

## West Nile Virus detection by RT-PCR from mosquitoes in a locality of Baja California, Mexico

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**ABSTRACT. Background:** West Nile virus (WNV) is an important public health flavivirus transmitted to humans by infected mosquitoes. Transmission involves birds and primarily *Culex* sp. mosquitoes, with humans as incidental hosts but with severe disease in some cases. **Methods:** A total of 2,333 mosquitoes distributed in 92 pools were sampled in a locality of Mexicali Valley, Baja California, Mexico, between September 2005 and August 2006 using CO<sub>2</sub> traps and aspiration tubes. Mosquito samples were identified, divided into groups by species and month of sampling and tested by reverse transcription-polymerase chain reaction (RT-PCR) in order to estimate the relative abundance of each mosquito species per month and the WNV infection rate respectively. **Results:** A total of seven mosquito species were identified and three of these were found to be infected with WNV: one in October of 2005 corresponding to *Aedes vexans*; two more in November 2005 and April 2006 corresponding to *Culex pipiens/quinqüefasciatus*; and one more in May 2006 corresponding to *Culex tarsalis*. **Conclusion:** The risk of WNV infection in human population in the study area exists, and actions to control mosquito populations for veterinary and public health authorities who conduct surveillance programs in the region are necessary, principally in spring and autumn.

**Key words:** West Nile Virus, vector transmitted diseases, RT-PCR detection, mosquitoes, infection rate.

**RESUMEN. Antecedentes:** El virus del Oeste del Nilo (VON) es un flavivirus de importancia en salud pública ya que puede provocar casos graves de encefalitis o meningitis en humanos. Es transmitido principalmente por mosquitos del género *Culex* y los hospederos más comunes son aves silvestres. **Metodología:** Se muestrearon con trampas de CO<sub>2</sub> y tubos de aspiración 2,333 mosquitos distribuidos en 92 grupos desde septiembre de 2005 a agosto de 2006. Las muestras de mosquitos se identificaron y se probaron por transcripción inversa acoplada a la reacción en cadena de la polimerasa (RT-PCR) agrupados por especie y por mes de colecta, con lo cual se estimó la abundancia relativa de cada especie de mosquito por mes y la tasa de infección por VON. **Resultados:** Se identificaron 7 especies de mosquitos, tres de las cuales fueron positivas a VON: *Aedes vexans* en el mes de octubre de 2005, *Culex tarsalis* en mayo de 2006 y *C. pipiens/quinqüefasciatus* en noviembre de 2005 y abril de 2006 respectivamente. La abundancia relativa de las especies encontradas positivas fue mayor durante los meses de primavera y otoño. **Conclusión:** El riesgo de infección en humanos por VON en el área de estudio existe, ya que el virus está presente, por lo tanto, es necesario tomar acciones para el control de las poblaciones de mosquitos por parte de las autoridades competentes, principalmente durante los meses de mayor riesgo, ya que la localidad de estudio está rodeada de zonas habitacionales y educativas.

**Palabras clave:** Virus del Oeste del Nilo, enfermedades transmitidas por vector, detección por RT-PCR, mosquitos, tasa de infección.

### INTRODUCTION

West Nile Virus (WNV) is a mosquito transmitted flavivirus that can cause fever and in some cases critical encephalitis or meningitis in humans (Reisen et al. 2004; Secretaría de Salud, México, n.d.; Centers for Disease Control n.d.). *Culex* species mosquitoes are the most efficient vectors of WNV, although various species of the *Aedes* genus are also capable of causing infection (Styer et al.

2007). In 1999, 62 cases of WNV encephalitis in humans occurred in New York, of which 7 died. Furthermore, the State of California reported 379 cases, with 16 deaths during 2007 (Centers for Disease Control, n.d.). In Mexico there has been growing concern about the appearance of the disease (Estrada-Franco et al. 2003). For example, in Baja California 95 birds were sampled during 2006 of which 7 were positive in serological tests, all within the municipality of Mexicali; also a total of 46 equine samples from 228 analyzed were positive to WNV of which 25 corresponded to the municipality of Mexicali. That same year, 31 people were sampled, but none were positive for WNV (Secretaría de Salud, México n.d.). In Baja California several species of the *Culex* and *Aedes* genera which are known to be vectors for the virus have been collected and identified.

