Evaluation of a Rapid Analyte Measurement Platform for West Nile Virus Detection Based on United States Mosquito Control Programs

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Abstract. The rapid analyte measurement platform (RAMP) system is an immunoassay test for West Nile virus (WNV) detection. Although reverse transcriptase-polymerase chain reaction (RT-PCR) methodology has been regarded as the gold standard for confirming WNV presence, usage of RAMP testing kits has increased in the past years. We collected RAMP test result data that were subsequently confirmed with RT-PCR methodology from mosquito control agencies to evaluate the efficacy of the RAMP testing. Results indicate that there are a high number of false positives (RAMP positive, RT-PCR negative) with RAMP testing. Correlation between RAMP unit values and RT-PCR cycle threshold values were varied depending on the primer/probe being compared. Comparison of RT-PCR results (on the same samples) between laboratories also indicates variation among the procedures and their potential to influence the RAMP testing efficacies. We discuss the potential issues and solutions that could prevent the high rate of false positives.

INTRODUCTION

West Nile virus (WNV), with its associated morbidity and mortality and potential for contamination of the blood supply and transplant tissues, is a serious and costly public health concern in the United States. The North American strain of the virus was first detected in New York in 1999, but it has since spread to Canada, Latin America, and the Caribbean and is now considered endemic throughout the continent.[1] Efficient and effective vector control is one of the most successful means of reducing disease transmission to humans, livestock, and wildlife. Thus, active vector surveillance through monitoring of mosquito populations and field infection rates is crucial for the success of abatement measures.

Most local mosquito abatement districts (MADs) or public health agencies participate in state-sponsored surveillance programs. Testing of mosquito pools for arbovirus presence is either through detection of live virus plaques by Vero cell culture assays or detection of viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Both systems have high sensitivity and specificity; however, they tend to have a high initial setup cost and require appropriate bio-safety features and specially trained staff members. Additionally, transport of samples to a testing center may introduce vital delays between the times that mosquitoes are collected in the field, diagnostic assays are conducted, results reported, and control measures implemented. To avoid the expense and delay often associated with cell culture and RT-PCR testing procedures, many MADs have adopted commercially available, on-site arbovirus testing kits to enhance or replace results from detection laboratories.

The rapid analyte measurement platform (RAMP), Response Biomedical Corporation (RBC), Vancouver, British Columbia, system is a commercially available immunoassay test for WNV detection that is increasingly used by MADs and local public health laboratories.[2,3] This method does not require the exten-

sive technical expertise or costly equipment and facilities inherent to cell culture and RT-PCR testing methods. Unlike RT-PCR, which is based on the nucleic acid, RAMP uses WNV-specific antibodies, conjugated to fluorescent latex particles, to determine the status of a sample. After mixing a homogenized sample with the conjugated antibody complex, a portion is added to the proprietary RAMP cartridge. As this sample migrates through the cartridge, antigen-bound particles are immobilized in the detection zone, whereas additional control particles are immobilized at an internal control zone. After drying, the RAMP reader measures the amount of fluorescence emitted by particles at each zone and displays a result as a relative value reflecting the ratio between the fluorescence values at the detection and internal control zones. Currently, a displayed test result of ≥ 30 relative units is the manufacturer's recommendation for classifying a sample as positive for WNV. The RAMP system allows MADs to collect mosquitoes from the field and rapidly (i.e., 2–4 hr) assay them for the presence of WNV in their local facilities. However, the accuracy of this technology and its ability to provide crucial information for rapid responses to mosquito populations has recently been under scrutiny. We undertook this study to investigate false-positive rates among RAMP-tested samples at local MADs across the United States to determine the use of this growing system for mosquito management decisions.

