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EVALUATION OF COMMERCIAL ASSAYS FOR DETECTING WEST NILE VIRUS ANTIGEN

KRISTEN L. BURKHALTER,[1] ROBBIN LINDSAY,[2] ROBERT ANDERSON,[3]
ANTONIA DIBERNARDO,[2] WHALLEY FONG[4] AND ROGER S. NASCI[1]

ABSTRACT. Two commercially available West Nile virus (WNV) detection assays (RAMP® WNV test, Response Biomedical Corp., Burnaby, British Columbia, Canada; and VecTest™ WNV antigen assay, Medical Analysis Systems, Inc., Camarillo, CA) were compared for sensitivity, specificity, and ability to detect WNV in field-collected mosquito pools. Serially diluted stock seed WNV and St. Louis encephalitis virus (SLEV) were used to determine sensitivity and specificity. The RAMP WNV test detected WNV at concentrations as low as 3.17 $\log_{10}$ plaque-forming units per milliliter (PFU/ml), whereas the VecTest assay detected WNV at concentrations as low as 5.17 $\log_{10}$ PFU/ml. Neither test cross-reacted with SLEV. A WNV-specific reverse transcriptase polymerase chain reaction was used to identify positives among field-collected mosquito pools. The RAMP WNV test detected 94% of positive pools and the VecTest assay detected 65% of the positive field-collected pools. Despite these differences, both assays have characteristics that make them useful in WNV surveillance programs.

KEY WORDS West Nile virus, VecTest™, RAMP® test, rapid detection, enzyme immunoassay, mosquito, vector

INTRODUCTION

As the range of West Nile virus (WNV) expanded across North America, health departments responded by establishing new arbovirus surveillance programs or expanding existing ones. Monitoring for virus activity in mosquito or bird populations or both has been central to many of these programs. The number of specimens being tested has increased dramatically, taxing the laboratory resources that support the surveillance efforts. To meet these demands, several procedures have been developed or modified to allow rapid, accurate detection of WNV in these samples. The most commonly used procedures include detection of live virus by using cell culture assays (Beaty et al. 1995), detection of viral RNA by using reverse transcriptase polymerase chain reaction (RT-PCR) (Lanciotti et al. 2000, Shi et al. 2001, Kauffman et al. 2003), and detection of virus antigen by using enzyme immunoassay (Hunt et al. 2002, Chiles et al. 2004). These tests are specific and very sensitive, but generally require well-equipped laboratories and specialized technical support staff. Recently, tests for detection of WNV in mosquito pools and bird samples have become commercially available. Two tests, the VecTest™ WNV antigen assay (Medical Analysis Systems, Inc., Camarillo, CA) and the Rapid Analyte Measurement Platform (RAMP®) WNV test (Response Biomedical, Corp., Burnaby, British Columbia, Canada) are modifications of the enzyme immunoassay protocols mentioned above. Many jurisdictions have adopted these commercial tests because their easy-to-use kit format eliminates the need to have highly trained technicians and comprehensive laboratory facilities to conduct testing. In this study we compared the ability of the two commercial WNV detection assays to detect WNV in serially diluted stocks of virus as well as in field-collected pools of infected mosquitoes. In addition, we examined the advantages and disadvantages associated with these assays.

MATERIALS AND METHODS

Detection assays for WNV: The VecTest WNV antigen assay is a dipstick-format, qualitative, immunochromatographic test that uses type-specific monoclonal antibodies to detect WNV antigen (Nasci et al. 2002, Ryan et al. 2003). For the VecTest assay, up to 50 mosquitoes are homogenized in 2.5 ml of grinding solution provided in the kit. The samples are homogenized at 25 cycles/sec for 4 min in a Qiagen Mixer Mill MM 300 (QIAGEN Inc., Valencia, CA) and centrifuged at 4,000 rpm for 4 min in a refrigerated centrifuge. After homogenization and centrifugation, 250 μl of the supernatant from the homogenate is placed in a 1.7-ml conical-bottom tube with one of the dipsticks. While the dipstick is immersed in the sample, the material migrates up the strip. Virus antigen, if present, reacts with type-specific, colloidal gold–conjugated antibodies contained in the bottom portion of the dipstick. The antigen–antibody–gold complexes then migrate past a zone containing immobilized antibody where they are bound and accumulate to form a visible pink–red line in positive tests. After a 15-min incubation period, the VecTest dipsticks are removed and results are interpreted (for the field-collected mosquito pool portion of the

