the Bioethics Committee of the University of Los Llanos. 613 samples of Creole and quarter-mile equine horses, intended for sports and work activities, with an age range of 2 to 15 years, were analyzed using serological and molecular tests in the transects: Villavicencio-Restrepo-Cumaral, Sanmartín- Castilla la Nueva-Granada and Puerto López-Puerto Gaitán, analyzed in 62 pools and 213 mosquitoes. The pool of sera of horses and mosquitoes were analyzed by ELISA and PCR. **Results**. No seropositive animals were found by the ELISA test and molecular tests were also negative. **Conclusions**. Although in this study the presence of IgM antibodies was not evidenced by the Elisa technique, and molecular tests (RT-PCR) were also negative for viral circulation, in the municipalities under study, it is important to indicate that the molecular detection in sera, it requires representative levels of viremia and that the animal is in the acute phase of the disease. Although it is possible that the equine population remains free of contact with the virus, epidemiological surveillance should be maintained against this important pathogen for human health, especially due to the outbreak of other zoonotic viruses such as Eastern Equine Encephalitis and Encephalitis Venezuelan Equine in the departments of Meta and Casanare, adjacent to this.

Keywords: Epidemiology; flavivirus; zoonoses; polymerase chain reaction (*Fuente: DeSC*).

RESUMEN

Objetivo. Identificar la presencia del virus del Oeste del Nilo en equinos y mosquitos en ocho municipios del departamento del Meta. **Materiales y métodos.** La investigación contó con el aval del Comité de bioética de la Universidad de los Llanos. Se analizaron mediante pruebas serológicas y moleculares 613 muestras de equinos criollos y de raza cuarto de milla, destinados a actividades deportivas y de trabajo, con un rango de edad de 2 a 15 años, en los transectos: Villavicencio-Restrepo-Cumaral, Sanmartín-Castilla la Nueva-Granada y Puerto López-Puerto Gaitán, analizados en 62 pool y 213 mosquitos. Los pool de sueros de equinos y mosquitos fueron analizados por ELISA y PCR. **Resultados**. No se encontraron animales seropositivos mediante la prueba de ELISA

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y las pruebas moleculares también fueron negativas. **Conclusiones.** Aunque en este estudio no se evidenció la presencia de anticuerpos IgM por la técnica de Elisa y las pruebas moleculares (RT-PCR) también fueron negativas para circulación viral, en los municipios objeto de estudio, es importante indicar que la detección molecular en sueros, requiere unos niveles de viremia representativos y que el animal se encuentre en la fase aguda de la enfermedad. Aunque es posible que la población equina se mantenga libre de contacto con el virus, se debe mantener la vigilancia epidemiológica frente a este importante patógeno para la salud humana, especialmente por la presentación de brotes de otros virus zoonóticos como la Encefalitis Equina del Este y Encefalitis Equina Venezolana en los departamentos del Meta y Casanare, contiguo a este.

Palabras clave: Epidemiología; flavivirus; zoonosis; reacción en cadena de la polimerasa (*Fuente: DeSC*).

INTRODUCTION

The West Nile virus (WNV), an emerging zoonotic pathogen affecting birds, is transmitted by mosquitoes, and has gained significant importance because of its impact on human and animal health (1). This positive-strand RNA virus belongs to the flavivirus genus consisting of more than 70 viruses, including dengue, yellow fever, tick-borne encephalitis, and St. Louis encephalitis viruses (2). Its icosahedral virion measures approximately 50 nm and is derived from host cell membranes (3). Humans and horses are the terminal incidental hosts of this virus, which causes a disease whose severity, may range from mild to highly fatal. (4). The infection is asymptomatic in humans, and approximately 20% of patients may show clinical signs of the flu accompanied by high fever; at least 1% of the infected individuals can have a highly fatal neuroinvasive disease (1). Horses can develop more severe clinical signs than humans, which are characterized by encephalitis, ataxia, weakness of the limbs, recumbence, and muscle tremor (5), reaching a mortality rate of up to 40%. Therefore, their epidemiological surveillance is a good predictor of human infection (6).

WNV first emerged in Uganda in 1937, from where it spread to Asia, Africa, the Middle East, Southern Europe, Australia, and America (7). It appeared in the US in 1999 (8) and was reported in Canada, Mexico, Guatemala, the Caribbean Islands, Argentina, and Venezuela within the following decade (9). At this time, the virus had spread throughout most tropical and subtropical regions of the world, and new, more pathogenic strains were detected in the new outbreaks (1). During the transmission cycle, a total of 59 species of mosquitoes infected by this virus were identified (10).