RAMP FALSE POSITIVES

Previous investigations showed the RAMP testing kit as highly reliable for detecting the presence of WNV in mosquito pools when compared with RT-PCR.[2,3] Given the believed superior sensitivity of RT-PCR testing, false-negative samples are expected using the RAMP kit. When verifying RAMP results with RT-PCR, there are four possible outcomes. A RAMP positive also tests RT-PCR positive or both assays are WNV negative. In either case the two tests are in agreement and can be considered true positives and true negatives. A negative RAMP and positive RT-PCR is expected for a portion of the results because of the lower sensitivity of RAMP compared with RT-PCR. When a RAMP is positive and the

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RT-PCR is negative, it is called a "false positive" and is an undesirable situation that may lead to unnecessary management decisions and unnecessary use of limited resources. Our goal was to evaluate the potential for false positives by a multistate review of field data.

PREVALENCE OF RAMP FALSE POSITIVES IN THE UNITED STATES

We acquired RAMP test result data from MADs and health departments across the United States for several surveillance seasons. Agencies from 12 states (Arizona, California, Illinois, New Jersey, Oregon, Pennsylvania, South Carolina, Texas, Utah, Virginia, Washington, and Wyoming) responded to our request, although the number of districts that provided data varied between states. We received a total of 4,579 RAMP results for samples that were also subsequently tested by the RT-PCR method. Burkhalter and others[2] reported that a RAMP unit value of > 15 was sensitive enough to accurately detect the presence of WNV, whereas Williges and others[3] were able to consistently confirm samples that had a RAMP unit value of > 30. Although the recommendation by Burkhalter and others[2] was based on infected mosquitoes in the laboratory, the study by Williges and others[3] was based on field-collected mosquito samples. Several state agencies have adopted different cut-off values for RAMP because of incongruencies in results and these are termed as grey zones. We reevaluated the data that we acquired by comparing the RAMP results, based on > 30 as a cut-off value for positives, to their respective RT-PCR results. With > 30 as a RAMP threshold value, 82% of RAMP results were congruent (either positive or negative) with their respective RT-PCR results (N = 3,758), whereas 18% of the results were incongruent (N = 821). In our comparison of all the RAMP data with RT-PCR results, 14% were false positives, correct positives were 15%, false negatives were 4%, and correct negatives were 67%. Although knowing whether a particular sample is a true negative for WNV is important, a positive sample typically elicits a series of management responses for MADs and local public health departments (LPHDs). This is especially true during the early stages of transmission when it is assumed rapid focal introduction of vector abatement may prevent or impede amplification within an area. If we reevaluated the results and consider only the positive samples, then 49% of the RAMP results were false positives compared with 51% correct positives. In contrast, 6% of all RAMP results were false negatives. This clearly indicates false positives were a major issue in our sample; however, there was considerable site-specific variation among states. Furthermore, some agencies did not confirm their samples if their RAMP unit values were above their individual grey zone value. For example, Pennsylvania did not confirm with RT-PCR those samples that had a RAMP unit value > 200, which could affect the overall false-positive rate. In California, New Jersey, and Pennsylvania over 90% of the RAMP results matched RT-PCR results, whereas in other states congruency rates were < 90% with Virginia having the lowest comparison with only a 37% matching rate (Figure 1). Many agencies do not confirm their RAMP negative samples with the RT-PCR method and hence we excluded some states from this analysis. Considering only the positives for states, false positives (RAMP positive samples that did not match with RT-PCR
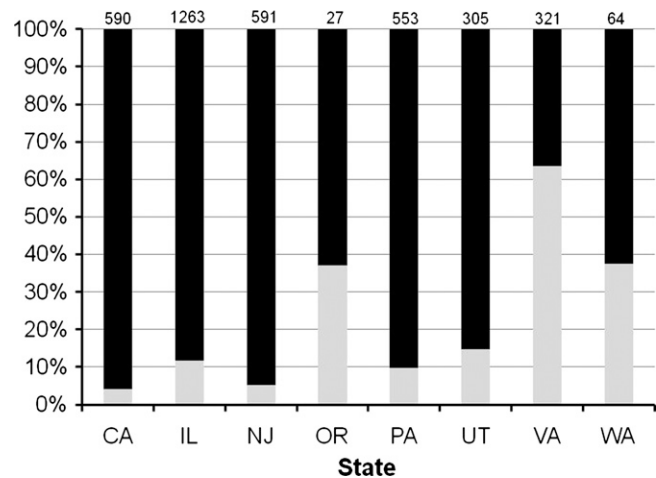

