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Full Length Research Paper

Evidence of the presence of West Nile Virus in mosquito pools in North Eastern Region of Venezuela

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The aim of this study was to determine the presence of West Nile Virus (WNV) in mosquitoes in northeastern Venezuela. Research was conducted in the Laguna de los Patos, Cumana, Sucre State to demonstrate the presence of WNV RNA in mosquitoes caught in traps of Light (CDC + CO_2) by RT-PCR, during the period July 2007 to February 2008. We found a positive mosquito pool for the species *Coquillettidia* (*Rhynchotaenia) venezuelensis* (Theobald, 1912). The minimum rate of infection of the caught was 0.16/ 1,000 individuals. Continued entomological surveillance is needed for establishing with conclusive criteria etiological responsibility of this and other species in these arboviruses.

Keywords: West Nile Virus, mosquitoes vector, RT-PCR, Venezuela.

INTRODUCTION

The West Nile Virus is primarily an infection of birds, but ornithophilic mosquitoes can transmit it to infect humans and animals. It is caused by an RNA virus of the Flaviviridae family. Ornithophilic mosquitoes (especially *Culex)* feed on these birds during their time of hibernation. Birds play an important role in maintaining the virus, since they act as reservoirs, amplify and spread the virus through their annual cycles of migration. There are more than 43 species of mosquitoes as vectors of WNV tested Granwehr *et al*., (2004) including species *Culex pipiens* and *quinquefasciatus.* The serological diagnosis of virus includes the detection of antibodies (Ac) IgG and IgM by ELISA capture, test plaque reduction neutralization (PNRP) reverse transcription reaction (RT-PCR). The purpose of this study was to determine the presence of natural infection in mosquitoes for WNV by RT-PCR. These studies are important because they allow to design early control strategies for this disease.

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MATERIAL AND METHODS

Study Area

The "Laguna de los Patos", located in the Sucre municipality, county Ayacucho, coordinates (10º 25' 80" N, 64° 11' 56" W) represented the sampling station,

Which was established between the communities, the "Lagunita" and the Malagueña". This lagoon is a migratory bird route wiyh WNV activity in birds (Bosch et al., 2007), and it is an ecotope located near the metropolitan area (campus) representing a potential risk for that urban area. The lagoon has ecological conditions favorable for the presence of birds, horses, and water bodies for mosquito breeding. The area covers 150 km^2 in area. The altitude of the study area is 3 m. In this sector of the lagoon water level decreases when the summer starts, however, in the lagoon there is a semi-arid vegetation with average annual rainfall of 104 mm and two seasons, a rainy season that starts in May and ends in November and a dry season, which begins in December and ends in April. The mean annual temperature is 28.8º C with a relative humidity of 65% Velásquez *et al.,* (2008). The climate according to Köppen climate classification is classified as tropical savanna (Aw) and warm steppe (BSH). Sampling was conducted in the period between July 2007 and February 2008. Adult mosquitoes were collected using light traps with carbon dioxide as an attractant. Mosquitoes were placed once in liquid nitrogen, transported to the laboratory and identified using taxonomic key (Lane, 1953; Cova-Garcia, 1966; Bram, 1967). They were then separated by species in groups of ≤ 150 per trap location, date and time of collection, and stored in microtubes (Eppendorf), 1.5 ml capacity, and stored at -70° C until they were processed for RT-PCR assay.

RT-PCR in mosquitoes

This was performed by phenol-chloroform method of guanidine thiocyanate according to the protocol modified by Urdaneta *et al*. (2005), which was conducted in three stages: maceration of the specimens through a denaturing solution and mechanical action, proper extraction and alcohol precipitation. Later were determined the resulting RNA concentrations. The reverse transcription reaction coupled to the chain reaction (RT-PCR) for amplification of certain regions of the genome of WNV (619 bp fragment of the NS5 gene region) was made possible by using specific oligonucleotides (Invitrogen™ Life Technologies, Brazil) and corresponded to the following sequences 5'GGGAAAGGACCCAAAGTC3'(sense) and GATGTCCTCTCGTTTTCCTG (anti sense). For this purpose we used the kit Access RT-PCR System (Promega™, USA). Viral RNA was converted to complementary DNA (cDNA) prior to enzymatic amplification of DNA using a reverse transcriptase (RT). The reaction (cDNA synthesis and amplification) was performed in a single microtube containing the reaction mixture. The reaction products were analyzed on an agarose 2% gel for visualization with UV lamp. Amplification of cDNA fragments was demonstrated through an agarose gel electrophoresis (Promega™, USA) for 45 minutes at 70 volts and revealed with ethidium bromide (Sigma Chemical Co., USA). The viewing and photographic record of the products of RT-PCR was performed on a UV transilluminator (Gel Doc 2000 Biorad, USA) to analyze the presence of DNA bands and their size.

