

## COMPARISON OF VERO CELL PLAQUE ASSAY, TAQMAN® REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION RNA ASSAY, AND VECTEST™ ANTIGEN ASSAY FOR DETECTION OF WEST NILE VIRUS IN FIELD-COLLECTED MOSQUITOES

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**ABSTRACT.** Mosquitoes collected during the epidemic of West Nile virus (WN) in Staten Island, NY, during 2000 were identified to species, grouped into pools of up to 50 individuals, and tested for the presence of WN by using TaqMan® reverse transcriptase polymerase chain reaction (RT-PCR) to detect West Nile viral RNA, Vero cell plaque assay to detect infectious virus, and VecTest™ WNV/SLE Antigen Panel Assay. A total of 10,866 specimens was tested in 801 pools. Analysis of results indicated that TaqMan RT-PCR detected 34 WN-positive pools, more than either of the other techniques. The plaque assay detected 74% of the pools positive by TaqMan, and VecTest detected 60% of the pools positive by TaqMan. The VecTest assay detected evidence of West Nile viral antigen in 67% of the pools that contained live virus detected by plaque assay. A WN enzyme immunoassay performed similarly to the VecTest WN assay. Differences in performance were related to relative sensitivity of the tests. Infection rates of WN in *Culex pipiens* and *Cx. salinarius* calculated by the 3 techniques varied, but each estimate indicated a high infection rate in the population. Positive and negative attributes of each procedure, which may influence how and where they are used in surveillance programs, are discussed.

**KEY WORDS** West Nile virus, surveillance, TaqMan®, reverse transcriptase polymerase chain reaction, plaque assay, VecTest™, enzyme immunoassay, mosquito, vector

### INTRODUCTION

During the 2 years after the introduction of West Nile virus (WN) into the USA in 1999 (Centers for Disease Control [CDC] 1999), the virus spread to 27 states and the District of Columbia in the eastern half of the country (CDC 2001). In anticipation of this expanding distribution, many state and local health agencies and mosquito control districts initiated new arbovirus surveillance programs or expanded existing programs. Four basic types of surveillance were recommended as important in monitoring WN activity (avian, mosquito, veterinary, and human), with mosquito-based surveillance (i.e., monitoring mosquito population density and virus infection rate) identified as a mainstay of the programs (Gubler et al. 2000).

The expanded surveillance programs and the markedly increased number of mosquitoes to be processed prompted development of efficient, rapid procedures for analyzing the specimens. As a result, automated RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) procedures for detecting West Nile viral RNA (Lanciotti et al. 2000, Hadfield et al. 2001, Shi et al. 2001) replaced standard cell culture assays for live virus (Beaty et al. 1989) as the preferred tools for screening large numbers of mosquito pools in

surveillance programs beginning in 2000. Subsequently, a commercially available dipstick test for detecting WN antigen in mosquito pools was developed (VecTest™ WNV/SLE Antigen Panel Assay, Medical Analysis Systems, Inc., Camarillo, CA) and was utilized in a few surveillance programs during 2001. Late in the course of this study, an antigen-capture enzyme immunoassay (AC-EIA) for WN was developed (Hunt et al. 2002).

Our preliminary evaluation of the VecTest kits indicated that they could detect and differentiate WN and St. Louis encephalitis virus (SLE) in seed virus stocks, in virus-positive mosquitoes produced by laboratory inoculation, and in field-collected WN-positive mosquito pools (Ryan et al., in press). The sensitivity and specificity characteristics of the WN AC-EIA (Hunt et al. 2002) seem to be similar to those of the VecTest WN assay.

Discrepancies in the number of WN-positive mosquito pools in field collections detected by RT-PCR, cell culture assay (Bernard et al. 2001, Nasci et al. 2001, White et al. 2001), and VecTest dipsticks (unpublished data) have occurred and are attributed to differences in the sensitivities of the procedures. The purpose of this research was to compare the ability of RT-PCR, cell culture, and WN VecTest assays to detect WN in a large number of field-collected mosquitoes and to evaluate their respective uses in mosquito-based WN surveillance programs. In addition, we were able to do a preliminary evaluation of the WN AC-EIA with field-collected WN-positive mosquito pools.