FIGURE 1. Bar graph showing percentages of the rapid analyte measurement platform (RAMP); results that were congruent with reverse transcriptase-polymerase chain reaction (RT-PCR). Black sections represent the percentages that match, whereas grey sections represent results that do not match.

results) were 13% or below in California, Illinois, New Jersey, and Oregon, whereas in other states the false positives were > 27% with South Carolina and Texas having the highest rate with 100% false positives (Figure 2). Thus, the RAMP test exhibited considerable state-specific variation with RT-PCR confirmation testing. These results indicated considerable variability in the efficacy with the recommended threshold of > 30.

Differences in RAMP cut-off points across the United States. Although RBC, the manufacturer of RAMP, recommends 30 as the cut-off point for detecting presence of WNV when using the RAMP testing kit, a technical bulletin by the company (no. 51138), released in March of 2009, describes the presence of a "Grey Zone" in some geographical areas caused by variability in preparation techniques of the WNV testing kit. According to RBC, a cut-off value of > 80 should be interpreted as WNV positive in those areas that have a grey zone, whereas if those values were between 30 and 80 they should be confirmed with RT-PCR. The technical bulletin further states, the user would be responsible for determining the presence and level of a grey zone based on their RT-PCR confirmations. We applied 80 (any sample that had a RAMP unit value of < 80 was considered negative for WNV) as the cut-off value for our data set and it showed that 89% of the RAMP test results were congruent with RT-PCR, whereas 11% were incongruent. The number of false positives decreased (when compared with the previous results with a RAMP cut-off value of 30) to 5%, correct positives to 13%, whereas false negatives increased to 6% and correct negatives to 76%. If only positives were considered, false positives dropped to 28% and correct positives increased to 72%. Among states, increasing the cut-off value to > 80 favored Pennsylvania and Washington by reducing the false-positive rate, however did not alter the outcomes for Arizona, South Carolina, Texas, Utah, Virginia, and Wyoming (Figure 2B). Comparison studies by Burkhalter and others[2] suggest that a RAMP unit value of > 15 may be sufficient for detecting the presence of WNV, which is contrary to the company's 2009 recommendation of > 80. Unlike Burkhalter and others[2]
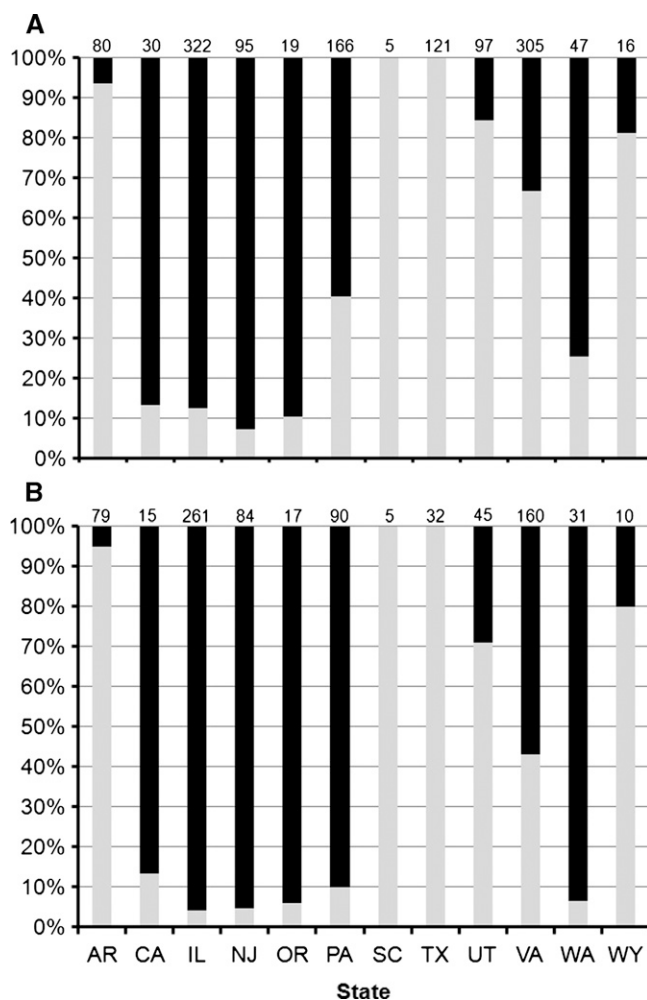
FIGURE 2.   Bar graph showing percentages of the rapid analyte measurement platform (RAMP); results that were false positives and correct positives. Black sections represent correct positives and grey sections represent false positives. (**A**) Positives based on a RAMP threshold value of 30. (**B**) Positives based on a RAMP threshold value of 80.

