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Detection of West Nile virus-specific antibodies and nucleic acid in horses and mosquitoes, respectively, in Nuevo Leon State, northern Mexico, 2006–2007

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Abstract. In the last 5 years, there has been only one reported human case of West Nile virus (WNV) disease in northern Mexico. To determine if the virus was still circulating in this region, equine and entomological surveillance for WNV was conducted in the state of Nuevo Leon in northern Mexico in 2006 and 2007. A total of 203 horses were serologically assayed for antibodies to WNV using an epitope-blocking enzyme-linked immunosorbent assay (bELISA). Seroprevalences for WNV in horses sampled in 2006 and 2007 were 26% and 45%, respectively. Mosquito collections in 2007 produced 7365 specimens representing 15 species. *Culex* mosquitoes were screened for WNV RNA and other genera (*Mansonia, Anopheles, Aedes, Psorophora* and *Uranotaenia*) were screened for flaviviruses using reverse-transcription (RT)-PCR. Two pools consisting of *Culex* spp. mosquitoes contained WNV RNA. Molecular species identification revealed that neither pool included *Culex quinquefasciatus* (Say) (Diptera:Culicidae) complex mosquitoes. No evidence of flaviviruses was found in the other mosquito genera examined. These data provide evidence that WNV is currently circulating in northern Mexico and that non-*Cx. quinquefasciatus spp.* mosquitoes may be participating in the WNV transmission cycle in this region.

Key words. Flavivirus, horse, mosquitoes, seroprevalence, surveillance, West Nile virus.

Introduction

West Nile virus (WNV; *Flaviviridae*, *Flavivirus*) is a member of the Japanese encephalitis complex, which also includes Japanese encephalitis virus, St. Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (Heinz *et al.*, 2000). The virus is transmitted in natural cycles that primarily involve *Culex* spp. mosquitoes and birds (Hayes, 1988; Burke & Monath, 2001). Humans and horses can develop clinical illness after WNV infections but are considered dead-end hosts for virus transmission (Hayes, 1988; Roehrig *et al.*, 2002).

The initial outbreak of WNV in North America occurred in New York City in 1999 and WNV has subsequently spread throughout the Americas (Lanciotti *et al.*, 1999; Gould & Fikrig, 2004; Cruz *et al.*, 2005; Mattar *et al.*, 2005). In the U.S.A., annual numbers of reported WNV disease cases during 2002 to 2008 ranged from 1356 to 9862 (http://www.cdc.gov/ncidod/dvbid/westnile/ surv&control.htm). The first evidence of WNV activity in northern Mexico occurred in 2002 when antibodies to WNV were detected in horses in the states of Coahuila, Chihuahua and Tamaulipas (Blitvich *et al.*, 2003a; Estrada *et al.*, 2003). Serological evidence of WNV activity

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has now been reported in almost every Mexican state (Komar & Clark, 2006; Blitvich, 2008). However, only eight human cases of WNV disease have been reported in Mexico. These cases occurred in the states of Chihuahua ($n = 4$), Sonora ($n = 1$) and Nuevo Leon ($n = 1$) in 2003, Sonora ($n = 1$) in 2004 and Nuevo Leon ($n = 1$) in 2009 (Elizondo Quiroga *et al.*, 2005; Komar & Clark, 2006; Blitvich, 2008; Rios-Ibarra *et al.*, 2010).

Several species of mosquitoes have been reported to be infected with WNV in Mexico, including *Culex nigripalpus* (Theobald) and *Cx. interrogator* (Dyar and Knab) in Chiapas State, *Cx. tarsalis* (Coquillett) in Baja California State, and *Cx. quinquefasciatus* (Say) in Nuevo Leon State (Elizondo *et al.*, 2005; Medina *et al.*, 2008; Ulloa *et al.*, 2009). However, there has been very limited success in obtaining isolates of WNV from any source in Mexico (Estrada *et al.*, 2003; Blitvich *et al.*, 2004; Elizondo *et al.*, 2005; Deardorff *et al.*, 2006). Indeed, the last WNV isolate from northern Mexico was obtained in Sonora State in 2004 (Elizondo *et al.*, 2005). The purpose of the present study was to test the hypothesis that WNV is still a cause of infection in horses and mosquitoes in Nuevo Leon State, northern Mexico.

To test this hypothesis, the seroprevalence for WNV in horses and the infection rate of WNV in mosquitoes in Nuevo Leon State was determined. Horses were sampled in the municipalities of Cadereyta, Montemorelos, Pesqueria and Villa Juarez in 2006, and Cadereyta, Pesquería, Monterrey and Santiago in 2007 (Table 1). Mosquitoes were collected in Ejido Francisco Villa in the municipality of Pesquería (25°4′11.9″N, 100°0′14.0″W). WNV had previously been isolated from a pool of mosquitoes collected in this site (Elizondo *et al.*, 2005). The average annual rainfall in all sites is approximately 550 mm and the mean annual temperature is 28 °C.