Infection by WNV can be detected through RT-PCR, serological ELISA tests and viral isolation in cell culture (Johnson et al. 2001 and 2003). RT/PCR has become

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widely used for quick identification of various viruses including WNV in different hosts, and it has been shown to have great sensitivity and specificity (Shi et al. 2001; Scaramozzino et al. 2001; Eisler et al. 2004). Until now no studies have been carried out on the infection rates in any species of mosquitoes in Baja California, nor has RT-PCR been used for detecting WNV.

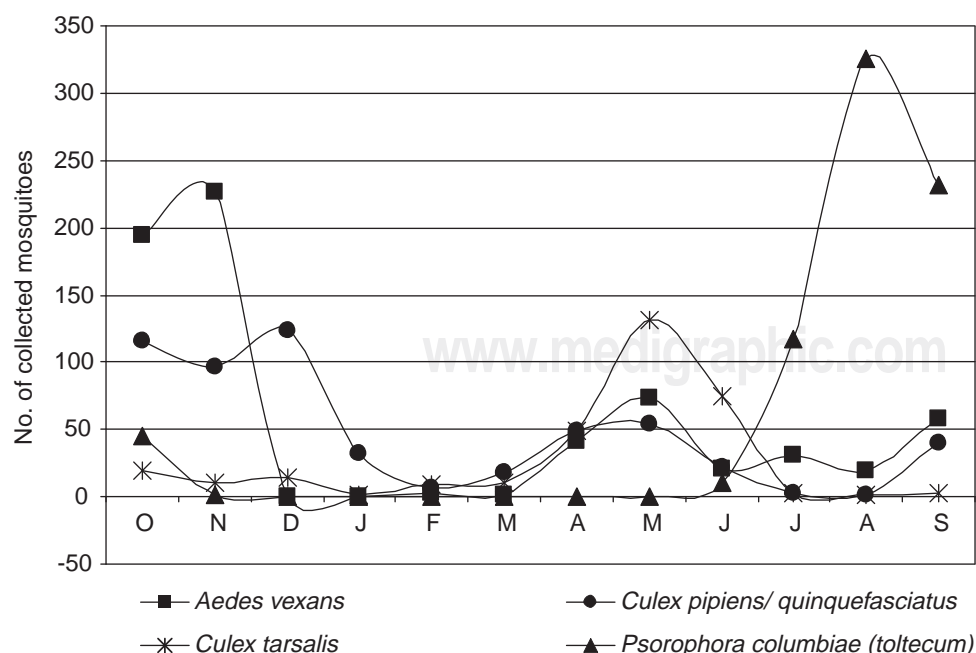
## MATERIALS AND METHODS

A total of 2,333 female mosquitoes were captured in a location within the Mexicali valley in Baja California, which is located at 32° 34' 10.4" latitude north and 115° 27' 3.7" longitude west. This location is characterized by its proximity to urban settlements with the presence of educational institutions and agricultural lands at less than 1 Km distance. Four monthly samplings were carried out from October 2005 to September 2006 using an EVS 2801A CO<sub>2</sub> trap (BioQuip Products Inc.) which was left at night, and two suction tubes were used in the afternoon for 1 hour located in three previously identified mosquito resting areas, at no more than 50 meters from the trap. Each mosquito sampled was identified according to a guide approved by Sanitary authorities in México (Meyer and Dhillon n.d., NOM-032-SSA2-2002) and pooled by species (with a maximum of 50 mosquitoes and a minimum of 1) and by month of capture in order to carry out RT-PCR. It is assumed that due to the sustained sampling effort and the fact that the

sampling methods used were not selective for some species, it is possible to determine the relative abundance of each mosquito species throughout the year.