[1] Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, PO 2087, Fort Collins, CO 80522.
[2] National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington Street, Winnipeg, Manitoba R3E 3R2, Canada.
[3] Department of Biology, University of Winnipeg, Winnipeg, Manitoba R3B 2E9, Canada.
[4] Response Biomedical Corp., 8081 Lougheed Highway, Burnaby, British Columbia V5A 1W9, Canada.

study, 1 individual interpreted results; for the sensitivity evaluation portion of the study, 3 individuals interpreted results). Results are reported as positive if there is a pink–red line at the appropriate position on the dipstick, and if the control line is visible (indicating that the sample material successfully migrated across the full length of the dipstick).

The RAMP WNV test utilizes WNV-specific antibodies conjugated to fluorescent latex particles. In the RAMP test, up to 50 mosquitoes are homogenized as described earlier in 1 ml of grinding solution provided in the kit. After centrifugation, 100 μl of the supernatant is mixed with the conjugated antibody complex, and 70 μl of the sample–conjugated antibody mix is added to the test cartridge that contains the test strip. As the mixture migrates along the strip within the cartridge, antigen-bound particles are immobilized at the detection zone. Additional control particles are immobilized at an internal control zone. After 90 min, the cartridge is placed into the RAMP reader, which measures the amount of fluorescence emitted by the particles bound at each zone. The reader displays the results in RAMP units, a relative value reflecting the ratio between the fluorescence values at the detection and internal control zones. Any RAMP unit values ≥ 15 are considered positive.

The WNV-specific RNA in samples was detected and quantified by using TaqMan RT-PCR performed as described by Lanciotti et al. (2000). To evaluate test sensitivity, RNA was extracted from serial dilutions of WN seed virus by using Qiagen's QIA amp Viral RNA minikit (QIAGEN). Five microliters of RNA extracted from each sample were added to primers and probe specific to the 3′ nontranslated region (NTR) of the WNV genome and reagents in Qiagen's Quantitect Probe RT-PCR kit. Samples were subjected to 45 amplification cycles in the Bio-Rad Icycler IQ™ Real-time Detection system (Bio-Rad, Hercules, CA) according to cycling conditions described previously (Lanciotti et al. 2000). The RT-PCR results are expressed as a cycle threshold (CT) number, the cycle at which fluorescence is detected above a fixed threshold level. The virus titer of each sample was quantified with a standard curve and expressed as plaque-forming units (PFU) per milliliter.

Viral RNA was extracted from the field-collected mosquito pools by using Qiagen's RNeasy 96-well kits. Five microliters of RNA from each pool were added to primers and probes specific to either the 3′ NTR or envelope (ENV) regions of the WNV genome and reagents in the PE Applied Biosystems TaqMan RT-PCR Ready Mix kit (PE Applied Biosystems, Foster City, CA). Specimens were screened with the 3′ NTR primer/probe and confirmed with the ENV primer/probe. Samples were subjected to 40 amplification cycles in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) according to the manufacturer's TaqMan RT-PCR cycling conditions. Field-collected pools were considered positive if the CT value was ≤ 37 in the RT-PCR protocol as described for these specimens.

Virus strains: West Nile virus strain NY99-6922 and St. Louis encephalitis virus (SLEV) strain TBH-28 were used in this study. Both were from high-titered stock grown in brains of suckling mice.