RESULTS AND DISCUSSION

Table 1 shows the composition of Culicidae fauna collected in the "Laguna de los Patos" Cumana, Sucre state, Venezuela. 13,294 mosquitoes were captured. The most abundant species was *Cq*. (*Ryn*.) *venezuelensis,* 6,215 specimens (46.7%). The same analysis explains that for the month of July 2007 (rainy season) the most abundant species was found to be *Cq. (Ryn.) venezuelensis* (65.8%), but not for the month of February 2008 (dry season), the abundance was higher for specimens of the genus *Cx*. (*Cux*.) *bidens* (53.2%). Likewise, for the months and years were evaluated in second order *Ma.* (*Man*.) *titillans,* (21.5%) and *Cq*. (*Ryn*.) *venezuelensis* (24.1%) and third order *De*. *atlanticus* (6.39%) *and Ma*. *titillans* (21.9%).

Importantly, the species *Cq. venezuelensis* has always been present in great abundance in the samples taken in the localities, the Lagunita and Malagueña. In this regard, Velásquez *et al.* (2008) report having collected the number of 5,039 mosquitoes (51%) of a total of 9,862 specimens collected during the years 2002 and 2004. This species thrives in farms near residences, bothers people because of aggressive bites to humans and pets, day and night (Consoli, 1994). Remarkably, the Lagoon is a ecosystem, located practically in the city, where residents of communities along the banks of the lagoon (Lagunita and Malagueña) conducted uncontrolled deforestation. Also, the locations under study are clearly urban communities, but poor economic conditions. Considering the proximity of houses to the lagoon, as well as other nearby urban areas and human-vector constant, preventive measures should be taken promptly to define its role as a transmitter of WNV in Venezuela. According *Cx.* (*Cux*.) *bidens* abundance found in the month of February 2008 could match the preference of the species in the environment modified by man. However, Visintin *et al.* (2006) concerns in Cordoba, Argentina, this species is abundant at the end of the spring and start the summer, coinciding in Venezuela when finish the dry season and early rainy season. In the collection efforts made in this study found most abundant

Table 1. Composition of Culicidae fauna collected in the "Laguna de los Patos" Cumana, Sucre state, Venezuela. Month July 2007 - Month of February 2008

Species	Year 2007 July Rainy season	%	Year 2009 february Dry season	%	Total	%
Aedes (Och.)taeniorhynchus	16	0,22	0	0,00	16	0,12
Anopheles (Yns.)aquasalis	0	0,00	5	0,08	5	0,03
Coquilletidea (Ryn.) venezuelensis	4750	65,8	1465	24,1	6215	46,7
Culex (Cux.) bidens	421	5,83	3228	53,2	3649	27,4
Culex (Cux.) mollis	17	0,24	0	0,00	17	0,13
Deinoceritis atlanticus	462	6,39	0	0,00	462	3,48
Mansonia (Man.) titillans	1555	21,5	1329	21,9	2884	21,7
Uranotaenia (Ura.)lowii	0	0,00	46	0,76	46	0,35
Total	7221	100	6073	100	13294	100

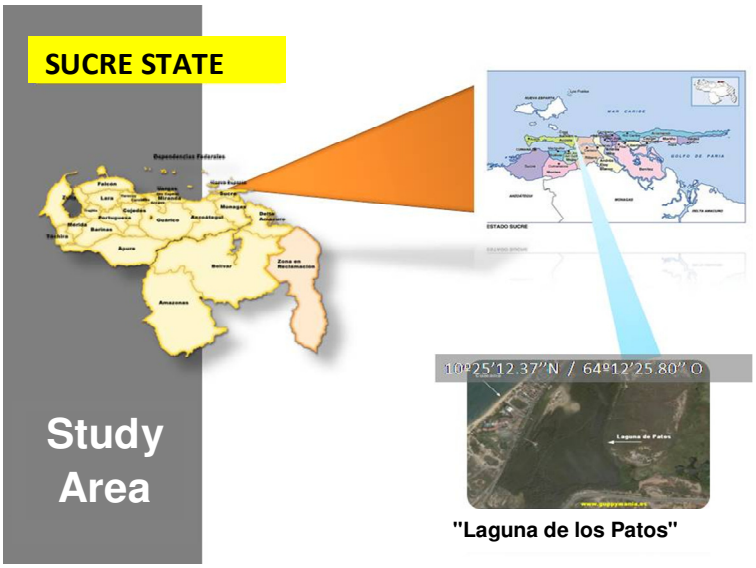
Table 2. Number of groups (pools) of mosquitoes processed by species, corresponding to the sampling point of the selected localities in Sucre state, Venezuela. Years 2007-2008

