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Sera of 624 blood donors were evaluated to determine seroprevalence of West Nile virus (WNV) antibodies following the 2003 WNV epidemic in Nebraska. Geographic factors contributing to differences in WNV seropositivity were evaluated. The overall prevalence of WNV in Nebraska was higher than reported previously in other U.S. locations (9.5% WNV immunoglobulin G seroprevalence rate), with the highest prevalence identified in the western part of the state (19.7%), followed by the central (13.8%) and the eastern (4.2%) parts. Regions of the state with the highest WNV-positive mosquito rates correlated with the highest human WNV seroprevalence rates. The results showed that both the western and central parts of the state, where mosquito positivity rates were highest, had significantly higher seroprevalence rates than the eastern region. Additional studies are needed to determine whether the high prevalence rates in Nebraska will be reflected in other states and what impact environmental and geographical factors may have on future outbreaks of WNV infection.

West Nile virus (WNV), a member of the *Flaviviridae* family, was first identified in the United States as a cause of disease in New York City (NYC), N.Y., in 1999 (11). WNV activity has through December 2005 been detected in all 48 continental states, as reported to the Centers for Disease Control and Prevention (CDC) through ArboNET (http://www.cdc.gov /ncidod/dvbid/westnile/surv&control.htm). The virus was first detected in Nebraska during the 2002 mosquito season (May through October) with 152 human cases of WNV-caused disease identified in the state. An epidemic occurred in 2003 with 1,937 cases reported which resulted in 29 deaths (ArboNET data). Human seroprevalence studies are limited within the United States, with a survey of New York City residents in October 1999 demonstrating that 2.6% of healthy individuals may have been infected with WNV without symptoms or exhibiting only mild symptoms and a study in one county in Wyoming estimating that 14% of the residents may have been infected (3, 13, 14). To evaluate seroprevalence on a statewide basis, this study determined the presence of WNV-specific antibodies in blood donors following the 2003 WNV epidemic in Nebraska. The results of testing were compared to data collected from mosquito surveillance studies by geographic regions within the state.

MATERIALS AND METHODS

Specimen collection. A 1-day statewide collection (1 July 2004) of 624 sera by the American Red Cross (Omaha, Nebraska, office) was used as a source of specimens for the study. Donors had been screened using a questionnaire modified to exclude donors with a history of possible acute meningitis to include that

caused by WNV. Sera were also tested for infectious agents including WNV RNA according to standard blood bank protocols (4, 21). Each donor serum was included only once. All specimens were deidentified and sorted into three geographical collection regions as defined by the American Red Cross prior to testing (Fig. 1). These regions were based on county borders. During the screening process, approximately 2 ml of excess sera was placed into sterile vials, coded by collection location, and stored frozen at −70°C until evaluated.

ELISAs. FDA-approved WNV immunoglobulin M (IgM) capture and IgG enzyme-linked immunosorbent assays (ELISAs) were performed on the specimens. An in-house protocol was used for WNV IgA ELISA. Both the WNV IgM capture ELISA and the IgG ELISAs (Focus Technologies, Cypress, CA) were interpreted according to package inserts. These assays were carried out using the MAGO Plus automated enzyme immunoassay analyzer (Diamedix, Miami, FL). A manual background subtraction assay, as described in the WNV IgM ELISA package insert, was performed on all IgM-positive samples. WNV IgA ELISA was done using the protocols described by Prince and Lape-Nixon (16). Briefly, the IgA assay was done using a 96-well polystyrene plate (Corning, Corning, NY) coated with 75 μl goat anti-human IgA (KPL, Gaithersburg, MD) at a concentration of 37.5 ng per well in 0.05 M carbonate buffer. This IgA-coated plate was incubated overnight at 4°C in a humidified chamber. After incubation, the plate was washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T20) (Sigma, St. Louis, MO) using the 96PW microplate washer (Tecan, Durham, NC). Previously tested patient sera were used as positive and negative controls. Patient sera and controls were diluted 1:100 in WNV ELISA diluent (Focus Technologies). Fifty microliters of diluted sample or control was added to wells of the plate in duplicate. The plate was incubated for 1 h at 37°C in a humidified chamber followed by five washes in PBS-T20. After the wash step, 50 μl of the WNV antigen (Focus Technologies) was added to the patient well and 50 μl WNV healthy control antigen (Focus Technologies) was added to the other well. The plate was subsequently incubated overnight at 4°C in a humidified chamber. Following the second incubation, the plate was washed five times and each well was inoculated with 50 μl of anti-WNV conjugate (Focus Technologies) and incubated for 1 h at 37°C. The plate was washed five times in PBS-T20; each well was inoculated with 75 μl of WNV substrate (Focus Technologies), and the plate was incubated for 10 min in the dark at room temperature. Seventy-five microliters of WNV stop reagent (Focus Technologies) was added to each well, and absorbance (A_{450}) was read on the ELx800UV plate reader (Bio-Tek, Winooski, VT). The data were analyzed as described previously (12).