### MATERIALS AND METHODS

Mosquitoes used in this study were collected with CO<sub>2</sub>-baited CDC miniature light traps and

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CDC gravid traps from June 2 through December 12, 2000, in Staten Island (Richmond County), NY, as part of the New York City Department of Health WN surveillance program (Kulasekera et al. 2001). These were extra specimens not tested during the regular surveillance season. Traps were set in the late afternoon or evening and retrieved the following morning. Specimens were immediately frozen and held at  $-70^{\circ}\text{C}$ . Before testing for virus, mosquitoes were identified to species at the New York City Department of Health by using morphological characteristics, and sorted into pools of up to 50 individuals, based on species, date, and site of collection. Identification and sorting were done on a chill table to help retain virus viability. Pooled mosquitoes were refrozen at  $-70^{\circ}\text{C}$  and shipped on dry ice to the CDC Division of Vector-Borne Infectious Diseases for testing.

Pooled mosquitoes were placed in 2.0-ml snap-cap microcentrifuge tubes (HS4264J, Daigger and Co., Lincolnshire, IL) with 1 copper-coated, 4.5-mm-diameter steel bead (Airgun Shot, Steel BB Cal, premium grade BBs, Copperhead, East Bloomfield, NY) and 1.7 ml of BA-1 diluent (1X M199 with Hanks' balanced salt solution, 0.05 M Tris buffer [pH 7.6], 1% bovine serum albumin, 0.35 g/liter sodium bicarbonate, 100 mg/liter streptomycin, and 1 mg/ml Fungizone [Sigma-Aldrich, St. Louis, MO]). The mosquitoes were then homogenized by placing the tubes in a Qiagen Mixer Mill MM 300 (QIAGEN Inc., Valencia, CA), which is designed to disrupt biological samples through the beating and grinding effect of the steel bead on the sample material as they are shaken together in the tube. The mixer mill can homogenize 48 pools at a time, and was operated in a high-efficiency particulate air-filtered, laminar flow biosafety cabinet at 25 cycles/sec for 4 min. After homogenization, the specimens were centrifuged at 4,000 rpm for 3 min with an aerosol-block rotor in a refrigerated microcentrifuge.

Each mosquito pool was tested for the presence of West Nile viral RNA by using a TaqMan<sup>®</sup> RT-PCR assay described by Lanciotti et al. (2000). Briefly, RNA was extracted from 140  $\mu\text{l}$  of the mosquito homogenate by using QIAamp viral RNA kits (QIAGEN). The RNA was eluted from the QIAGEN columns in a final volume of 100  $\mu\text{l}$  of elution buffer and stored at  $-70^{\circ}\text{C}$  until used. For the TaqMan test, 5  $\mu\text{l}$  of RNA was combined with the appropriate primers and probes by using the TaqMan RT-PCR Ready-Mix Kit (PE Applied Biosystems, Foster City, CA). The samples were subjected to 45 cycles of amplification in an ABI Prism 7700 Sequence Detection System instrument (PE Applied Biosystems) according to the manufacturer's protocol for TaqMan RT-PCR cycling conditions. All pools were screened with the ENV primers/probe (genome positions: forward 1,160–1,180; reverse 1,209–1,229; probe 1,186–1,207) and those producing positive results were confirmed as posi-

tive with the 3'NC primers/probe (genome positions: forward 10,668–10,684; reverse 10,770–10,756; probe 10,691–10,714). Pools were considered positive only if they gave positive reactions with both probes.