Materials and methods

Serum samples were collected from 203 available selected horses in every locality. Of these, 62 horses were sampled from April to August 2006, and 141 horses were sampled in September and October 2007. None of the horses had been vaccinated against WNV or had any travel history.

Table 1. Study sites in Nuevo Leon State and numbers of horses sampled during 2006–2007.

Study site	Location	No. of horses sampled (%)
2006		
Cadereyta	25°36′32.8″N; 100°02′19.5″W	22 (35.5)
Montemorelos	25°11′01.4″N; 99°49′46.2″W	5 (8.1)
Pesquería	37°25′81.8″N; 122°05′36″W	12 (19.3)
Villa Juarez	25°38′50.4″N; 100°05′40.5″W	23 (37.1)
TOTAL		62 (100)
2007		
Cadereyta	25°36′32.8″N; 100°02′19.5″W	56 (39.7)
Monterrey	25°35′45.4″N; 100°15′0″W	58 (41.1)
Pesquería	25°42′56.0″N; 100°01′04.6″W	2 (1.5)
Santiago	25°28′08.2″N; 100°10′26.9″W	25 (17.7)
TOTAL		141 (100)

Additionally, all were asymptomatic at the time of sampling. Serum samples were screened at the Universidad Autonoma de Nuevo Leon (UANL) for antibodies to WNV using an epitope-blocking enzyme-linked immunosorbent assay (bELISA) as previously described (Blitvich *et al.*, 2003b,c). The ELISA was performed with WNV-specific monoclonal antibody (MAb) 3.1112G (Vector Laboratories, Burlingame, CA, U.S.A.). The ability of the horse serum to block the binding of MAb to WNV antigen was compared with the blocking ability of horse serum without antibody to WNV. Data were expressed as relative percentages, and inhibition values ≥30% were considered to indicate viral antibodies (Blitvich *et al.*, 2003b,c).

Mosquitoes were collected in Ejido Francisco Villa in September and October 2007 using CO_2-baited CDC miniature light traps (John W. Hock Company, Gainesville, Florida, U.S.A.). Mosquitoes were transported on dry ice to the UANL then transported on dry ice to Colorado State University (CSU) for species identification and testing for WNV RNA. Mosquitoes were identified to species on a chill table using a published identification key (Darsie & Ward 2005) and sorted into pools of 1–20 according to species, sex and collection date. A total of 7365 mosquitoes from 6 genera and 15 species were collected. The most common species were *Mansonia titillans* (Walker) (24.1%), followed by *Cx. quinquefasciatus* (12.7%), *Cx. coronator* (Dyar and Knab) (7.7%) and *Cx. erraticus* (Dyar and Knab) (6.9%; Table 2) although 1618 (22.0%) mosquitoes were damaged and could only be identified to genus.

Mosquito pools were triturated for 45 s with a vortex mixer in a 5-mL round-bottom polypropylene tube (Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) that contained 1.5 mL of diluent (1× minimum essential medium supplemented with 2% fetal bovine serum, penicillin/streptomycin, L-glutamine and non-essential amino acids) and four copper-coated steel shot (4.5-mm diameter; 0.177″ caliber). Suspensions were centrifuged at 12,000 **g** for 10 min at 4 °C, and supernatants were collected. Total RNA was extracted from supernatants using the QIAamp viral RNA Mini kit (Qiagen Inc., Valencia, California, U.S.A.). All non-*Culex* spp. mosquitoes were analysed by RT-PCR using previously described flavivirus-specific primers (Scaramozzino *et al.*, 2001). Mosquito pools with *Culex* spp. mosquitoes were tested using WNV-specific primers developed and recommended by the United States Centers for Disease Control and Prevention for use in WNV surveillance (Gubler *et al.*, 2000; Lanciotti *et al.*, 2000). Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, California, U.S.A.) and PCRs were performed using GoTaq DNA polymerase (Promega, Madison, WI, U.S.A.). PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen Inc.) and sequenced at the CSU Proteomics and Metabolomics Facility which uses the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, U.S.A.).

Results and Discussion

Eighty-three (41%) horses had antibodies to WNV by bELISA. Of the 62 horses sampled in 2006, 16 (26%) were seropositive

Table 2. Mosquitoes collected in Nuevo Leon State, Mexico in September and October 2007 and examined for flaviviruses.

