LABORATORY AND FIELD EVALUATIONS OF A COMMERCIALY AVAILABLE REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR THE DETECTION OF WEST NILE VIRUS IN MOSQUITO POOLS

KRISTEN L. BURKHALTER,1 MICHAEL O’KEEFE,2 ZACHARY HOLBERT-WATSON,2 THEODORE GREEN,2 HARRY M. SAVAGE1 AND DANIEL M. MARKOWSKI2

ABSTRACT. Although the specific cDNA amplification mechanisms of reverse-transcriptase polymerase chain reaction (RT-PCR) and RT loop-mediated isothermal amplification (RT-LAMP) are very different, both molecular assays serve as options to detect arboviral RNA in mosquito pools. Like RT-PCR, RT-LAMP uses a reverse transcription step to synthesize complementary DNA (cDNA) from an RNA template and then uses target-specific primers to amplify cDNA to detectable levels in a single-tube reaction. Using laboratory-generated West Nile virus (WNV) samples and field-collected mosquito pools, we evaluated the sensitivity and specificity of a commercially available WNV real-time RT-LAMP assay (Pro-AmpRT™ WNV; Pro-Lab Diagnostics, Inc., Round Rock, Texas) and compared the results to a validated real-time RT-PCR assay. Laboratory generated virus stock samples containing ≥2.3 log10 plaque-forming units (PFU)/ml and intrathoracically inoculated mosquitoes containing ≥2.4 log10 PFU/ml produced positive results in the Pro-AmpRT WNV assay. Of field-collected pools that were WNV positive by real-time RT-PCR, 74.5% (70 of 94) were also positive by the Pro-AmpRT WNV assay, resulting in an overall Cohen’s kappa agreement of 79.4% between the 2 tests. The Pro-AmpRT WNV assay shows promise as a suitable virus screening tool for vector surveillance programs provided agencies are aware of its characteristics and limitations.

KEY WORDS Arbovirus detection, mosquito, RT-LAMP, West Nile virus

INTRODUCTION

Since its introduction in 1999, West Nile virus (WNV) has dispersed across the USA and emerged as the main arbovirus threat in the country (McDonald et al. 2019). Due to the nature of its enzootic transmission cycle, in which WNV is amplified in susceptible avian hosts and transmitted by mosquitoes to other vertebrates including humans, virus surveillance in vector populations is a critical component of vector control programs that seek to detect active circulation of WNV, determine the risk of human illness, and inform prudent vector control measures (Kwan et al. 2012, Kilpatrick and Pape 2013, DeFelice et al. 2017).

Methods of arbovirus detection commonly employed in the USA include live virus detection, nucleic-acid–based detection assays such as standard and real-time reverse transcriptase polymerase chain reaction (RT-PCR), or antigen-based qualitative assays such as the Rapid Analyte Measurement Platform (RAMP) (Burkhalter et al. 2014) or VectorTest (Varnado and Goddard 2016) and compared the results to a validated real-time RT-PCR assay. Laboratory generated virus stock samples containing ≥2.3 log10 plaque-forming units (PFU)/ml and intrathoracically inoculated mosquitoes containing ≥2.4 log10 PFU/ml produced positive results in the Pro-AmpRT WNV assay. Of field-collected pools that were WNV positive by real-time RT-PCR, 74.5% (70 of 94) were also positive by the Pro-AmpRT WNV assay, resulting in an overall Cohen’s kappa agreement of 79.4% between the 2 tests. The Pro-AmpRT WNV assay shows promise as a suitable virus screening tool for vector surveillance programs provided agencies are aware of its characteristics and limitations.

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Several RT-LAMP assays have been developed for human clinical diagnostics to detect arboviruses such as WNV, Japanese encephalitis virus (JEV), dengue virus (DENV), Chikungunya virus, yellow fever virus, Zika virus (ZIKV), and Rift Valley fever virus (Parida et al. 2004, 2005, 2007; Le Roux et al. 2009; Toeh et al. 2013; Wang et al. 2016). However, there have been few reports on the utilization of RT-LAMP assays for mosquito pool testing. In one such assessment, Wheeler et al. (2016) developed an in-house RT-LAMP protocol to detect WNV, western equine encephalitis virus, and St. Louis encephalitis virus in mosquito pools and found the sensitivity limit to be \(~2–3\) log_{10} (plaque forming units) PFU/ml more sensitive than antigen-based assays and \(~1\) log_{10} PFU/ml less sensitive than real-time RT-PCR. Evaluations of JEV, DENV, and ZIKV RT-LAMP assays showed they performed similarly in mosquito pools (Li et al. 2011, Liu et al. 2012, da Silva et al. 2019). These results, as well as the simplicity and speed of the method, suggest that RT-LAMP could be a viable option for vector-based pathogen surveillance.