Experimental design: Sensitivity of the VecTest and RAMP WNV tests was evaluated by making 10-fold or 2-fold serial dilutions of high-titered WN seed virus in the grinding buffers provided in the respective test kits, and testing the dilutions as described in the instructions provided by the manufacturers. Because detergents contained in the test buffers rendered the WNV noninfectious and titration of infectious virus could not be conducted, the titer of virus in each sample was determined from a standard curve developed by using the viable virus stocks diluted in BA-1 medium, cell culture assay and RT-PCR to correlate the RT-PCR CT value to the sample titer (Lanciotti et al. 2000). Each dilution was tested in duplicate with the VecTest and RAMP WNV assays.

Specificities of the VecTest and RAMP WNV tests were determined by diluting a high-titer suspension of SLEV 1:10 in the respective kit buffer and testing it according to the manufacturers' instructions. The resulting sample contained SLEV at approximately 8 $\log_{10}$ PFU/ml. The sensitivity and specificity evaluations were performed at the U.S. Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, in Fort Collins, CO.

Ability of the RAMP and VecTest assays test to detect WNV in mosquito pools was evaluated by using mosquitoes collected in Manitoba and Saskatchewan, Canada, during 2003. Mosquitoes were sorted into groups of up to 50 individuals and ground in 1 ml of BA-1 buffer. The RNA was extracted from the pools and tested for the presence of WNV-specific sequences by using TaqMan RT-PCR as described above. Of the 325 RT-PCR-positive pools collected during 2003, 100 pools covering a range of positive CT values were selected for subsequent testing with the RAMP and VecTest assays. Mosquito species represented in the 100 positive pools are *Aedes vexans* (Miegen) ($n = 4$ pools), *Coquillettidia perturbans* (Walker) ($n = 2$), *Culiseta inornata* (Williston) ($n = 3$), *Culex restuans* Theobald ($n = 11$), and *Cx. tarsalis* Coquillett ($n = 80$). Because these pools had been ground in BA-1 buffer (a medium compatible with tissue culture assay, but not optimal for the RAMP and VecTest assays), the mosquito homogenates in BA-1 were mixed 1:1 with the grinding medium from the commercial assays to provide the required volumes (i.e., 50 μl BA-1 supernatant : 50 μl RAMP buffer; and 125 μl BA-1 supernatant : 50 μl VecTest buffer). This resulted in a 50% reduction in the virus titers in the samples, and a 50% reduction in the detergent concentrations in the samples. There-

Table 1. Sensitivity of the RAMP® West Nile virus (WNV) test determined from serial dilutions of WN seed virus in RAMP grinding medium. Results are shown as the WNV titer (determined from reverse transcriptase polymerase chain reaction [RT-PCR] standard curve), the RT-PCR cycle threshold (CT) value for the sample, and the duplicate RAMP test results.

Sample titer[1] (log_{10} PFU/ml)	CT value	Ramp units (A)	Ramp units (B)
6.56	14.72	>640[2]	>640[2]
5.96	16.70	>640[2]	>640[2]
4.99	19.95	>640[2]	>640[2]
4.14	22.76	251.5[2]	135.1[2]
3.17	25.99	18.7[2]	15.9[2]
2.80	27.23	17.8[2]	14.1[2]
2.47	28.34	16.3[2]	9.3
2.40	28.60	8.6	8.7
2.18	29.29	9.0	14.3
2.03	29.79	5.6	15.5[2]
1.91	30.19	9.8	5.3
1.88	30.31	11.7	6.4
1.70	30.79	11.8	6.8
1.60	31.10	8.8	5.6
1.10	32.73	4.1	4.7
RAMP buffer control	40	10.4	7.0

[1] Determined from RT-PCR standard curve. PFU, plaque-forming units.
[2] Positives (≥15 RAMP units).

fore, the mosquito pool sample preparation was not optimal for use in the RAMP and VecTest assays, but still provided results enabling comparison of the tests. Once the mosquito pool sample had been prepared by using BA-1 and test kit buffer, the assays were conducted according to the manufacturer's instructions as described earlier. The evaluations using field-collected, WNV-positive mosquito pools were conducted at the Health Canada, National Microbiology Laboratory, Winnipeg, Manitoba, Canada.