Species	Total of mosquitoes collected	Percentage of total mosquitoes collected	Number of pools (total number of mosquitoes) examined for WNV (*Culex*) or flavivirus (other genera)
Mansonia titillans	1778	24.14	91
Culex spp.	1228	16.67	62*
Culex quinquefasciatus	934	12.68	48
Culex coronator	567	7.69	30
Culex erraticus	509	6.91	27
Anopheles crucians	495	6.72	26
Anopheles quadrimaculatus	491	6.66	26
Aedes taeniorhynchus	488	6.62	26
Anopheles spp.	276	3.74	14
Aedes scapularis	220	2.98	12
Psorophora columbiae	145	1.96	9
Psorophora ciliata	22	0.29	7
Aedes spp.	114	1.54	6
Aedes vexans	39	0.52	3
Anopheles pseudopunctipennis	34	0.46	3
Uranotaenia lowii	15	0.2	1
Psorophora cyanescens	3	0.04	1
Aedes albopictus	7	0.09	1
Total	7365	100	393

*Two pools were positive for WNV RNA; minimum infection rate of 0.61 per 1000 *Culex* mosquitoes.

for WNV. Of the 141 horses sampled in 2007, 67 (48%) had evidence of WNV exposure. Rates of WNV exposure were compared by sex and age using the SAS 9.1 statistical software package (SAS Institute Inc., Cary, NC). There were no significant differences in the seroprevalence for WNV in female and male horses in 2006 (27% of 37 females vs. 20% of 25 males with evidence of WNV exposure; $\chi^2 = 1.36$, $P = 0.24$) or in 2007 (50% of 54 females vs. 46% of 87 males with evidence of WNV exposure; $\chi^2 = 0.32$, $P = 0.57$). However, there were significant differences in the seroprevalence for WNV in horses <10 years and >10 years in 2006 (25% of 61 younger horses vs. 0% of 1 older horses with evidence of WNV exposure; $\chi^2 = 28.57$, $P < 0.001$) and 2007 (51% of 119 younger horses vs. 27% of 22 older horses with evidence of WNV exposure; $\chi^2 = 12.11$, $P < 0.001$). The youngest seropositive horse was a 12-month-old colt sampled in Cadereyta in October 2007 suggesting that the most recent WNV infection occurred in late 2006 or in 2007.

A total of 3238 *Culex* mosquitoes were screened using RT-PCR with WNV-specific primers. WNV RNA was detected in two pools consisting of *Culex spp*. mosquitoes (Minimum Infection Rate per 1000 *Culex* mosquitoes = 0.61). Sequencing of the PCR products (GenBank accessions GQ862373 and GQ862374) revealed that they are identical to the homologous regions of multiple isolates from both the NY99 or WN02 genotypes and thus, we could not identify the WNV genotype responsible for these infections. The mosquitoes in these two pools could not be identified to species based on morphological characteristics and therefore we attempted to determine their identity using a molecular species identification technique. Briefly, genomic DNA was extracted from the mosquito pools using the Qiagen DNeasy blood and tissue kit (Qiagen Inc.) and analysed by PCR using previously described *Cx. quinquefasciatus* complex-specific primers (Aspen *et al.*, 2003). This analysis revealed that both pools were negative for *Cx. quinquefasciatus* and *Cx. pipiens* (Linnaeus) DNA. Flavivirus RNA was not detected in any of the pools comprised of *Mansonia, Anopheles, Aedes, Psorophora* and *Uranotaenia* spp. mosquitoes.

Taken together, the findings from the current serological investigation and entomological study indicate that WNV remains a current cause of infection in horses and mosquitoes in northern Mexico. Seroprevalence for WNV in horses in Nuevo Leon State in 2006 and 2007 was moderate to high (26% and 48%, respectively). Moderate to high rates of seropositivity for WNV were also reported in horses in Coahuila State in 2002 (62%), Nuevo Leon State in 2003 (23%), Quintana Roo State in 2003 (52%) and Chiapas State in 2006 (53%) (Blitvich *et al.*, 2003a; Marlenee *et al.*, 2004; Farfan-Ale *et al.*, 2006; Alonso-Padilla *et al.*, 2009). WNV RNA was detected in two pools of *Culex* mosquitoes in Nuevo Leon State in 2007. Unfortunately, both mosquito pools containing WNV RNA were comprised of *Culex* spp. mosquitoes. Using a molecular species identification technique, it was determined that these pools did not contain *Cx. quinquefasciatus* or *Cx. pipiens*. This is important because *Cx. quinquefasciatus* was found to be infected with WNV in a previous study in Nuevo Leon (Elizondo *et al.*, 2005). Additionally, *Cx. quinquefasciatus* is the principle vector of WNV in neighbouring Texas (Lillibridge *et al.*, 2004; Vanlandingham *et al.*, 2007). Other *Culex* species collected in the present study, and thus likely to be represented in the *Culex* spp. pools, included *Cx. erraticus* and *Cx. coronator*. Both species have been reported to be naturally infected with WNV in the U.S.A. (http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm#99). Furthermore, Ulloa *et al.* (2009) detected WNV in pools of *Cx. nigripalpus* and *Cx. interrogator* from Chiapas State in southern Mexico, and Medina *et al.* (2008) reported WNV in *Cx. tarsalis* from Baja California State in northwestern Mexico. None of these three species were collected in the present study. Further studies on *Culex* mosquitoes in different parts of Mexico are needed to determine which species potentially serve as locally important WNV vectors to humans and domestic animals. Indeed, much remains to be discovered in northern Mexico about enzootic WNV transmission cycles, including determination of locally important mosquito vectors and microhabitats where humans and horses are at an elevated risk of exposure to WNV-infected mosquitoes.

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