A commercial RT-LAMP assay has been developed specifically for the detection of WNV in mosquito pools, in which all the necessary reagents to perform the assay are included in a single kit. We describe a laboratory evaluation to assess the sensitivity and specificity of the Pro-AmpRT™ WNV RT-LAMP assay (Catalog no. PLM-1008, Pro-Lab Diagnostics, Inc., Round Rock, TX), as well as an application of this assay to screen field-collected mosquitoes in comparison to the results of the real-time RT-PCR assay routinely used by the Centers for Disease Control and Prevention (CDC) to detect WNV RNA in mosquito pools. A distinctive feature of the Pro-AmpRT WNV assay is the inclusion of a DNA intercalating dye in the reaction mixture, which facilitates the fluorescent detection of the amplified cDNA products. To that end, the assay has been designed to be used on the OptiGene vector-based surveillance (Lanciotti et al. 2000). Cells were incubated for 60 min at 37°C and 5% CO₂, after which a 1% agarose overlay was applied. On day 2 postinfection (dpi), a 1% agarose overlay with neutral red was applied, and the cells were observed for plaques on 3–7 dpi.

The RT-LAMP assay was carried out in a 20-µl total reaction volume containing the kit-supplied Pro-AmpRT isothermal mastermix, Pro-AmpRT WNV primer mix, and RT enzyme according to the manufacturer’s instructions. The reaction mixture was aliquoted into wells of Genie tube strips (Pro-Lab Diagnostics, Inc.), which are provided in the kit or can be purchased separately. A 5-µl aliquot of sample RNA was added to the reaction mixture. A positive control of either previously extracted WNV RNA or the positive control provided in the kit and a negative water control were included in each run. The run was deemed valid if the positive and negative controls produced expected results. The samples were run in the Genie II instrument, which was programmed as follows: 1) 66°C for 30 min and 2) an annealing gradient from 98°C to 80°C at 0.05°C/sec. Samples that returned a single annealing peak between \(-86\) and \(88\)°C were considered positive. To test the RT-LAMP assay on a conventional real-time thermocycler, an aliquot of RNA from each virus dilution was added to RT-LAMP assay as described in a PCR reaction plate and sealed with optical film. The reactions were run on a Bio-Rad CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA) using the following protocol: 1) 66°C for 30 sec × 60 cycles, each cycle followed by a plate read using the FAM detection channel, and 2) a melt curve from 65 to 95°C in increments of 0.5°C/sec, concluding with a final plate read.

All samples were tested with the validated real-time RT-PCR assay used by the CDC for WNV vector-based surveillance (Lanciotti et al. 2000). Briefly, a 5-µl aliquot was added to WNV real-time RT-PCR primers and reagents of the Qiagen Quantitect Probe RT-PCR kit and subjected to 45 amplification cycles as recommended by the manufacturer on the Bio-Rad CFX96 Touch Real-Time PCR detection system. Samples returning a cycle threshold (CT) value of \(\leq 37.0\) were considered positive.

**MATERIALS AND METHODS**

**Laboratory evaluation:** For the sensitivity evaluation, stock WNV derived from a positive *Culex tarsalis* Coquillett mosquito pool collected in Colorado (CO08-13382) was 10-fold serially diluted in bovine albumin (BA)-1 cell culture medium. A 100-µl aliquot of each dilution was removed and extracted for viral RNA using Qiagen’s Qiamp Viral RNA extraction kit (Qiagen, Inc., Valencia, CA) on the Qiagen BioRobot Universal according to manufacturer’s protocol, then tested with real-time RT-PCR and RT-LAMP assays in parallel (described below). Three aliquots per dilution were tested with the RT-LAMP assay. An aliquot of each dilution was also applied to monolayers of Vero cells in 6-well plates to determine titer as previously described (Beaty et al. 1995). Cells were incubated for 60 min at 37°C and 5% CO₂, after which a 1% agarose overlay was applied. On day 2 postinfection (dpi), a 1% agarose overlay with neutral red was applied, and the cells were observed for plaques on 3–7 dpi.
temperature was calculated using Vero cell plaque assay. The RT-LAMP assay was performed in triplicate for each dilution; the results are displayed as the number of samples that were positive out of the 3 replicates.