Studies on WNV conducted in Colombia have indicated that the virus antibodies and their natural circulation are present in horses in the Departments of Córdoba (11) and Antioquia (12). Furthermore, a virus obtained from captive flamingos in the Department of Antioquia was isolated and molecular and phylogenetic characterization was performed (13). A new virus was later isolated with phylogenetic and phylogeographic characteristics similar to those of the strain isolated in Texas (14). Despite these important findings, no fatal cases have been reported in humans and horses, and thus, uncertainty about the epidemiological behavior of this virus has increased.

WNV is now considered an important pathogen by the Colombian Ministry of Health, which recommends its epidemiological surveillance (15). In the Department of Meta and in most of the Orinoquia region, the geographical and epidemiological characteristics are combined to initiate this surveillance, which includes high biological diversity, location of birds in the migration route as they travel toward the south of the continent, where epidemic outbreaks have occurred (16), high agglomeration of birds at certain times of the year, and a close relationship between birds, mosquitoes, horses and other wild mammals. The purpose of this study was to identify WNV in horses and mosquitoes in eight municipalities of the department of Meta using serological and molecular tests.

MATERIALS AND METHODS

Approval from the Ethics Committee. Because this research included animal experimentation, it was previously approved by the Bioethics Committee of Universidad de los Llanos. The methods for collecting, processing, preserving, and submitting samples were consistent with those included in the biosafety guidelines suggested by WHO as well as those suggested by the Animal Research Resources Committee of the University of Wisconsin.

Study area and socioeconomic and climatic conditions. The study was conducted in eight municipalities of the Department of Meta that are connected to its capital, Villavicencio, by three transects. The first included samples of horses located in Villavicencio, Restrepo, and Cumaral; the second in San Martín, Castilla la Nueva,

and Granada; and the third in Puerto López and Puerto Gaitán, from June to December 2014 and from January to June 2015 (Figure 1).

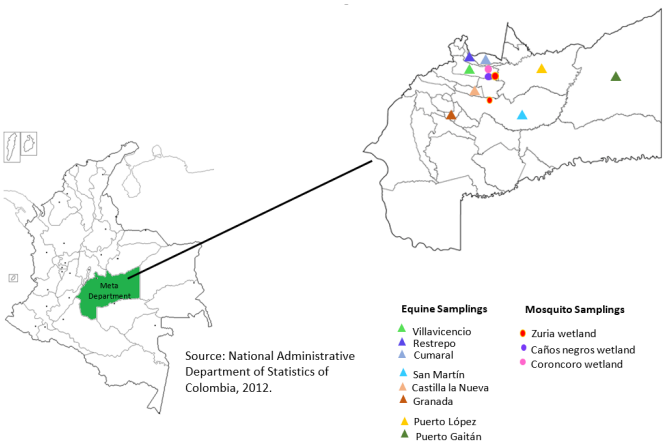

Figure 1. Sampling areas - Epidemiological surveillance of the West Nile virus in the department of Meta, Colombia.

The major economic activities of these municipalities include agriculture and animal husbandry, mainly cattle ranching and rice cultivation. These areas also include abundant tributaries, wetlands, *moriche* palm swamps, and estuaries which are inhabited by a wide variety of wild species most of the year. They have a tropical humid forest ecosystem at an altitude of 200–437 m above sea level, with a relative humidity of 80% during the rainy season (from April to November), 50%–60% humidity during the dry season (from December to March), and an average annual rainfall between 2.000 and 3.800 mm.

Sample selection. A sample consisting of 613 creole and quarter-mile horses was selected, and the calculation was performed based on an expected prevalence of 6.7% (17), with a 95% confidence and 2% accuracy, as described in the guidelines of Peña et al (18), for a total population of 24,480 equines (19). The animals were between 2 and 15 years old.

Obtaining samples from horses. A volume of 5.0 ml of jugular vein blood was obtained from apparently healthy horses destined for sports and work activities, placed in vacutainer tubes without anticoagulant (BD), and transported to the Animal Reproduction and Genetic Laboratory of Universidad de Los Llanos in cold chain at 4°C. They were centrifuged at 5000 g for 10 min and the serum obtained was separated into 1.0 ml aliquots and stored at −70°C until they were tested.

Analysis of equine sera by enzyme-linked immunosorbent assay and polymerase chain reaction. The equine sera were analyzed in pools (1 pool <10 sera) using a commercial IgM antibody capture kit (ID Screen® West Nile IgM capture. ID VET Innovative Diagnostics, Montpellier, France) according to the manufacturer's instructions.