HAI testing. Hemagglutination inhibition (HAI) assays using WNV- and Saint Louis encephalitis virus (SLEV)-specific antigens were performed on all IgG ELISA WNV-positive sera to confirm results (20). Briefly, the sera were treated with kaolin to remove nonspecific inhibitors (5). Gander red blood cells (G-RBC; Colorado Serum Company, Denver, Colorado) were prepared by washing the

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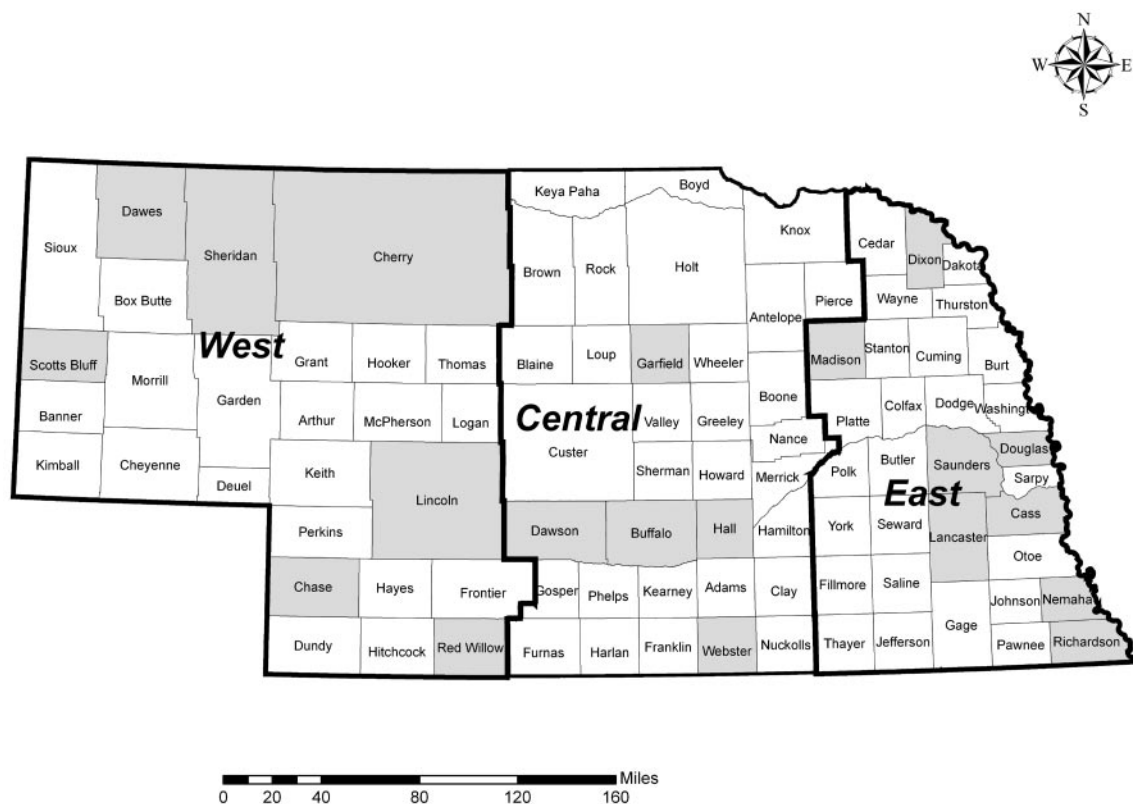
FIG. 1. Geographic divisions of Nebraska by county. The shaded areas represent the counties where mosquito testing was performed.