where the mosquitoes were infected with WNV in the laboratory, technical bulletin (no. 51138) published by the RBC reports that their suggested value is based on field data with 716 data points that they had collected from a few MADs. Therefore, it remains possible that variations in infected field-collected mosquitoes could demand the increase in the RAMP cut-off value suggested by RBC. However, Mercer County Mosquito Control in New Jersey conducted a study from 2005 to 2008 where they sent all field collected/RAMP tested mosquito pools for confirmation with RT-PCR and detected no false positives using > 30 as the cut-off value.[3] Their sample size was 431, among which 21% were correct positives, 6% were false negatives, and 73% were correct negatives. Mercer County mosquito control's data after 2008 continued to follow a similar pattern in 2009 and 2010 (Farajollahi A, unpublished data). Moreover, MADs in California continue to use > 30 as their cut-off value and still have consistent results when compared with other states (Figure 2). The technical bulletin released by RBC (no. 51138) argues that the increase in the RAMP cut-off value is necessary because of variations in mosquito, bird, and virus populations. Nucleotide sequence

comparison studies comparing the New York (northeastern United States) WNV strain to nine WNV strains isolated in south Texas (southern United States) indicate limited evolution of WNV during its southwesterly spread in the United States.[4] *Culex* spp. were the principal mosquitoes in the pools from all agencies, but unfortunately not many agencies identified the specimens beyond the generic level. Nevertheless, it is difficult to comprehend that a few states can still use > 30 as a cut-off value for RAMP and have a higher RT-PCR confirmation, whereas other states need to increase the cut-off value to get similar results. We found that increasing the cut-off value did not always reduce the false-positive rate for some states, thereby strengthening the argument that the underlying cause for such variation needs to be investigated.

CORRELATION BETWEEN RAMP AND RT-PCR VALUES

The use of RT-PCR as a confirmatory test for the presence of WNV is widespread throughout North America, but the methods vary between agencies. The original recommendation by Lanciotti and others[5] was to use two or more primer sets that amplify different parts of the flavivirus genome (e.g., NS5, ENV, and 3′NC). Presently, there are many commercially available primer/probe sets available for WNV-RNA detection. Our collaboration with the various states allowed us to address two questions:

1. How well do the cycle thresholds (CT) of different primer sets correlate to RAMP values? If the RAMP response is quantitative, we anticipate a significant correlation between the values for the two assays.
2. Do the different RT-PCR primer/probe sets correlate well to each other? Although there may be some variation in the sensitivity of primer/probe sets for RT-PCR,[5] we expect the data sets to be highly correlated and significant.

Users of RAMP often assume there is a relationship between the amount of virus and RAMP test value. In Illinois, a bivariate plot of mean RAMP value (x) to mean CT (y) for every 100 RAMP units (0 to 99, 100–199, 200–299, 300–399, 400–499, and 500–599) was significant ($y = -0.01x + 32.04$; r2 = 0.80; df = 1.5; F = 15.8; P = 0.016). Thus, the mean RAMP values appear to have a good quantitative relationship based on the RT-PCR CTs. However, the correlation of each RAMP value to a single CT value displayed considerable variation. The Illinois data from two MADs indicated only 34% of the variation in the RAMP value was explained by the CT value ($y = -0.01x + 33.82$; r2 = 0.34; df = 1,229; F = 116.54; P < 0.0001). Illinois had the largest number of WNV-positive mosquito samples and the RAMP comparison to RT-PCR was by one agency, thus this data played a key role in our analyses. Furthermore, we were also able to evaluate the RT-PCR reliability of Illinois Natural History Survey (INHS) by comparing the results of field samples with another agency.