Vero cell plaque assay in 6-well plates (Beatty et al. 1989) with a double agar overlay was used to detect live virus in the pools. Supernatant from the homogenized mosquito pool (100  $\mu\text{l}$ ) was inoculated into each of 2 wells on a 6-well plate containing a confluent Vero cell monolayer and held for 1 h at  $37^{\circ}\text{C}$ ; the cells then were overlaid with 3 ml of agar (1% Noble agar in M199, 0.35 g/liter sodium bicarbonate, 100 mg/liter streptomycin, 1 mg/ml Fungizone, and 150 mg/ml diethylaminoethyl-dextran) and incubated at  $37^{\circ}\text{C}$ . On the 4th day after inoculation, a 2nd agar overlay was placed on each well (3 ml/well identical to the 1st overlay except for the addition of 4 ml/liter 1% neutral red to the agar). The plates then were observed daily for 10 days for evidence of plaques. The number of plaques observed in each positive well was recorded and cell sheets in wells showing plaques were harvested and tested for virus identification by using the WN-specific, TaqMan real-time RT-PCR described above.

The VecTest WNV/SLE Antigen Panel Assay was used to detect West Nile viral antigen in the mosquito pools. This is a qualitative, immunochromatographic test that uses type-specific monoclonal antibodies to detect WN or SLE antigen in mosquito pools. According to the package instructions, mosquito pools containing up to 50 individuals are ground in 2.5 ml of the grinding solution provided with the kit. After grinding, the homogenate may be centrifuged to remove debris (an optional step, not required for the test to run), and 250  $\mu\text{l}$  of the homogenate is dispensed into a 1.7-ml conical-bottom tube provided in the kit. A test strip is inserted into the tube for 15 min. After 15 min, the strip is removed from the tube and examined. As the mosquito homogenate migrates up the strip, viral antigen, if present, reacts with type-specific, colloidal gold-conjugated monoclonal antibodies contained in the bottom portion of the strip. The antigen-antibody-gold complexes then migrate through the test zone containing immobilized antibody where they are bound and accumulate to form a visible, reddish purple line (Fernandez et al. 1994). The test zone has 2 virus-specific areas, 1 for WN and 1 for SLE (Fig. 1). Unbound dye complexes migrate out of the test zone and are captured in the control zone, which shows a line indicating that the homogenate has migrated through the test zone. Unless the control line is present, the test is invalid. Preliminary tests with laboratory-infected, WN and SLE-positive and -negative mosquito pools demonstrated the ability of the VecTest assay to detect and differentiate the virus antigens in mosquito pools (Ryan et al., in press).

We departed from the VecTest recommended

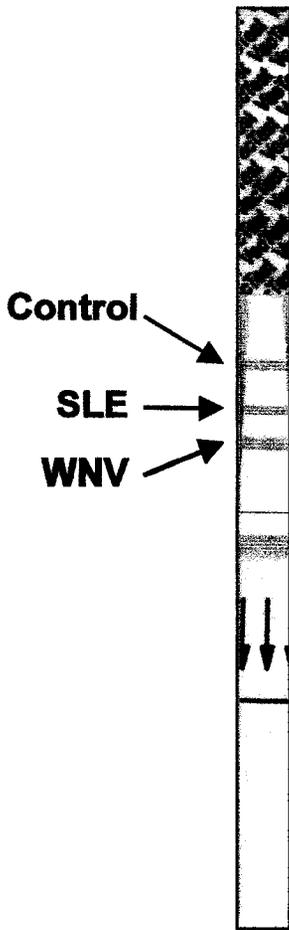


Fig. 1. A VecTest<sup>®</sup> WNV/SLE Antigen Panel Assay Test strip indicating relative position of West Nile virus positive indicator line, St. Louis encephalitis virus positive indicator line, and control zone line, demonstrating that the sample migrated through the test zone.