cells three times in dextrose-gelatin-Veronal (Cambrex, Walkersville, MD) and then resuspending them in a pH 6.4 working buffer to a final concentration of 8% G-RBC (5, 19). A working dilution of 1:24 was made of the G-RBC solution. WNV and SLEV antigens were obtained from the CDC (Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado) and rehydrated as directed. The WNV and SLEV antigens were diluted 1:15 and 1:40, respectively, using a pH 6.4 working buffer, creating a suspension of 4 to 8 hemagglutination units. Prior to testing, the sera were diluted 1:40 in 0.4% bovine albumin borate saline for screening purposes (5, 19). Equal parts (25 μl) of the diluted sera, G-RBCs at a working dilution, and diluted antigens at a pH of 6.4 were placed into a round-bottomed 96-well microtiter plate (Nalgene Nunc, Rochester, NY) and incubated overnight at 4°C. Results were interpreted as described by Casals and Brown (2).

Mosquito testing. Mosquitoes were trapped biweekly using CDC light traps from June to October 2003 in 20 Nebraska counties (Fig. 1) (15). Trapping of mosquitoes for 2004 began on 1 June. Female *Culex* species (*C. tarsalis*, *C. restuans*, *C. pipiens*, and *C. salinarius*) were identified (by W.L.K.) and combined into pools of up to 50 mosquitoes and stored frozen until evaluated. At the time of testing, the samples were thawed and phosphate-buffered saline, pH 7.4 (at a ratio of 1.5 ml per 50 mosquitoes), was added to each pool. The pools were disrupted using the Mixer Mill 300 (QIAGEN Inc., Valencia, CA) at 20 cycles per second for 4 min. The samples were subsequently centrifuged at 14,000 rpm for 3 min. A total of 150 μl of mosquito supernatant was used for total RNA extraction on the MagNA Pure LC instrument (Roche, Indianapolis, Indiana) using the Roche RNA Isolation Kit III with the fresh/frozen protocol and a final elution volume of 75 μl. Reverse transcription-PCR was performed using the Lightcycler models 1.2 and 2.0 (Roche, Indianapolis, Indiana) with primers WN9483F (5'-CAC CTA CGC CCT AAA CAC TTT CAC C-3') and WN9794R (5'-GGA ACC TGC TGC CAA TCA TAC CAT C-3') (WNV training course material; CDC Division of Vector-Borne Infectious Diseases, Fort Collins, CO, February 2001). The Roche RNA master amplification kit utilizes SYBR green as the fluorescent detector. Two microliters of extracted RNA was combined with 0.19 μl of each primer (0.38 μM concentration), 0.5 μl of Mn(OAc)$_2$ (2.5 mM concentration), 3.7 μl of Roche enzyme, and 3.42 μl of water to bring the final volume to 10 μl. The reverse transcription-PCR consisted of an initial

reverse transcription of the RNA at 61°C for 20 min and denaturation of the cDNA for 95°C for 2 min followed by 40 amplification cycles. Each amplification cycle consisted of a denaturation step at 95°C for 1 s, an annealing step at 69°C for 5 s, and an extension step at 72°C for 16 s. The annealing temperature was adjusted from 69°C to 62°C over the first 12 amplification cycles. Melting temperature analysis was used to identify WNV RNA-positive pools. The melting curve assay was performed by increasing the temperature from 65°C to 95°C at 0.1°C per second. A mosquito pool known to be positive for WNV was included in each run for comparison of the melting temperature curve of the unknown pool with the melting curve of the control pool. Melting curve temperatures of approximately 85°C were seen with WNV. The minimum infectivity rate (MIR) was calculated by dividing the number of positive mosquito pools by the number of mosquitoes tested and expressing this as the number of positive mosquitoes per 1,000 mosquitoes tested.

Statistical analysis. Ninety-five percent confidence intervals (CIs) were calculated using SPSS software (SPSS, Inc., Chicago, Illinois). A comparison of prevalence rates between geographical regions was made using Fisher's exact test. A P value of <0.05 was considered significant.