In New Jersey where two primer sets were used, one exhibited a significant correlation with RAMP values ($y = -0.01x + 31.88$; r2 = 0.30; df =1.51; F = 21.90; P < 0.0001) similar to that observed in Illinois. Unfortunately, the other primer/probe set did not correlate with RAMP values ($y = -0.003x + 31.13$; r2 = 0.02; df = 1.51; F = 1.12; P = −0.29).

These comparisons were for RAMP values > 0.0 and < 640. Although a bivariate comparison of the two CTs from each primer set per sample in New Jersey (of all samples regardless of RAMP value) indicated the two significantly correlated, only 44% of the CT variation in one primer/probe set was explained by the variation in CTs of the other primer/probe set (primer set 1 CT = 0.60 (primer set 2 CT) + 110.46; r2 = 0.44; df = 1,103; F = 106.63; P = 1.5 E-17). The choice of primer/probe sets for comparison to RAMP could greatly impact the interpretation of RAMP efficacy.

Several states such as Utah classify a sample as WNV positive only if two primer/probe sets agree. If only one of the primer/probes shows positive for WNV, the test is then considered as indeterminate. The previous results indicate that the RAMP unit value could have significant or non-significant correlations with CT values depending on the type of primer/probes used to confirm the RAMP results of a sample. This shows that variation between the primer/probe sets could lead to a higher rate of false negatives when used for confirming RAMP tests. We suggest that the potential variation caused by differences in RT-PCR techniques needs to be investigated or else it may not always be a justified "gold standard" to test the efficacy of RAMP testing.

CORRELATION IN RT-PCR VALUES BETWEEN TWO LABORATORIES

Analyzing differences in RT-PCR procedures for all the agencies was beyond the scope of this study; however, we were able to directly compare the RT-PCR results of RAMP samples by two laboratories, Unified State Laboratories (USL) and, INHS Medical Entomology Program. These laboratories located in Utah and Illinois, respectively, process RAMP and other mosquito samples from MADs in their respective states for WNV-RNA. The Illinois group tends to analyze over 5,000 mosquito pools per year by RT-PCR. To minimize costs and reduce turnaround time for such a large number of samples, INHS uses just the ENV primer for detection of WNV-RNA. In contrast, USL uses two primer/probe sets for RT-PCR confirmation, ENV and NS1 or 3′UTR. The primer sets that both laboratories use are based on the publication by Lanciotti and others.[5] The main scope of this comparison was to investigate the percentage of inconsistency between the two laboratories in detecting WNV-positive samples that were previously tested using RAMP. In 2010, INHS sent 199 samples (extracted RNA) considered WNV positive by their RT-PCR analysis to be retested at USL, and USL sent 29 samples (extracted RNA) to INHS. Among the 199 samples from INHS, 94% were reconfirmed WNV-RNA by USL and 86% of the 29 USL samples were reconfirmed by INHS, indicating that there is variation in RT-PCR techniques between these two laboratories.

IMPROPER RAMP TESTING AND SAMPLE STORAGE

The RAMP testing involves grinding mosquitoes with the RAMP buffer, centrifuging the ground product, transferring the supernatant to the test cartridge, and finally inserting the test cartridge into the reader to obtain the RAMP unit value. The RAMP cartridge that is inserted into the RAMP machine for reading has a test strip to which the sample is transferred. Once transferred, the sample needs to migrate through to the end of the strip, which is marked by a line that is visible on the back of the cartridge. If the sample does not reach the end of the strip then the RAMP testing may not yield reliable results. Some of the problems that could cause such an incident would be an inadequate amount being transferred or inadequate centrifugation, which might result in transferring mosquito body fragments that could prevent the sample from migrating. Pooled samples may accidentally include blood-fed mosquitoes, which are known to interfere with RAMP results. The RBC recommends 50 mosquitoes per sample, whereas Sutherland and Nasci[6] reported that samples larger than 50 mosquitoes could still be used but the sensitivity might decrease with larger pools.