Table 1. Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and Pro-AmpRT™ West Nile virus (WNV) real-time loop-mediated isothermal amplification (RT-LAMP) assay results of a WNV serial dilution series. Virus titers were calculated using Vero cell plaque assay. The RT-LAMP assay was performed in triplicate for each dilution; the results are displayed as the number of samples that were positive out of the 3 replicates.

| WNV calculated titer (log10 PFU/ml) | Real-time RT-PCR CT1 | Real-time RT-PCR result | Real-time RT-PCR positives2 | RT-LAMP positives3 |
|-----------------------------------|----------------------|-------------------------|----------------------------|---------------------|
| 6.3                               | 21.7                 | Positive                 | 3/3                        |
| 5.3                               | 24.2                 | Positive                 | 3/3                        |
| 4.3                               | 27.7                 | Positive                 | 3/3                        |
| 3.3                               | 30.8                 | Positive                 | 3/3                        |
| 3.2                               | 34.0                 | Positive                 | 3/3                        |
| 1.2                               | 36.8                 | Positive                 | 0/3                        |
| 0.2                               | 40.4                 | Negative                 | 0/3                        |

1 PFU, plaque-forming units.
2 CT, cycle threshold.
3 RT-LAMP results were considered positive if the annealing temperature was ~86–88°C.

To determine specificity, high-titered isolates of nontarget viruses were prepared in BA-1, extracted for viral RNA, and tested with real-time RT-PCR and RT-LAMP as described above.

Prior to the field evaluation, laboratory-generated mosquito pools were tested with each assay to determine the effect of mosquitoes on the Pro-AmpRT WNV assay performance. Female laboratory-reared Cx. quinquefasciatus Say were intrathoracically inoculated with 0.3 μl of ~4.7 log10 PFU/ml WNV stock strain CO08-10142 (isolated from a pool of Cx. tarsalis collected in Colorado) as described previously (Rosen and Gubler 1974). Pools consisted of 1 infected mosquito and 49 uninfected laboratory-reared mosquitoes, to which 1 copper-clad BB and 1 ml BA-1 were added. Pools were homogenized by vortex for 1–2 min and centrifuged for 3 min at 4000 rpm in a refrigerated centrifuge. Pools of 50 uninfected mosquitoes were processed to serve as negative controls. An aliquot of clarified supernatant from each pool was removed and extracted for viral RNA and tested with real-time RT-PCR and RT-LAMP (in triplicate) as described above. Another aliquot of clarified supernatant from each pool was titrated on Vero cell plaque assay as described above.

Field evaluation: Mosquitoes used for the field evaluation were collected as part of routine arbovirus surveillance by the Franklin County Public Health Department (FCPHD; Franklin County, OH) during epidemiological weeks 30–36 in the summer of 2017. Standard gravid traps (John W. Hock Company, Gainesville, FL) baited with a grass and hay infusion were set at dusk, picked up the next morning, and transferred to the laboratory. Mosquitoes were identified and sorted into pools of 1–50 individuals by collection site, collection date, genus, and sex. Pools of female Culex mosquitoes were homogenized as described above in 1.5-ml bovine albumin fortified with 1% Triton X. The samples were stored at –80°C immediately after an aliquot was taken for routine virus screening by the FCPHD field staff. A total of 278 frozen pools were shipped overnight on dry ice to the CDC in Fort Collins, CO, where they were processed to extract RNA and tested with real-time RT-PCR and RT-LAMP as described above.

Using the CDC’s verified real-time RT-PCR WNV assay as the gold standard, the validity of the Pro-AmpRT WNV assay was determined by calculating sensitivity, specificity, and positive and negative predictive values. The degree of agreement between the 2 assays was measured by the Cohen’s kappa statistic (κ) (Watson and Petrie 2010). Kappa values are typically interpreted as follows: κ < 0.00 (poor agreement), 0.00 ≤ κ ≤ 0.20 (slight agreement), 0.21 ≤ κ ≤ 0.40 (fair agreement), 0.41 ≤ κ ≤ 0.60 (moderate agreement), 0.61 ≤ κ ≤ 0.80 (substantial agreement), and κ > 0.80 (almost perfect agreement).