grinding procedure because we wanted to be able to test the pools for live WN with the plaque assay, and preliminary tests indicated that the grinding solution provided with the VecTest kit rendered WN in the mosquito pools noninfectious in cell culture (we were unable to recover infectious virus from WN and SLE seed stocks mixed 1:10 with the VecTest grinding buffer then serially diluted with BA-1 medium, but were able to recover infectious eastern equine encephalomyelitis and western equine encephalomyelitis viruses treated similarly [unpublished data]). We used the mosquito homogenate ground in BA-1 medium described above, mixed 1:1 with the VecTest grinding medium (125  $\mu$ l of mosquito homogenate and 125  $\mu$ l of VecTest grinding medium) in the conical-bottom tube before inserting the VecTest strip. As a result, the material tested with VecTest strips was diluted 1:1 in comparison to the concentration of homogenate tested

with TaqMan and cell culture assays (i.e., virus concentration in the positive pools was reduced by 0.3 log<sup>10</sup> plaque-forming units [PFU]/ml), and components of the VecTest grinding medium designed to optimize test performance (e.g., detergents) also were diluted 1:1.

All of the pools were tested with TaqMan RT-PCR and the positives from that test were subsequently tested with the plaque and VecTest assays. In addition, 135 TaqMan-negative pools were tested with the plaque and VecTest assays to examine the potential for the VecTest assay to produce false-positive results. All tests were done blind, without the investigator having prior knowledge of the results from the other tests.

Mosquito pools positive by RT-PCR with sufficient material remaining after other testing was completed also were tested with a WN AC-EIA (Hunt et al. 2002). This test is essentially identical to the AC-EIA test for SLE (Tsai et al. 1987), but replaces the SLE-specific capture and detection antibodies with WN-specific antibodies. The AC-EIA protocol consists of an antigen-capture assay followed by a confirmatory inhibition assay. Specimens were called positive only if confirmed in the inhibition assay.

The impact of variation in number of positives detected by the different tests on the data used by mosquito-based surveillance systems was evaluated by estimating the WN infection rate (IR) with the results from each of the assays for the *Culex pipiens* L. and *Culex salinarius* Coquillett collected during August, and correcting for variable pool sizes (Walter et al. 1980). Meaningful IRs could not be calculated for other species or other months because fewer than 1,000 specimens were available for testing for the month or no positives were found during the month with any of the assays. The estimated IR was expressed as the number of positive pools collected per 1,000 specimens tested (Nasci and Mitchell 1996).

## RESULTS

A total of 10,866 mosquitoes in 801 pools, representing 23 mosquito species, was provided by the New York City Department of Health (Table 1). The TaqMan assay detected WN RNA in 34 pools, most of which contained *Cx. pipiens* or *Cx. salinarius*. Of the 34 TaqMan-positive pools, 25 (74%) pools were positive in cell culture and 18 (60%) of 30 were WN-positive in the VecTest WNV/SLE Antigen Panel Assay (4 of the TaqMan-positive pools tested with plaque assay were not tested with VecTest because of insufficient volume remaining after other manipulations).

Table 2 shows the results from each of the 3 tests. When using TaqMan results as the basis for comparison, no false positives were found in the plaque assay or VecTest. Pools positive with plaque assay, VecTest, or both also were TaqMan-positive.

Table 1. Mosquitoes tested for evidence of West Nile virus (WN) by using TaqMan® reverse transcriptase polymerase chain reaction and results of testing the TaqMan WN-positive pools with Vero cell culture plaque assay and VecTest™ WNV/SLE Antigen Panel Assay.