Mosquito pools were homogenized to a final volume of 100 µl with DNAase and RNAase free water (Sigma, Inc.), using sterilized pestles in 1.6 polypropylene tubes. The commercial reagent Trizol® LS Reagent (Invitrogen Inc.) was used in accordance to manufacturer's guidelines on the homogenized pools described above. Total RNA extracted was left in 75% ethanol at -20°C until it was checked in a 1% agarose gel stained with ethidium bromide in UV lamp in order to determine its integrity and later use in RT-PCR.

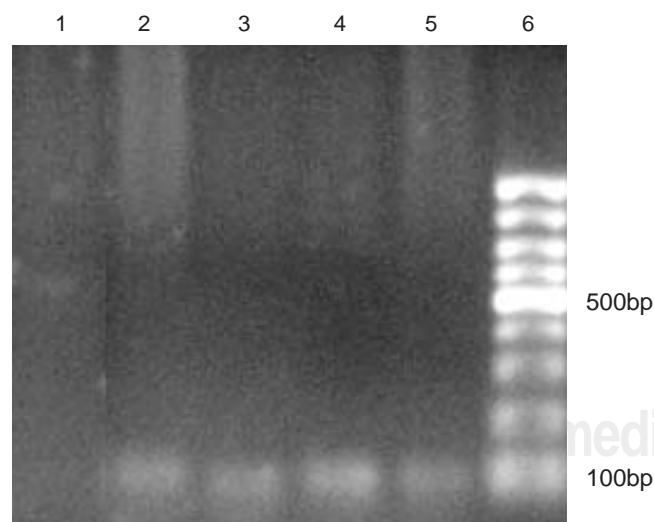
A 408 base pair fragment, which corresponds to the terminal carboxyl of gene C and to the amino terminal end of the pRM gene of WNV was amplified through RT-PCR in a single step, and a nested PCR was carried in order to obtain a 103 base pair fragment as described previously (Shi et al. 2001). Briefly, the primers used were VNO1: 5'-TTG TGT TGG CTC TCT TGG CGT TCT T-3' and VNO2: 5'-CAG CCG ACA GCA CTG GAC ATT CAT A-3' for the first reaction; VNO3: 5'-CAG TGC TGG ATC GAT GGA GAG G-3' and VNO4: 5'-CCG CCG ATT GAT AGC ACT GGT-3' for the second reaction. The RT-PCR reaction was performed using a one step amplification kit (Qiagen Inc.) according to the manufacturer instructions. The nested PCR reaction was performed using a Taq DNA polymerase under the same conditions (Invitrogen Inc.).



**Figure 1.** Relative abundance of mosquito species per sampling month showing the quantity of mosquitoes for each of the four more abundant species collected. Four monthly samplings were carried out from October 2005 to September 2006 using a CO<sub>2</sub> trap which was left at night, and two suction tubes used at dusk for 1 hour located in three previously identified mosquito resting areas, at no more than 150ft. of the trap.

The RT-PCR and nested PCR reactions were made in an "iCycler" thermocycler (Biorad Inc.) under the following conditions: for RT-PCR reaction tubes were incubated at 50°C for 30 min and at 95°C for 15 min followed by 35 cycles at 94°C for 45 seg, 56°C for 45 seg and 72°C for 1 min. The final extension step was at 72°C for 10 min. For nested PCR the reaction tubes were incubated at 94°C for 3 min, followed for 22 cycles at 94°C for 45 seg, 58°C for 45 seg and 1 min at 72°C. Finally, the reaction tubes were amplified at 72°C for 10 min. 15 µl of the products were analyzed in 1.5% TBE 1X agarose gels, stained with 0.5 µg/ml of Ethidium Bromide and observed under ultraviolet light (UV Transilluminator 2000, Biorad, Inc.). All reactions were performed in a PCR enclosure cabinet (Labconco, Inc.) and the all laboratory material was DNase, RNase, Pyrogen, DNA and RNA certified free (CLP, Inc.). Each group of reactions included a negative control with only water, and a positive RT-PCR product was sequenced (Davis sequencing Inc.) using the primers of the second reaction in order to ascertain that the fragment corresponded to gene C of the WNV.