Mosquitoes should be tested on the same day of collection, if possible, or else be stored at –20°C to preserve the viral RNA (RBC, technical bulletin no. 51173). After RAMP testing, only the supernatant, without the homogenates and bbs (added to aid in mosquito tissue grinding), are recommended to be stored for future RT-PCR testing in a –70°C freezer to preserve the viral RNA if they are not being shipped the same day. The RBC recommends the use of coolers with dry ice for shipping the supernatants. Unfortunately, dry ice shipping requires special training and packaging because it is listed as a hazardous substance by the United States Department of Transportation.

Failing to follow the recommended sample preparation and storage instructions could lead to erroneous results both with RAMP testing and confirmations with RT-PCR. During the months of June and July in 2010, ADAPCO, the distributor of the RAMP testing kits in the United States, and RBC provided RAMP test cartridges to the Salt Lake City Mosquito Abatement District to evaluate the performance of RAMP test kits in Utah, which had large numbers of false positives (Figure 2A). The investigator (BK) of the project attended the training seminar given by the RBC staff and followed the sample preparation and storage instructions. In total, 196 mosquito pools were tested with RAMP kits and subsequently with RT-PCR. The false positives were 3% (N = 6), correct negatives were 97% (N = 190), and there were no false negatives if the cut-off value was set at > 30. There were no false positives and 100% of the RAMP tested samples were correct negatives if the cut-off value was set at > 80. Unfortunately, during the months of June and July in 2010, there were no WNV-positive mosquito pools from the area and hence the beneficial effect of following the manufacturer's instructions on WNV-positive mosquito pools could not be evaluated.

CONCLUSION

The RT-PCR equipment is expensive and requires special expertise, therefore MADs and LPHDs often transport their mosquito samples to a central facility for testing. During periods of high surveillance or increasing positives, samples may overwhelm a facility's capacity to provide a rapid turnaround time for results. Williges and others[3] reported that the average time to receive results from RT-PCR in New Jersey was 10 days. The RAMP test kits could be a valuable tool for making management decisions within a few hours of sample collection. Early targeting of WNV transmission could prevent or ameliorate WNV outbreaks. Inconsistency in the RAMP test kit results across the United States casts a shadow on the efficacy of the kit in detecting WNV positives. Increasing the RAMP cut-off point to > 80 could help some states,

but there are several states where the false positives are still high despite increasing this value. According to RBC, areas where a RAMP cut-off point of > 80 still results in false positives the cut-off values should be increased to accommodate the grey zone. Although most of the laboratories use the TaqMan RT-PCR method, they use different RNA extraction methods. California uses ABI lysis buffer following ABI guidelines, INHS uses QIAmp virus Biorobot 9604 kit, whereas USL uses QIAmp viral RNA mini kit and Ambion MagMAX viral RNA isolation kit. These differences in RNA extraction could also have contributed to the incongruencies.

In summary, the potential problems with the high false positives could be caused by one or a combination of the following:

1. Improper sample preparation, RAMP testing, storage, and shipping.
2. Improper RT-PCR testing and variation among WNV primer/probe sets.
3. Variation in mosquito species and/or physiological stages causing variation in RAMP and RT-PCR results.
4. Variation in the sensitivity of the RAMP kits.

The RBC should conduct training seminars, webinars, or distribute educational material regarding proper usage of RAMP test kits. There were several MADs in our survey that had not received any technical bulletins from RBC. The manufacturer and/or distributor should provide their future bulletins through better mediums such as the American Mosquito Control Association or the Centers for Disease Control and Prevention (CDC). The Center for Vector-Borne Diseases at the University of California in Davis provides WNV proficiency panels to the MADs and laboratories that use RAMP and RT-PCR. Although providing proficiency panels for RT-PCR and RAMP may not be logistically feasible for every state testing agency, they would be useful in specific areas to narrow down the source of false positives. In particular, this might be more feasible if the proficiency panels could be provided by the CDC or RBC. Finally, advising some states to increase the cut-off value, whereas other states efficiently use lower cut-off values is arbitrary unless the underlying cause for the variation is explained to support such discrepancies.

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