RESULTS

The real-time RT-PCR and RT-LAMP results of the virus stock serial dilutions are presented in Table 1. Although the Genie II returned a “time to amplification” result for most of the positive pools, the manufacturer cautioned that a time result may not be displayed and recommended that the positive determination be derived from the presence of an annealing curve of ~86–88°C, regardless of the numerical value of the time result or whether a value appears at all. In addition, the “time to amplification” did not necessarily correspond to viral titers (i.e., shorter amplification times were not consistently congruent with higher titers, and vice versa; data not shown), and thus time to amplification for a sample could not be considered an indirect quantitative value. Therefore, we report the results as a qualitative positive or negative based on the presence or absence of a single annealing peak within the proper temperature range. For all 3 panels of WNV serial dilutions, the Pro-AmpRT WNV assay detected samples containing at least 2.3 log10 PFU/ml, corresponding to an RT-PCR CT value of 34.0 (Table 1). RT-LAMP samples run on the Bio-Rad CFX96 returned the same results as those run on the Genie II (data not shown).

The results from the ITI mosquito pools, nontarget viruses, and negative controls are presented in Table 2. None of the nontarget viruses produced positive results; likewise, no amplification was detected in the negative controls. The RT-LAMP detected ITI pools containing 2.4 log10 PFU/ml (CT = 32.5) in 3 of 3 replicates, and 2.3 log10 PFU/ml (CT = 32.4) in 1 of 3 replicates.

Of the 278 field-collected mosquito pools from Franklin County, 94 (33.8%) were positive for WNV by real-time RT-PCR, and 70 pools (25.2%) were positive by RT-LAMP. Sensitivity, specificity, predictive values, and Cohen’s kappa results are shown...
in Table 3. Overall, compared to RT-PCR, the RT-LAMP assay had a sensitivity of 74.7% (95% confidence interval [CI]: 64.43–82.91%) and a specificity of 100%. The RT-LAMP assay produced a positive predictive value (PPV) of 100%, in that all positive samples that were identified by RT-LAMP were true positives. The Cohen’s kappa statistic (κ) showed an overall agreement of 79.4% (95% CI: 71.7–87.1%) between the 2 tests. Qualitative (i.e., positive/negative) results of the RT-LAMP assay are plotted against their correlating real-time RT-PCR positive CT values in Fig. 1. Positive RT-LAMP samples corresponded to real-time RT-PCR CT values ranging from 20.3 to 36.0; however, the assays were in perfect agreement at CTs, 31 (κ = 1.0). Of the 24 samples that were real-time RT-PCR positive but RT-LAMP negative, 24 (100%) returned CT values > 31, including 23 (95.8%) with CT values > 32, and 19 (79.2%) with CT values > 33 (Fig. 1). Assay characteristics for samples that returned CTs ≤ 31 are also found in Table 3: notably, the RT-LAMP sensitivity dropped to 41.46% (95% CI: 26.32–57.89) and κ = 53.7% (95% CI: 38.1–69.2%).

**DISCUSSION**

In the absence of specific medical treatments or vaccines for WNV, personal protection measures such as the use of insect repellent and vector control constitute the most effective means of defense against human illness (Petersen 2019). The surveillance of WNV in mosquito pools is an essential component of a vector control program, as the detection of virus in the mosquito population combined with monitoring vector abundance has been repeatedly shown to be a reliable predictor of human WNV disease (Chung et al. 2013, DeFelice et al. 2017, Karki et al. 2020). The most effective control decisions are made when surveillance data are acquired in a timely fashion (DeFelice et al. 2019), which can be accomplished if the centralized testing facility (such as a state health department) is efficient in returning results, or if the vector control district conducts their own testing on site. Agencies that perform vector control vary in their testing capacities and access to resources, compelling the need for a diverse portfolio of virus screening options that balance the aptitudes of various agencies with useful and timely mosquito infection data. Sensitive real-time RT-PCR assays are idealized as the gold standard; however, they require sophisticated equipment and specially trained personnel to ensure valid results. Antigen-based assays are simpler and quicker to perform but have limited sensitivities compared to real-time RT-PCR, and the results can be difficult to interpret. The innovation of LAMP chemistry provides mosquito control districts and other public
health entities a new alternative for fast and efficient screening of mosquito pools for WNV in addition to well-established assays. LAMP assays have been shown to be highly specific due to multiple primers incorporated into the reaction, and tolerant to inhibitory substances as demonstrated in previous studies (Waters et al. 2014, Francois et al. 2011). The lack of thermal cycling facilitates rapid amplification of DNA template, and often the reaction time is completed within 30–60 min.