The sera and controls were transferred to 96-well microplates and covered with an anti-equine IgM antibody. The plates were washed automatically (Bioteck Elx50 Instruments Inc, Winooski, VT, USA) with 20× wash solution, and the virus antigen was added, followed by another wash and the addition of a second antibody against WNVE protein labelled with radish peroxidase. After performing a third wash in order to remove the excess conjugate, TMB substrate solution was added and the plates were incubated in a dark room for 15 min. The breaking solution was added, and the plates were subsequently read in a reader at a wavelength of 450 nm (EON-Bioteck Instruments Inc, Winooski, VT, USA). Sera that presented an S/P% ratio of ≤35% were considered negative, those between 35% S/P and 45% S/P were considered doubtful, and those with S/P% of ≥45% were classified as positive. Sera with OD ≥1.45 were considered positive.

Nucleic acid was extracted with Trizol LS, (20). One-step reverse transcription-polymerase chain reaction (RT-PCR) was performed using the One-Step RT-PCR kit (Qiagen®, Valencia, CA), with universal primers specific for flavivirus: FU2 and cFD3, which amplify a 1084 bp fragment of the region of the NS5 gene (21). The RT-PCR conditions were: 50°C x 50 min; 95°C x 5 min, 30 cycles of 94°C x 30 sec, 60°C x 30 s, 72°C x 1 min, and a final extension of 72°C x 10 min (21). The negative control used for RT-PCR was molecular grade water (Gibco®) and the positive control was RNA obtained from an inactivated isolated WNV (access No. JN716372.1), kindly provided by the University of Wisconsin.

The protocol intended for electrophoresis was not used because no amplifications were obtained.

Capture, classification, and PCR in mosquitoes. Mosquitoes were captured at three different sites: the Zuria and Caños Negros wetlands (from September to October 2014, during the transition from winter to summer, and in winter) and the Coroncoro wetlands (from March to April, winter 2015). These sites were located within a 40-km radius from Villavicencio, and two CDC and 1 Shanon light traps were installed for the collections, located 20 m from each other and activated from 6:00 p.m. to 6:00 a.m. (Figure 1).

Adult mosquitoes were collected in entomological boxes with silica gel, while immature individuals were collected in plastic jars with water from ponds, lagoons, wetlands, or *moriche* palm swamps near the adult capture areas. Mosquitoes were morphologically identified at the species level using the taxonomic codes of Lane (22) and Pecor (23), and the provisions of the taxonomic codes in the WRBI (Walter Reed Biosystematic Unit) (24,25). Once classified, they were grouped into pools according to the capture site, species, and date, with a maximum of five mosquitoes per pool and were stored at −80°C until use.

The abdomen of each mosquito was removed and used for RNA extraction. The mosquito pool was macerated manually in 500 µl of PBS. RNA was extracted with Trizol LS. For the molecular detection of the virus, the RT-PCR technique was used with the Qiagen® One-Step RT-PCR kit, both for reverse transcription and for PCR, with the same primers described above.

Sequencing. Correspondingly and independently to the RT-PCR, sera samples were sent to the Biotechnology Center of University of Wisconsin for sequencing using the Sanger method, according to this institution's protocols. Attempts were made to amplify the NS5 gene using the universal flavivirus primers described above (21).

Statistical analysis. Proportional abundance (26), Wealth, Dominance (Simpson Index), and Equity (Shannon–Wiener Index) indices were used to analyze data from the captured mosquitoes. A comparison of diversity indices was performed using Student's t-test, as proposed by Moreno (27).

Statistical analyses for laboratory results were performed using descriptive methods, and measures of central tendency and dispersion, and frequency calculations were made.

RESULTS

In 613 equine sera analyzed in 62 pools, no positivity against IgM antibodies was found using the ELISA technique. Similarly, the results for molecular tests (RT-PCR) in mosquitoes and in equine sera were also negative. No genomic information was obtained for the virus in the sera analyzed with Sanger sequencing of the NS5 gene of WNV. Regarding mosquitoes, 213 were captured in 2014 and 222 in 2015, which were classified into 18 different species, and showed no differences in diversity indices according to sampling time ($p \geq 0.5$). The highest percentages of abundance during 2014 were those of *Culex quinquefasciatus* (53.5%), *C. melanoconion* (18.3%), and *Coquillettidia Rhynchotaenia* (13.1%), whereas in 2015, the most abundant species were *C. quinquefasciatus* (27.4%), *C. nigripalpus* (23.8%), and *C. melanoconion* (10.3%) (Tables 1 and 2).