RESULTS

Sensitivity

The RAMP test produced duplicate positive results (i.e., RAMP units ≥ 15 in both tests run on the same dilution) in samples that contained WNV concentrations as low as 3.17 log_{10} PFU/ml (CT values as high as 25.9) (Table 1). Equivocal positive results (where only 1 of the 2 samples tested produced RAMP units ≥ 15) were detected down to a virus concentration of 2.03 log_{10} PFU/ml, with a CT value as high as 29.79. The RAMP WNV assay produced negative results when tested against a sample of SLEV containing 8 log_{10} PFU/ml.

The VecTest WNV assay detected WNV in samples containing concentrations as low as 5.17 log_{10} PFU/ml in duplicate samples (CT values up to 19.36) (Table 2). In this evaluation, no equivocal positive VecTest WNV assay results were detected (i.e., where less than 3 of 3 test observers agreed that there was a discernable pink line at the appropriate position on the dipstick or where only 1 of the duplicate tests was positive). The VecTest WNV assay produced negative results when tested against a sample of SLEV containing 8 log_{10} PFU/ml.

Detection of WNV in field-collected mosquito pools

Of the 100 mosquito pools positive for WNV by RT-PCR, the VecTest WNV assay detected evidence of WNV in 65 pools, and the RAMP WNV test detected evidence of WNV in 94 pools. False-negative rates were 6% for the RAMP WNV test and 35% for the VecTest WNV assay. The predictive value of a positive test (i.e., the percentage of positive results which are actually positive) was 100% for both the VecTest and RAMP WNV assays. The predictive value of a negative test could not be determined because no RT-PCR negative samples were tested in this evaluation.

By using the RT-PCR results, we were able to determine the detection threshold and the error rate of the assays on samples that should have been detectable (Table 3). The RAMP WNV test detected WNV in pools with RT-PCR CT values ≤ 36 (the highest CT value in the positive samples used in this assay). The VecTest WNV assay could not detect virus in pools with RT-PCR values > 30. All 100 mosquito pools fell within the range that should have been detectable by RAMP but 6 (6%) of the pools with CT values ≤ 36 were not detected. A total of 83 pools fell within the range that should have been detectable by VecTest (CT ≤ 30) but 18 (21.7%) of these were not detected. The reason for false-negative results from samples with CT values within the detection ranges is not clear. Using these unexplained false negatives, we can predict that RAMP will miss 6% of pools with a CT ≤ 36 and VecTest will miss 21.7% of pools with a CT ≤ 30 (with the RT-PCR parameters used for this assay). This is in addition to those positive pools that are not detected because they contain too little virus to produce a positive result in these as-

Table 2. Sensitivity of the VecTest™ West Nile virus (WNV) test determined from serial dilutions of WN seed virus in VecTest grinding medium. Results are shown as the WNV titer (determined from reverse transcriptase polymerase chain reaction [RT-PCR] standard curve), the RT-PCR cycle threshold (CT) value for the sample, and the duplicate VecTest test results.

Sample titer[1] (log_{10} PFU/ml)	CT value	VecTest (A)[2]	VecTest (B)[2]
7.11	12.88	Positive	Positive
6.35	15.40	Positive	Positive
5.17	19.36	Positive	Positive
3.93	23.47	Negative	Negative
3.20	25.90	Negative	Negative
2.76	27.38	Negative	Negative
2.59	27.94	Negative	Negative
2.40	28.56	Negative	Negative
2.28	28.98	Negative	Negative
2.20	29.22	Negative	Negative
2.20	29.11	Negative	Negative
2.14	29.45	Negative	Negative
2.10	29.60	Negative	Negative
2.00	29.79	Negative	Negative
VecTest™ Buffer control	40	Negative	Negative

[1] Determined from RT-PCR standard curve. PFU, plaque-forming units.
[2] VecTest results were observed by 3 individuals and considered positive if all 3 independently agreed that a pink-red line was present at the appropriate location on the VecTest dipstick.

says, with CT values above the thresholds of detection.