Species	Sucre The "Laguna de los Patos"	
	N° Mosq	Pool
Adeomyia (Aed.) squamipennis	0	-
Aedes (Och.) serratus	0	-
Aedes (Och.) taeniorhynchus	16	1
Anopheles (Nys.) aquasalis	5	1
Anopheles (Nys.) marajoara	0	-
Anopheles (Ano.) mattogrossensis	0	-
Anopheles (Nys.) oswaldoi	0	-
Coquillettidea (Ryn.) venezuelensis	6,215	42*
Culex (Cux.) bidens	3,649	25
Culex (Cux.) declarator	0	-
Culex (Cux.) mollis	17	1
Culex (Cux.) quinquefasciatus	0	-
Culex (Mel.) dunni	0	-
Culex (Mel.) inhibitator	0	-
Culex (Mel.) spissipes	0	-
Deinoceritis atlanticus	462	4
Mansonia (Man.) titillans	2,884	20
Psorophora (Jan.) albipes	0	-
Psorophora (Jan.) cingulata	0	-
Uranotaenia (Ura.) colosomata	0	-
Uranotaenia (Ura.) lowii	46	1
Uranotaenia (Ura.) pulcherrima	0	-
Total	**13,294**	**95**

Legend (*) Pool Positive for WNV

in the middle of the dry season so monthly samples would be needed to verify this observation.

We processed a total of 13,294 mosquitoes grouped into 95 pools of different species collected in "The "Laguna de los Patos", Cumana, Sucre state in the months of July and February of 2007 and 2008, using the molecular test of RT-PCR (Table 2). The greatest number of mosquitoes tested by the technique as expected corresponded to *Cq. venezuelensis* (42), followed by *Cx. bidens* (25) and *Ma. titillans* (20). The smaller number of mosquitoes examined belonged to the species *Aedes taeniorhynchus*, *An. aquasalis, Cx. mollis*, and *Ur.lowii* (1). The number of mosquitoes present in the



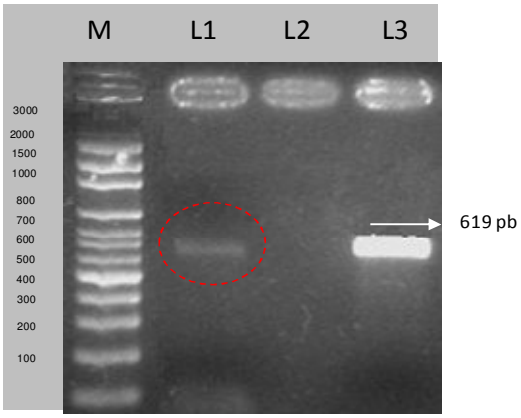


Figure 1. Agarose gel showing the amplified bands containing the positive sample. M: molecular marker 100 bp to 3000 bp. L1 Positive Pool Cq. venezuelensis 10G (619 bp), L2: Negative Control, L3: WNV NS5 plasmido

group of mosquitoes evaluated was from 1-150. We obtained a pool of mosquitoes positive for WNV belongs to the genus *Cq. venezuelensis.* This positive pool contained 153 mosquitoes of species (*Cq.venezuelensis*), confirming that the possibility of finding a pool positive for the virus in question could not be directly associated with the abundance of the species of mosquitoes, but also the amount of viral genetic material present in the sample.

However, other studies in the U.S.A confirm that in evaluating fewer pools of mosquitoes in an investigation, there is a risk of finding WNV-negative pools (Bernard and Kramer, 2001; Kulasekera el al., 2001) so it is advisable to analyze more of these, because the percentage of naturally infected mosquitoes is very low.

In this regard, there are controversies regarding the exact number of mosquitoes that must be analyzed to ensure the probability of finding infected mosquitoes with WNV using RT-PCR Meece et al. (2002) say they have used to carry out the study between 1 to 50 mosquitoes for WNV in the demonstration of mosquitoes captured Sutherland and Nasci (2007) evaluated four different methods, the sensitivity of detection of WNV in mosquito pools of various sizes. They concluded that had the highest sensitivity of real-time PCR, which was able to detect the virus in 100% of the samples containing a single infected mosquito in a pool of 500 mosquitoes.