Table 1. Mosquito captured in 2014 in Caños Negros and Suria de Villavicencio.

Species	Total	%A	Pi	λ = Σpi ²	H
Culex melanoconion	39	18.3099	0.1831	0.0335	-0.3109
C. quinquefasciatus	114	53.5211	0.5352	0.2865	-0.3346
Psorophora confinis	8	3.7559	0.0376	0.0014	-0.1233
P. ferox	2	0.9390	0.0094	0.0001	-0.0438
Anopheles darling	3	1.4085	0.0141	0.0002	-0.0600
Coquillettidia Rhynchotaenia	28	13.1455	0.1315	0.0173	-0.2667
Uranotaenia Lowii	9	4.2254	0.0423	0.0018	-0.1337
Uranotaenia spp.	3	1.4085	0.0141	0.0002	-0.0600
Wyeomyia spp.	7	3.2864	0.0329	0.0011	-0.1122
Total Mosquitoes	**213**				

%A= % Abundance

Table 2. Mosquitos captured in 2015 in Universidad Cooperativa wetland forest in Villavicencio.

Species	Total	%A	Pi	λ = Σpi ²	H
Aedes howardina	2	0.9009	0.0090	0.0001	-0.0424
A. scapularis	4	1.8018	0.0180	0.0003	-0.0724
A. taeniorhynchus	2	0.9009	0.0090	0.0001	-0.0424
Anopheles darling	5	2.2523	0.0225	0.0005	-0.0854
Culex melanoconion	23	10.3604	0.1036	0.0107	-0.2349
C. nigripalpus	53	23.8739	0.2387	0.0570	-0.3420
C. quinquefasciatus	61	27.4775	0.2748	0.0755	-0.3550
Culicidae spp.	1	0.4505	0.0045	0.0000	-0.0243
Culiseta spp.	2	0.9009	0.0090	0.0001	-0.0424
Psorophora cillianta	19	8.5586	0.0856	0.0073	-0.2104
P. confinis	21	9.4595	0.0946	0.0089	-0.2231
P. ferox	6	2.7027	0.0270	0.0007	-0.0976
Psorophora spp.	8	3.6036	0.0360	0.0013	-0.1198
Shannoniana spp.	1	0.4505	0.0045	0.0000	-0.0243
Uranotaenia sp.	6	2.7027	0.0270	0.0007	-0.0976
Wyeomyia sp.	8	3.6036	0.0360	0.0013	-0.1198
Total Mosquitoes	222				

%A= % Abundance

DISCUSSION

Epidemiological surveillance of WNV is an important activity for human and animal health in Colombia, especially facing the recent outbreaks of other zoonotic viruses, such as the Eastern Equine Encephalitis and Venezuelan Equine Encephalitis viruses, in the Department of Meta, where this study was conducted, and in Casanare, which is an adjacent department (28). The results of this study indicate the absence of recent punctual infection in horses from the municipalities analyzed. It is also important to highlight that serum molecular detection requires representative levels of viremia and an animal in the acute phase of the disease.

IgM is the first immunoglobulin that appears during the process of immune response to an infection between 4 and 7 days, and IgG arises during this time. However, in a study on this virus, IgM could persist for a longer period of 1 year (29), which was not observed in our study despite the number of animals analyzed. Other research based on IgG response suggests a limited value in the initial diagnosis of WNV (30).

The presence of IgG antibodies was not determined in this study because of the complementary use of molecular tests in equine serum and in mosquitoes to determine the presence of viral circulation. However, it is possible that the equine population remains free of contact with the virus, as was seen 5 years ago in a study with animals from the same region where the plaque reduction neutralization test was used, which is considered a reference for the specific diagnosis of WNV (12). It is also known that an avian host is required to maintain the transmission and amplification cycles of the virus (31). Therefore, more detailed studies on the avian species susceptible to the virus in this region are suggested because this characteristic may be one of the possible explanations for the absence of virus circulation.

In epidemic outbreaks due to WNV, there is a high rate of infection among birds, which transmit the virus to mosquitoes, which in turn transmit the virus to humans by biting them (32).

The presence of this virus in horses and birds in places such as the Atlantic Coast (11,33,34) and the Department of Antioquia (12), unlike in other places in Colombia, has been reported (35). Furthermore, seroconversion was confirmed against both West Nile and St. Louis encephalitis viruses in horses in the Department of Bolívar (36).