Species	No. pools	No. specimens	No. positive pools <sup>1</sup>		
			TaqMan	Plaque assay	VecTest
<i>Aedes albopictus</i> (Skuse)	3	3			
<i>Ae. vexans</i> (Meigen)	96	834	1 <sup>2</sup>		
<i>Anopheles barberi</i> Coquillett	1	1			
<i>An. crucians</i> Wiedemann	4	4			
<i>An. punctipennis</i> (Say)	26	49			
<i>An. quadrimaculatus</i> Say	12	28			
<i>An. sp.</i>	7	18			
<i>Coquillettidia perturbans</i> (Walker)	35	103			
<i>Culex erraticus</i> (Dyar and Knab)	2	8			
<i>Cx. pipiens/restuans</i> mixed pools	20	229	2	2	
<i>Cx. pipiens</i> L.	133	1,928	15 <sup>2</sup>	13	9
<i>Cx. restuans</i> Theobald	32	210			
<i>Cx. salinarius</i> Coquillett	212	6,144	14 <sup>3</sup>	8	8
<i>Cx. sp.</i>	21	284	2	2	1
<i>Cx. territans</i> Walker	11	14			
<i>Ochlerotatus atropalpus</i> (Coquillett)	1	1			
<i>Oc. canadensis</i> (Theobald)	3	3			
<i>Oc. cantator</i> (Coquillett)	27	192			
<i>Oc. dorsalis</i> (Meigen)	2	12			
<i>Oc. grosbecki</i> (Dyar and Knab)	2	3			
<i>Oc. sollicitans</i> (Walker)	16	284			
<i>Oc. sp.</i>	37	241			
<i>Oc. taeniorhynchus</i> (Wiedmann)	1	1			
<i>Oc. triseriatus</i> (Say)	55	177			
<i>Oc. trivittatus</i> (Coquillett)	29	69			
<i>Psorophora ferox</i> (Von Humboldt)	8	17			
<i>Uranotaenia sapphirina</i> (Osten Sacken)	5	9			
Total	801	10,866	34	25	18

<sup>1</sup> Only the TaqMan-positive pools and 135 negative pools were tested with plaque assay and VecTest assay.

<sup>2</sup> One of the TaqMan-positive pools for this species was not tested with the VecTest strips because of insufficient volume.

<sup>3</sup> Two of the TaqMan-positive pools for this species were not tested with the VecTest strips because of insufficient volume.

The 3 assays agreed in 16 (53%) of 30 TaqMan-positive pools. In 8 (27%) of 30 pools, the plaque assay and TaqMan were positive but the VecTest was negative, and in 2 (6%) of 30 pools, the VecTest and TaqMan were positive but the plaque assay was negative. The VecTest detected 16 (67%) of 24 of the pools that were positive in plaque as-

say, and also detected WN antigen in 2 pools that were TaqMan-positive but plaque assay-negative.

No evidence of WN was found in the 135 pools that were negative by TaqMan when tested with plaque assay or VecTest. None of the pools produced an SLE-positive reaction in the VecTest assay.

Table 2. Results from TaqMan® reverse transcriptase polymerase chain reaction (RT-PCR); Vero cell culture plaque assay, and VecTest™ WNV/SLE Antigen Panel Assay on 801 field-collected mosquito pools.

WN assay results			
TaqMan RT-PCR (34 positives)	Plaque assay (25 positives)	VecTest (18 positives)	No. pools
+	+	+	16
+	+	-	8
+	+	Not tested	1
+	-	+	2
+	-	Not tested	3
+	-	-	4
-	-	-	135
-	Not tested	Not tested	632
Total			801

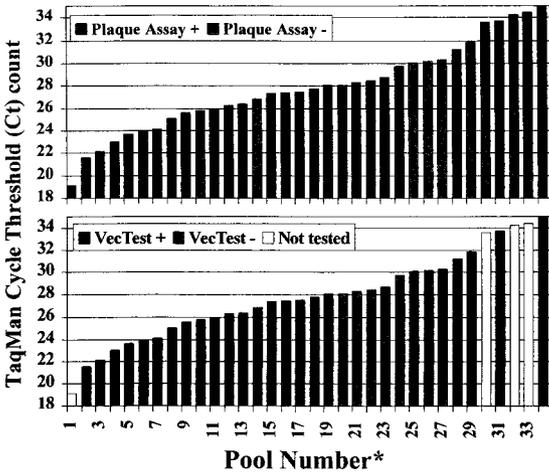


Fig. 2. The TaqMan-positive pools ranked by cycle threshold (Ct) number from lowest to highest (highest to lowest West Nile virus titer), indicating positive (black bars), negative (hatched bars), and not tested (white bars) pools for pools tested with Vero cell plaque assay (top panel) and VecTest assay (bottom panel).