RT-PCR for detection of WNV in mosquitoes

Figure 1 show the agarose gel revealing the amplified bands containing the positive sample. Was found in one of the pools a fragment of similar size to the NS5 region encoding prM (band corresponding to the 619 bp). Figure indicate the band corresponding to the positive sign of

WNV in *Cq. venezuelensis*, collected in the locality the Lagunita, "Patos Lagoon", Cumana, Sucre state in July 2007.

These findings differ from those obtained by Jaramillo (2005) in a study conducted in six sampling sites of the Cordoba Department, Colombia, where 4,942 mosquitoes were processed. Of these total 61 pools correspond to the genera *Culex* and *Coquillettidea,* but of different species found in our study, however, no found positive pools, a total of 99 pools tested. A similar case occurred in a study conducted during the outbreak of WNV in 1999 in the states of New York and New Jersey where it were colected 32,814 specimens of 25 species, distributed in 1,853 pools, that were found with viral RNA, all belonging to the genus CulexNasci *et al.* (2001). Also, Elizondo-Quiroga (2002) found one positive pool *Cx. quinquefasciatus* collected in field in Nuevo Leon, Mexico, processed by RT-PCR. We identified a total of 2,297 mosquitoes, represented in four genera and eleven species grouped in 238 pools of mosquitoes, not showing their collections in the species referred to as positive for WNV in this research.

It is the first time that this mosquito could is implicated as a possible vector of WNV in Venezuela and worldwide. This positive pool of individuals captured alive, was obtained from the trap of light Nº. 11 (CDC + CO_2) placed in the Lagunita sector in Epidemiological Week Nº. 29 MPPS (2007), in July 2007. Additionally, nine species were collected during the epidemiological week, including from 15 July to 21 July of that year. This species is found in Venezuela in the eastern tropical areas of the country and is one of the most abundant of the studied area Velásquez et al. (2008), Cova- Garcia (1966), Sutíl (1980). It has been reported by Garcia (2007) in Chiapas, Mexico, as one of the main species collected, but analysis of the pools by RT-PCR was negative.

In this regard, Diéguez-Fernández *et al*. (2003), in a study conducted in Cuba, indicated that the species transmitting WNV are not the same in all geographical areas, so we believe there is a barrier to the adaptation of viral strains to populations of culicids in certain sampling areas. Similar case could be occurring in the study area of this research. Jaramillo *et al*. (2005), indicate that the lack of positive results in certain areas should be interpreted carefully as there are many variables which can be influenced, among which they mentions, the number of traps used, sampling intensity, sampling specific points, time of year when the catches were taken and the time when the virus was active and was leaving the bird cycle. Moreover, we consider relevant to note the role of storage temperature of samples (specimens) to achieve viral detection; Garcia (1977) notes the importance of the storage temperature of the specimens as one of the most important impact on the quality of the sample for virus isolation. In this respect, we recommend that any sample to be analyzed for virus detection in mosquitoes using RT-PCR technique should be stored at 70 ° C for the better preservation of virus particles.

Minimum Infection Rate (MIR)

The overall infection rate was 0.06 /1,000 mosquitoes captured. The minimum rate of WNV infection for *Cq. venezuelensis* was 0.16 / 1000. The overall infection rate for *Cq. venezuelensis* in June 2007 was 0.21 per 1000 individuals (positive pool). On the contrary the rate of infection for subsequent sampling was of zero for the other species.

These results can suggest that the species *Cq. venezuelensis* is a low transmission rate. Garcia (2007) indicated for *Cx. interrogator* an MIR of 0.5 /1000 mosquitoes. This species being one of the most abundant in the studied community of the city of Reynosa, Tamaulipas, Mexico, which is related to the abundance of the species *Cq. venezuelensis* in the sampling area, but for 2008 positive pools were not obtained for this species, which could be because the virus was not present for that year, the specimens were not infected, or perhaps motivated to birds showed no viremia high enough to allow its bird cycle exit. Bernard and Kramer (2001) says about it for *Cx. pipiens* that when there is active transmission of the disease rates are high, but these are increased even more when it coincides with peak transmission. However, in the U.S.A., it has being reported infection rates of 10.9 / 1,000 for *Coquillettidea* species (Godsey et al., 2002).

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