RESULTS

A total of 624 sera were collected from various regions within Nebraska (Table 1). In the east region, 15 of 359 samples were positive for WNV-specific IgG (4.2%; 95% CI, 2.1 to 6.3) and two samples were positive for WNV-specific IgM (0.6%; 95% CI, 0.0 to 1.3). A significant increase in WNV-specific IgG positivity was noted for the central region, where 19 of 138 sera were IgG positive (13.8%; 95% CI, 8.0 to 19.6) compared to the east region ($P < 0.001$). Also, there were significantly more positive WNV IgM samples in the central region than in the east region ($P = 0.01$) with a total of six positive samples (4.3%; 95% CI, 0.9 to 7.8). In comparing the

TABLE 1. Comparison of West Nile virus-positive patient sera with positive mosquito pool samples by region

Region	Patient sera[a]					Mosquito pools[b]		
	No.	IgG		IgM		No.	No. positive (%)	95% CI
		No. positive (%)	95% CI	No. positive (%)	95% CI			
East[c,e]	359	15 (4.2)	2.1–6.3	2 (0.6)	0.0–1.3	725	75 (10.3)	8.1–12.6
Central[c,d]	138	19 (13.8)	8.0–19.6	6 (4.3)	0.9–7.8	1,163	528 (45.4)	42.5–48.3
West[d,e]	127	25 (19.7)	12.7–26.7	4 (3.2)	0.1–6.2	928	333 (35.9)	32.8–39.0
Total	624	59 (9.5)	7.2–11.8	12 (1.9)	0.8–3.0	2,816	936 (33.2)	31.5–35.0

[a] Patient sera were collected on 1 July 2004 from healthy blood donors.
[b] Mosquito pools were collected during June to October 2003. Each pool consisted of up to 50 female *Culex* species.
[c] East compared to central: IgG ($P < 0.001$), IgM ($P = 0.01$), and mosquito pools ($P < 0.001$).
[d] Central compared to west: IgG ($P = 0.25$), IgM ($P = 0.50$), and mosquito pools ($P < 0.001$).
[e] West compared to east: IgG ($P < 0.001$), IgM ($P = 0.12$), and mosquito pools ($P < 0.001$).

west region to the east region a significant increase in WNV-specific IgG-positive results was also observed, with 25 of 127 (19.7%; 95% CI, 12.7 to 26.7) donors positive in the west ($P < 0.001$). However, no statistical difference in the prevalence of WNV-specific IgM samples was noted in comparing the west to the east region ($P = 0.12$). Additionally, no statistical difference was seen in the number of WNV-specific IgG-positive ($P = 0.25$) or IgM-positive ($P = 0.50$) donors in comparing the central to the west region.

Overall statewide, 59 sera (9.5%; 95% CI, 7.2 to 11.8) were positive for WNV-specific IgG and 12 sera (1.9%; 95% CI, 0.8 to 3.0) were positive for WNV-specific IgM. HAI testing confirmed that all positive sera contained WNV-specific IgG antibodies. In each donor with a positive WNV-specific IgM, a corresponding positive result for WNV-specific IgG was noted. None of the WNV-specific IgM-positive samples were positive for WNV-specific IgA antibodies (results not shown).

A total of 2,816 *Culex* species mosquito pools were tested during the 2003 WNV season in Nebraska (Table 1). The first positive pool was collected on 19 June 2003, and the last positive pool was collected on 29 September 2003. WNV RNA was detected in 75 of 725 pools from the east (10.3%; 95% CI, 8.1 to 12.6), 333 of 928 pools from the west (35.9%; 95% CI, 32.8 to 39.0), and 528 of 1,163 pools from the central (45.4%; 95% CI, 42.5 to 48.3) region of the state. A significant difference in mosquito positivity was noted in comparing the east or west to the central region ($P < 0.001$) and in comparing the east and west regions ($P < 0.001$). Of the 528 positive pools

collected in the central region, 260 (49.2%) were collected from Dawson County, adjacent to the west region (Fig. 1). The first WNV-positive mosquito pool for 2004 was collected after the date of serum collection.

The population of Nebraska according to the 2000 U.S. census was 1,711,263 people with a concentration of people in the east region of the state (1,234,018 or 72.1% of the population) (Table 2). The population in the central section of the state was 304,884 (17.8% of total) and in the west was 172,361 (10.1% of total). The overall population density of Nebraska averaged 22.3 people per square mile. The population density of the east region was over three times the state average, with 73.7 people per square mile. The central and west regions of Nebraska were sparsely populated with population densities of 11.7 and 5.2 persons per square mile, respectively. The overall seroprevalence for WNV in Nebraska statewide of 9.5% (95% CI, 7.2 to 11.8) suggested that possibly 161,800 individuals (95% CI, 127,531 to 209,009) may have developed a subclinical WNV infection during 2003.