Sufficient sample material to allow testing with the WN AC-EIA remained from only 15 of the RT-PCR-positive pools. Of these, plaque assay detected 13 (87%), AC-EIA detected 13 (87%), and VecTest detected 11 (73%) pools. No difference was found in the proportion of positive pools detected by AC-EIA and VecTest assays ( $\chi^2 = 0.003$ ,  $P > 0.05$ ).

The association between TaqMan cycle threshold (Ct) number in the 34 RNA-positive pools (when using the 3'NC probe) and detection by cell culture or VecTest is shown in Fig. 2. The TaqMan Ct is the cycle number at which fluorescence increases above the background threshold. The Ct value is directly related to the amount of RNA in the sample and is correlated with the titer of viable virus in the sample (Lanciotti et al. 2000). Lower Ct values indicate higher concentrations of viral RNA, and higher virus titers in the sample. The VecTest assay detected West Nile viral antigen in 17 of 17 TaqMan positives with Ct < 28, but only detected

1 (8%) of 13 with Ct > 28. Vero cell culture plaque assay detected live virus in 16 (89%) of 18 TaqMan-positive pools with Ct ≤ 28, and 9 (56%) of 16 pools with Ct > 28. In this assay, a TaqMan Ct of 28 correlated with approximately 3.5–4.0 log<sup>10</sup> PFU/ml in the Vero cell plaque assay (Lanciotti et al. 2000).

The WN infection rate (IR) estimated for mosquitoes collected during August ranged from 5.2 (VecTest) to 8.8 (TaqMan) for *Cx. pipiens* and from 7.1 (VecTest) to 12.1 (TaqMan) for *Cx. salinarius* (Table 3).

DISCUSSION

Mosquito-based arbovirus surveillance programs traditionally have used detection of infectious virus in cell culture or vertebrate test systems for determining the presence and relative abundance of virus in a mosquito population. In our evaluation of WN in field-collected mosquitoes, Vero cell culture plaque assay was not as sensitive as a procedure to detect West Nile viral RNA in mosquito pools, but was more sensitive than a procedure to detect West Nile viral antigen in the pools. These results are consistent with published accounts of the performance of viral RNA (Lanciotti et al. 2000, Bernard et al. 2001) or viral antigen detection systems (Hildreth et al. 1984, Tsai et al. 1987, Hunt et al. 2002) in the laboratory and field. In this project, the TaqMan real-time RT-PCR procedure detected evidence of West Nile viral RNA in 36% more pools than plaque assay detected live virus, and the VecTest WNV/SLE assay detected evidence of West Nile viral antigen in only 67% of the pools that contained live virus. Stated another way, plaque assay identified 74% of the TaqMan-positive pools and VecTest identified 60% of the TaqMan positives.

The difference in ability to detect evidence of WN in a mosquito pool is primarily related to the sensitivity of the tests. The TaqMan Ct values of the plaque assay and VecTest positive and negative pools demonstrated that pools containing higher amounts of viral RNA were more likely to be detected with the other techniques. The VecTest

Table 3. Number of positive pools and estimated infection rate from pools of *Culex pipiens* and *Cx. salinarius* collected during August 2000 on Staten Island, NY, tested with 3 assays.

Species	No. specimens	No. pools	No. positive pools Infection rate <sup>1</sup> (95% confidence interval)		
			TaqMan®	Plaque Assay	VecTest®
<i>Cx. pipiens</i>	1,675	85	13	11	8
			8.8 (5.0–14.6)	7.2 (4.0–12.6)	5.2 (2.6–10.0)
<i>Cx. salinarius</i>	1,115	65	11	8	7
			12.1 (6.5–21.3)	8.3 (4.0–16.0)	7.1 (3.3–14.2)

<sup>1</sup> IR, estimated infection rate, expressed as number of positive pools per 1,000 specimens tested.