Infection rate was calculated using the Excel® add-on Pooled Inf Rate version 3.0 (Biggerstaff, 2006) for the whole sampling, for total species monthly, for each species and for each species monthly.



**Figure 2.** Nested PCR of WNV RNA positive samples and a negative control. Lane 1. Negative control. Lane 2, *A. vexans* pool sampled in October of 2005. Lane 3. *C. pipiens/quinquefasciatus* pool sampled in November of 2005. Lane 4. *C. pipiens/quinquefasciatus* pool sampled in April of 2006. Lane 5. *C. tarsalis* pool sampled in May of 2006. Lane 6. 100bp Weight marker (CLP inc.).

## RESULTS

The most abundant species sampled were *Psorophora columbiae* (31.29%), *Aedes vexans* (28.68%), *Culex pipiens/quinquefasciatus* (24.09%), and *Culex tarsalis* (14.02%), while the least abundant species were *Culex erythrothorax* (1.29%), *Aedes dorsalis* (0.43%) and *Culiseta inornata* (0.21%).

The relative abundance of the major species collected throughout the year can be seen in Figure 1. It was found that *A. vexans* is very abundant in the autumn months of October and November, as well as in May during spring, but has low numbers in summer and is absent during winter. In contrast, *C. pipiens/quinquefasciatus* can be found throughout the year, even though populations do decrease during the hottest and coldest months. Similarly, *C. tarsalis* can also be found throughout the year but particularly from April to June. *P. columbiae* is a mosquito that is highly abundant in the area but only from July to September when average temperatures reach 39°C, making it a summer species.

A total of 92 mosquito pools were analyzed by RT-PCR, of which four were positive (4.35%). In Figure 2 are presented the 103 bp fragments corresponding to the positive samples to WNV nested PCR. The positive RT-PCR product corresponding *C. pipiens/quinquefasciatus* pool from April of 2006 was sequenced (Davis sequencing Inc.) confirming that the amplicon really correspond to WNV genetic material. The general infection rate was 1.77 per 1,000 mosquitoes with a minimum of 0.57 and a maximum of 4.26 (95% CI). The infection rate per species infected with WNV was as follows: *C. tarsalis* with 3.16, *C. pipiens/quinquefasciatus* with 3.70 and *A. vexans* with 1.51. In the same manner the infection rate was calculated monthly for all species and for each species. The general infection rate in October 2005 was 2.68 per 1,000 individuals but the infection rate for *A. vexans* in this month (one positive pool) reached 5.16, furthermore during November 2005 the general infection rate reached 2.97 but for *C. pipiens/quinquefasciatus* it was 9.17. During April 2006 the general infection rate was 7.24, even though specifically for *C. pipiens/quinquefasciatus* it was 19.58. Finally, in May 2006, the general infection rate was 3.99 while specifically for *C. tarsalis* it was 8.09. In the rest of mosquito species the infection rate was zero.

## DISCUSSION

In Imperial County in California, which is adjacent to the Mexicali Municipality and has similar climatic characteristics, it has been found that 6.72% of sampled groups were infected with WNV (Reisen et al. 2004) which is sim-

ilar to the results in this study. Although that study found only *C. tarsalis* groups infected, while in the present study *C. pipiens/quinquefasciatus* and *A. vexans* also were found to be infected. This is understandable as *C. pipiens* has been found to be one of the main vectors for the disease in other counties in California and the rest of the United States (Reisen et al. 2004) while *A. vexans*, although considered a moderate vector, has been founded with WNV in some other areas in the United States (Molaei and Andreadis 2006., Reisen et al. 2004). This study demonstrates the presence of important vectors infected with WNV in a Baja California, México locality, mainly in spring and autumn. This points to the fact that the human populations in the locality are at risk and actions necessary to prevent cases of meningitis due to WNV in humans such as surveillance programs, fumigation, elimination of mosquitoes breeding areas, and population information campaigns about the hours of highest activity of mosquitoes, use of repellents and water container elimination, should be strengthened and increased specifically during these seasons.

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