The MIR overall for the mosquitoes tested in Nebraska was 5.8 infected per 1,000 mosquitoes tested (Table 3). The MIR for the east region was 1.4, that for the central region was 7.7, and that for the west region was 4.9.

DISCUSSION

Following an epidemic of confirmed WNV infections in Nebraska during the 2003 mosquito season, a seroprevalence study was performed to determine the extent of individuals who devel-

TABLE 2. Estimation of the seroprevalence of West Nile virus in Nebraska regions

Region	Population[a] (%)	Population density[b]	Seroprevalence estimate	
			No. positive[c]	95% CI
East	1,234 (69.7)	73.7	51.8	25.9–77.7
Central	305 (17.2)	11.7	42.1	24.4–59.8
West	172 (13.1)	5.1	34.0	21.9–46.2
Total	1,711	22.3	161.8	127.5–209.0

[a] Based on 2000 U.S. census, presented in thousands.
[b] Number of people per square mile.
[c] Calculated by multiplying the WNV-specific positive IgG rate for each region by total population, presented in thousands.

TABLE 3. Mosquito testing for the presence of West Nile virus RNA in regions of Nebraska

Region	No. of mosquitoes[a]	MIR[b]
East	34,554	1.4
Central	64,104	7.7
West	62,758	4.9
Total	161,416	5.8

[a] Includes the total number of all *Culex* species tested.
[b] MIR was calculated by dividing the number of positive mosquito pools by the number of mosquitoes tested and expressing this as the number of positive mosquitoes per 1,000 mosquitoes tested.

oped WNV-specific antibodies from various regions within the state. To date, only limited seroprevalence studies have been performed in areas of the United States as WNV moves across the nation (13, 14). The broad expanse of Nebraska with multiple environments and the large number of WNV cases that occurred during 2003 provided the opportunity to study in more detail the impact that geographic factors may have had on human seroprevalence rates.

Of the 624 healthy blood donor sera collected about 8 months after the 2003 epidemic, 9.5% were positive for WNV-specific IgG antibodies. In comparing the results for each region of the state, the prevalence of WNV IgG antibodies was lower in donors from the east than in those from the central or west region. In comparison, a report from the evaluation of NYC residents following a 1999 outbreak in that area showed that 2.6% of the population developed antibodies to WNV (13).

The greater-than-3.5 times increase in the percentage of the population that had detectable WNV-specific antibodies within Nebraska compared to NYC may be due to multiple variables. Vector exposure and the species of mosquito vector would be major variables to consider. Janousek and Kramer showed that floodwater mosquitoes (*Aedes* sp.) were more prevalent in the eastern areas of Nebraska, while the standing-water mosquitoes (*Culex* sp.), which are more likely to transmit WNV, were more prevalent in western portions of the state (8). The effectiveness of virus transfer by the different species within the genus varies, with *Culex tarsalis* considered one of the most efficient vectors within the group (1, 6, 15, 22). During the present study, *C. tarsalis* was the most common *Culex* mosquito detected in western Nebraska (data not shown). A major difference in prevalence between NYC and western Nebraska may have been the species of mosquito vector present in each area. *Culex tarsalis* was not found in large numbers in NYC or in eastern Nebraska but was the predominant species in the central and west regions of Nebraska (8, 13, 15).

A similar serosurvey was performed on residents from Goshen County, Wyoming (14). In this study, 14% of the population was considered to be infected with WNV. Since this county borders western Nebraska, a similar geography and mosquito population were present. The CI obtained from testing in the western region of Nebraska was 12.7 to 26.7, so it is reasonable to assume that the 14% seroprevalence rate detected in Wyoming corresponds to the rates detected in western Nebraska as a part of the present study (14). No mosquito data were reported from the Wyoming project for comparison purposes.

Twelve of the 624 donor sera (1.9%) were positive for WNV-specific IgM. These sera were also positive for WNV IgG antibodies. In most other serological applications, the presence of specific IgM antibodies suggests acute infection. Previous research, however, has shown that WNV-specific IgM can persist in humans up to 500 days after onset of symptoms (9, 17, 18). The presence of IgM antibodies in conjunction with WNV-specific IgG, therefore, does not necessarily indicate acute infection. The results for the IgM-positive samples were close to the negative cutoff, while the corresponding IgG results were well above the negative cutoff as described by the assay procedures.