The sensitivity thresholds and error rates were used to estimate the number of positives each assay would detect of the total 325 RT-PCR-positive pools found in 2003. Of the 325 RT-PCR-positive pools, only 1 had a CT value above the level detectable by the RAMP test. A total of 28 were outside the threshold detectable by VecTest, leaving 297 that should have been detectable with this assay. By multiplying the number of detectable pools by the error estimate for each assay, we estimated that the RAMP assay would miss 19 of the detectable positive pools and VecTest would miss 64. By subtracting this error from the detectable pools we estimated that of the 325 positive pools detected by RT-PCR, the RAMP test would detect 304 (93.5%) and VecTest assay would detect 233 (71.7%).

DISCUSSION

Results from serial dilutions containing known concentrations of WNV demonstrated that the RAMP test reliably indicated the presence of WNV in samples containing as little as 3.17 log_{10} PFU/ml, similar to the sensitivity of an enzyme-linked immunosorbent assay for WNV (Hunt et al. 2002). In our evaluations, the RAMP test was approximately 100-fold more sensitive than VecTest. The VecTest WNV assay required 5.17 log_{10} PFU/ml to produce an unequivocal positive result, although previous studies demonstrated that the VecTest assay could detect WNV in samples with 1.5–2 log_{10} PFU/ml lower titers than in the current study (Nasci et al. 2002, Ryan et al. 2003, Chiles et al. 2004). Neither assay gave positive reactions against high-titered SLEV preparations, demonstrating that both assays are able to differentiate between the 2 mosquito-transmitted flaviviruses of public health importance in North America.

West Nile virus–positive mosquito pools from field collections vary in the amount of WNV they contain. Both assays were able to detect WNV in field-collected mosquito pools that were demonstrated to contain WNV by using RT-PCR. As was suggested by the results of the sensitivity trials, the

Table 3. West Nile virus (WNV) assay error rates based on the number of positive pools with cycle threshold (CT) values within the limits of detection that should have been detectable by the assays, and the number of pools within the detection limits that gave negative results.

WNV assay	CT value cutoff[1]	No. of pools with CT values within detection limits[2]	No. false negatives within detection limits[3]	Error rate (%)[4]
VecTest™	30	83	18	21.7
RAMP®	36	100	6	6

[1] Reverse transcriptase polymerase chain reaction CT above which no positives were detected by the indicated WNV assay.
[2] Samples with CT values below the listed cutoff for detection.
[3] Samples with CT values within the range of detection by each test, but that were negative.
[4] Percentage of samples within detection limits giving false-negative results.

RAMP test detected WNV in more of the RT-PCR-positive pools than did the VecTest WNV assay. The RAMP test detected WNV in 96% of the positive pools that were evaluated. The VecTest WNV assay detected WNV in 65% of the pools, which was consistent with previously reported results of this test with field-collected samples (Nasci et al. 2002). Although a portion of the false negatives resulted from the samples containing too little virus protein to produce a positive result, sensitivity did not appear to account for all of the false negatives. According to the RT-PCR CT values, all of the RAMP false negatives appeared to contain sufficient virus to be detectable by this assay. Of the 35 false-negative pools in the VecTest WNV assay, 17 appeared to be outside the detection threshold of the test and 18 appeared to contain sufficient virus to be detectable. The reason for the failure of these tests to detect WNV in pools that RT-PCR indicated are within their detection thresholds is unclear. One explanation is that the measurable amount of RNA that is detected by RT-PCR assays may not correspond to the amount of detectable WNV antigen at certain points in the extrinsic incubation period in the mosquito.