Although this study did not include birds for determining the presence of the virus in this group of animals, it is possible that the virus is maintained in an enzootic cycle in viremic birds and ornithophilic mosquitoes as was demonstrated in another study (37). However, not all epidemiological conditions have converged in these municipalities, including climatic conditions, abundance of vectors in contact with birds and humans, and the presence of infected migratory birds, thus requiring new studies. The spatial and temporal prevalence of the virus is known to depend on intrinsic and extrinsic factors, including host, vector, food preference of the mosquito, and longevity, which combine with the weather to determine the epidemiological patterns of the infection (38,39). Special surveillance has been suggested, particularly in the case of the studied municipalities, which are located in the Eastern Plains area, because of the presence of migratory birds and large bodies of water at certain times of the year, from May to November (11).

Although it was not the subject of this study, it has been suggested that some migratory birds whose transit route is the Orinoquia region can serve as a mechanism for the introduction or dissemination of WNV as they fly toward the south of the continent. This is based on the fact that it was suggested that rapid dissemination to other areas occurred through migratory birds, after WNV emerged in the US for the first time (40). A total of 284 infected bird species have been identified in North America (41), and some of these species migrate to the south of the continent.

The municipalities sampled in our study are within this migratory route (42). Therefore, it can be hypothesized that if this event has occurred in areas close to the municipalities included in our study, it is possible that these birds have not had contact with the virus, or that local conditions did not favor the epidemiological cycle of transmission. This would in a way explain the absence of antibodies in horses and/or the absence of the viral genome in its main virus amplifying agent—mosquitoes—or in its accidental host—horses. However, it must be emphasized that the sample size analyzed in our study does not represent the entire equine population or the entire population of mosquitoes in the region.

These results are different from those of a study where seropositive equines were found, but without evidence of the virus circulating in pools of 99 different species of analyzed mosquitoes (43). This also applies to the way the virus has been spreading throughout the other countries of South America (9).

Regarding captured mosquitoes, most belonged to the *Culex* genus; however, it was not possible to determine the genome of the virus using PCR. It is known that the species *C. pipiens* is the largest transmitter of the virus (44,45).

The capture ELISA test used in this study is considered a reliable and indicative test of infection (33), even though no serological reactivity was observed against the virus. Although serological reactors were found, a careful interpretation of the results has been suggested, given the close antigenic relationship with other flaviviruses which may be circulating (46,47). Based on this, it is believed that the available diagnostic tests have not been the most appropriate in the regions of Colombia where the virus is endemic, which limits serological diagnosis especially where other flaviviruses circulate (11,47,48).

Conversely, the use of universal primers for flavivirus and non-specific primers for WNV does not invalidate the results of this study because we sought to use it as a screening test and then use specific primers if we obtained positive results.

Furthermore, there is no clear explanation for the negative results obtained for flavivirus because the municipalities analyzed have environmental conditions that are favorable for the circulation of dengue, Zika, and chikungunya, or other flaviviruses not yet identified within the region.

The non-evidence of viral circulation obtained in this study does not imply that guard against epidemiological surveillance should be lowered because the environmental transformation resulting from the fragmentation of the ecosystem that the region is suffering, motivated by large-scale business agriculture, can generate new risk factors in the future, including biotic and abiotic risk factors that could favor the presentation of epidemic outbreaks. An example of this condition has been observed through the relationship between the abundance of the virus-transmitting mosquito (*C. pipiens*) and an invasive aquatic plant (*Ludwigia grandiflora*) (48). Likewise, the effects generated by global climate change could cause changes in weather patterns and in the migratory routes of birds in the future, which would lead to outbreaks in horses and humans in unexpected places, a situation for which Colombia is not sufficiently prepared in terms of diagnostic infrastructure and, much less, regarding knowledge of this important disease.

Equal importance should be given to the transmission that may exist through airline flights which have shortened distances between regions, countries, and continents (48).

Lack of evidence about the presence of this virus forces us to continue the search for serological reactors in other municipalities within the Orinoquia region or in other regions of the country where epidemic outbreaks of diseases such as Venezuelan equine encephalitis and eastern equine encephalitis that cause mortality in horses have occurred (28). However, a hypothesis has recently emerged that the absence of severe cases in horses and humans in Colombia can be explained by the fact that the circulating virus is an attenuated strain which is similar to the strain isolated in Texas in 2002 (14).

Finally, we conclude that WNV circulation is absent from the municipalities analyzed, but this does not exempt the country's health authorities and research centers from lowering their guard against the epidemiological surveillance of this virus.

Conflicts of interest

The authors declare no conflicts of interest.

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