To evaluate further the significance of a WNV-specific IgM titer, IgA antibodies were also considered as a possible predictor of acute disease (16, 17). None of the IgM-positive sera in the present study were positive for WNV-specific IgA. These results compared with the findings of Lanciotti, who presented data to indicate that WNV IgA was rarely detected in serum samples collected from WNV-infected patients >50 days after onset (10). Prince et al., however, showed that both the WNV IgM and IgA persisted for at least 6 months after infection (17). They, however, suggested that IgA detection did not appear to be a useful adjunct to WNV IgM detection to identify very recent WNV infection. The discrepancy in IgA levels between studies may be due to multiple issues including the study group tested and the immunological assays used, since no commercial product is available for the testing of WNV IgA and the methods were developed in house. More studies are needed to determine the relevance of detecting IgM antibodies long after the onset of acute WNV illness and the role that WNV-specific IgA may have in the serological evaluation for WNV disease.

The ELISA-based test utilized in the present study does not differentiate between infections caused by WNV and those caused by SLEV, and further testing of positive samples was done to confirm the presence of WNV-specific antibodies. HAI testing of all IgG-positive samples verified the presence of WNV-specific neutralizing antibodies. This was not unexpected, since SLEV infection cases have rarely been reported as a cause of human disease in Nebraska (7).

Mosquito surveillance for the presence of arboviruses has been done in most states, and the results of testing have been made electronically available as a means to predict WNV activity for control of disease outbreaks (http://westnilemaps.usgs.gov). Testing results have shown that the MIR necessary to cause outbreaks of human disease varies among mosquito species (6). In the present study, the MIRs of WNV-infected mosquitoes ranged from 1.4 to 7.7 among the three regions of Nebraska. This correlates with what had been seen during previous years of the WNV outbreak in other states, where the MIR ranged from 0.07 to 5.7 (6). The prevalence of WNV-positive mosquitoes was significantly higher in the central region of Nebraska than in either the west or east area, correlating with the higher WNV antibody rates.

An observation of note concerning WNV seroprevalence was that the rate was higher in the west than in the central region, while mosquito WNV-positive rates were higher in the central than in the west region. One explanation for the discrepancy was the high number of infected mosquitoes collected in Dawson County (accounting for 49.2% of the total positive mosquito pools from the central region), a county which is adjacent to the western region (Fig. 1). The prevalence of WNV-positive mosquitoes in this county was reflective of the prevalence rate for the west region, illustrating one of the limitations to conducting this type of comparative analysis. This county has geological and environmental aspects similar to those of counties to the west, which may account for similarities in the number of positive mosquitoes detected.

The population density in NYC was 26,403 people per square mile compared to 73.7, 11.7, and 5.1 people per square mile in the east, central, and west regions of Nebraska, respectively. Additionally, the MIR of mosquitoes in NYC ranged from 0.7 to 57.1 while similar surveillance in Nebraska found the MIRs to range from 1.4 to 7.7. The presence of an efficient vector, even in an area with a low population density, appears to put people at a

higher risk of infection. These results show that population density does not always correlate with higher risk, but other factors such as mosquito species and exposure to these vectors need to be considered. No other studies to our knowledge have evaluated population density and similar MIRs in comparison with mosquito species and human WNV seroprevalence rates.

In consideration of the number of individuals who were identified as positive for WNV-specific IgG and comparing this with the total state population, it seems highly possible that the number of individuals in Nebraska who may have developed a silent infection with WNV may be large (ranging from 127,500 to 209,000 individuals). Since the risk of exposure to infected mosquitoes appeared to be less in the east, where the population density was greatest, the detection of WNV-specific antibodies in humans could have theoretically been higher if the population density of the east were present in the western region of the state. Additional studies are needed to further evaluate population densities and the risk of WNV exposure.

This study was conducted to determine the seroprevalence of WNV antibodies in the human population following an epidemic. The results showed that a high percentage of individuals may have developed subclinical infections leading to the production of WNV-specific antibodies. This evaluation also confirmed, as expected, that regions of the state with high mosquito WNV infectivity have a higher risk of individuals developing WNV infections, even in areas where the population density is low. Additional studies are needed to determine what effects high WNV seroprevalence may have on the future incidence of WNV-caused infections and what impact environmental and geographical factors may have on outbreaks of disease.

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