Our estimates of the assays' performances on a large number of RT-PCR-positive field-collected mosquito pools took into consideration the sensitivity of the assays and the likelihood that they will not detect WNV in some of the pools that appear to be within their sensitivity range. The results suggested that the RAMP test would detect approximately 20% more of the positive pools than would the VecTest assay. The impact of this difference on mosquito infection rates calculated for surveillance purposes is not known, but previous analyses suggest the differences would have negligible impact (Nasci et al. 2002). However, we suggest that surveillance data should always be interpreted with knowledge of and in respect to the limitations of the surveillance protocol and diagnostic tests used.

Although both assays appear useful in WNV surveillance programs, users should be aware of some of the assays' characteristics. Both assays are easy to use and require minimal laboratory facilities and technical experience, particularly in comparison to the RT-PCR assays. However, both tests require facilities for homogenizing and centrifuging samples of mosquitoes, and both have the potential to produce aerosols during the procedure. Although the tests could be performed under field conditions, both are better conducted in laboratory settings with provisions for use of personal protection measures (e.g., restricted access to the area, ability to sanitize work surfaces when finished, and use of gloves, eye protection, lab coat and N-95 mask by the operator, or use of a biological safety cabinet, if available) because there is no guarantee that all virus contained in positive samples is rendered noninfectious by the grinding buffers. The RAMP test is more sensitive than VecTest, but its test protocol takes longer, requiring more sample preparation steps and a 90-min incubation. After the incubation, the cartridges are read individually by the RAMP reader, which takes approximately 1 min per cartridge. The VecTest protocol is considerably faster, requiring fewer sample preparation steps and a 15-min incubation period, after which many tests can be ''read'' in quick succession. One major difference between the tests is related to how the results are obtained. The VecTest assay strips are read by visual observation of pink–red bands at given locations on the strip, and are easy to interpret when distinct bands are present on the strip. However, the subjective interpretation of bands on VecTest strips introduces the potential for human error. For example, false negatives may occur when bands are very light when the sample's virus titer approaches the test's sensitivity threshold, or false positives may occur when very narrow, incomplete bands are formed (e.g., as reported for some avian tissue and oral swab samples) (Stone et al. 2004). Potential for errors can be reduced by having several people interpret the results independently and through the accumulation of experience in observing positive and negative strips. The RAMP reader measures emitted fluorescence at the test and control sites on the strip, objectively assigning a number value that is easy to interpret by referring to the manufacturer's suggested cutoff level for positive tests. This removes the potential for misinterpretation of results by observers.

With the exception of a few common laboratory items, such as pipettes and a centrifuge, everything needed to perform each assay is found packaged in its kit. At the time of this publication, the cost of using the RAMP system includes an initial investment of U.S. $3,500 for the reader, and U.S. $1,500 for the test kit (100 assays per kit). The RAMP kit includes test cartridges, grinding buffer, assay tips, and other components of the assay. The VecTest assay's kit is U.S. $400 for 50 tests, and includes dipsticks, grinding buffer, and other components of the assay. No initial investment is required to perform the VecTest assay.

In summary, our results indicate that both the RAMP WNV test and the VecTest WNV assay have characteristics that make them useful in WNV surveillance programs. Understanding the limitations of these tests will assist in interpreting results. Further experimentation is required to better document the tendency of these assays to produce false-negative results with a variety of field-collected mosquitoes (e.g., different mosquito species or blood-engorged specimens), and how the RAMP assay performs with other specimens such as oral swabs from dead birds or tissue homogenates. As a final caution, the grinding buffers used in the RAMP and VecTest assays contain a detergent that can inhibit the polymerase reaction in RT-PCR. If RT-PCR is used as a confirmation for these tests, RNA extraction protocols should be enhanced with

additional wash steps to ensure that detergent is removed from the sample.

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