WNV/SLE assay detected West Nile viral antigen in all of the pools containing a minimum of  $3.5\text{--}4.0 \log^{10}$  PFU/ml of virus (estimated Vero cell plaque assay equivalent). The plaque assay was capable of detecting infectious virus in pools with the highest Ct values (i.e., lowest titer pools detected by TaqMan), but was less likely to do so as Ct values increased. This is related to the probability of virus being present in the aliquot being tested. Our standard plaque assay protocol tests approximately 10% of the total mosquito pool homogenate. The plaque assay would probably detect virus in greater proportion of the low-titer pools if a greater proportion of the total pool material was tested.

Although less sensitive than the plaque assay, the VecTest assay detected viral antigen in 2 pools that did not produce live virus in plaque assay. This indicates that the VecTest assay is similar to other antigen detection systems in its ability to detect viral antigen from mosquito pools containing killed virus (Tsai et al. 1987, Hunt et al. 2002), and that specimens need not be maintained in a continuous cold chain to detect the presence of viral antigen with this test. Standard RT-PCR and TaqMan RT-PCR assays share this characteristic, and do not require a cold chain to be effective (Kramer et al. 2001, Turell et al. 2002). Although this characteristic may be used to reduce cost associated with surveillance programs, it should be noted that lack of a cold chain compromises the ability to isolate infectious virus for further study and to detect any virus that will grow in cell culture.

Although differences were demonstrable in sensitivity and in the number of positive pools detected by the 3 techniques, the effect on the estimated IRs calculated during August 2000 for 2 species was negligible. The highest IRs, calculated with the TaqMan results, were 1.5- to 1.7-fold greater than the lowest IRs calculated with the VecTest results, and considerable overlap occurred in the 95% confidence intervals around the IR estimates. Therefore, analysis of the data indicated a high infection rate in the mosquito populations regardless of the test system used.

In summary, TaqMan RT-PCR, cell culture, and VecTest WNV techniques detected evidence of WN in field-collected mosquito pools, and despite differences in sensitivity, each provided evidence of high infection rates in key mosquito populations. The WN AC-EIA performed similarly to the VecTest WNV assay in the limited testing that was possible. Although appearing to be relatively equal in ability to provide an index of virus activity in a mosquito population, TaqMan RT-PCR, cell culture, and VecTest WNV have positive and negative attributes that should be considered when deciding which to use in a surveillance program. The TaqMan RT-PCR is extremely sensitive, provides the highest probability that early-season transmission will be detected when IRs in the mosquito populations are low, and does not require a cold

chain. However, TaqMan is very costly and requires specialized equipment and trained personnel. Plaque assay is also very sensitive, and it has the added benefit of being able to detect any virus that will grow in Vero cells with a single test. This trait that makes plaque assay very attractive to surveillance programs that must detect several potential arboviruses. However, viruses that are isolated with plaque assay must be identified by using different techniques, and plaque assay is costly, requiring specialized equipment and trained personnel. The VecTest assay is less sensitive than TaqMan RT-PCR and plaque assay, but is relatively inexpensive, doesn't require costly equipment or specially trained personnel, and can test for WN and SLE on the same strip. The VecTest SLE assay and the WN AC-EIA share many of the positive aspects of the VecTest WNV assay, but they have not been adequately field tested yet. The comparatively lower sensitivity of the VecTest means that a greater likelihood exists of not detecting early season transmission when IRs are low. This may be overcome by testing larger numbers of specimens early in the season. Further investigations of how these assays perform, particularly in other geographic regions and with other mosquito communities, are needed to provide a basis for determining their use in surveillance programs designed to monitor the events and conditions that may lead to epizootics and epidemics (Moore et al. 1993).

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