To characterize the performance of the Pro-AmpRT WNV assay, we conducted several evaluations. The laboratory evaluation using virus stocks determined the detection limit and specificity. To that end, the assay was extremely specific (100%), as it produced no false positives with negative control samples or nontarget viruses. Its limit of detection of \(~ 2.3 \text{ log}_{10} \text{PFU/ml}\) falls in between the sensitivity limits of real-time RT-PCR (\(~ 1 \text{ log}_{10} \text{PFU/ml}\); Lanciotti et al. 2000) and antigen-based assays such as RAMP (\(~ 4 \text{ log}_{10} \text{PFU/ml}\); Burkharter et al. 2014) and VectorTest (\(~ 5 \text{ log}_{10} \text{PFU/ml}\); Burkharter et al. 2006). We also tested mosquito samples created in the lab and collected from the field to determine whether the presence of mosquitoes would inhibit assay performance before applying the assay to a field evaluation. The laboratory-generated pools produced virus titers well within the limit of detection of the Pro-AmpRT WNV assay and were diluted to create samples of lower titers. The assay was reliable (i.e., all 3 replicates were positive) down to 2.4 \text{ log}_{10} \text{PFU/ml}, and samples were detectable with titers as low as 2.3 \text{ log}_{10} \text{PFU/ml} as observed in the limit of detection.

| Table 3. Sensitivity, specificity, predictive values, and Cohen’s kappa analysis results of the Pro-AmpRT West Nile Virus (WNV) real-time loop-mediated isothermal amplification (RT-LAMP) assay to detect WNV from field-collected mosquito pools \((n = 278)\) compared to the gold standard (real-time reverse-transcriptase polymerase chain reaction (RT-PCR)). Assay characteristics were also calculated for pools returning cycle threshold (CT) values \(>31\) \((n = 225)\).1

| Assay | Sensitivity (95% CI), % | Specificity, % | PPV, % | NPV, % | Cohen’s kappa, % |
|-------|-------------------------|----------------|--------|-------|-----------------|
| All samples | 74.7 (64.43–82.91) | 100 | 100 | 88.46 (84.44–91.55) | 79.4 (71.7–87.1) |
| Positive | 70 | 184 | 208 | 278 | 100 | 100 |
| Negative | 24 | 184 | 208 | 225 |

| CT \(>31\) | Sensitivity (95% CI), % | Specificity, % | PPV, % | NPV, % | Cohen’s kappa, % |
|------------|-------------------------|----------------|--------|-------|-----------------|
| Positive | 17 | 184 | 208 | 225 |
| Negative | 24 | 184 | 208 | 225 |

1 CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

Fig. 1. Qualitative results (positive/negative) of the Pro-AmpRT West Nile Virus (WNV) real-time loop-mediated isothermal amplification (RT-LAMP) assay are plotted against the cycle threshold (CT) results of the same samples tested by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Pro-AmpRT WNV assay samples that produced a single annealing peak between \(\sim 86 \text{ and } 88\) were considered positive; samples that did not produce an annealing peak were considered negative. Real-time RT-PCR samples that produced CT \(< 37\) were considered positive.
assessment. Finally, we evaluated the assay for its intended purpose. When used to screen field-collected pools for WNV, the overall agreement (79.4%) between real-time RT-PCR and the Pro-AmpRT WNV assay indicates substantial agreement (Watson and Petrie 2010). For samples with CTs of 31 or lower, the RT-LAMP assay correctly identified 100% of the positive and negative pools. Samples with CTs ≥ 31, even as high as 36.0, were also detected, albeit with less reliability. Taking into account only samples returning CT values higher than 31, the agreement between the assays was reduced to 53.7%, which corresponds to moderate agreement.

The Genie instruments are compact, lightweight, portable, and easy to operate. They are programmed in advance, facilitating efficient program loading for subsequent runs. The Genie II has a 16-sample capacity and 1 fluorescence channel; the Genie III has an 8-sample capacity and the ability to read 2 fluorophores. Although the LAMP assay finishes within ~35 min compared to 1–2.5 h real-time RT-PCR reaction times, agencies with high sample volumes may find their screening efficiency limited by the small sample capacities of the Genie instruments. Therefore, we tested the assay on a conventional real-time thermocycler with a 96-sample capacity. Unsurprisingly, we found that the assay performed the same on both machines. As no other modifications to the sample preparation or reaction mixture were needed, the Pro-AmpRT WNV kit is easily adapted to a high-capacity platform if necessary.

In this evaluation, we tested the Pro-AmpRT WNV RT-LAMP assay’s ability to detect WNV in mosquito pools and found the assay simple to perform, and the Genie II intuitive to operate. The Pro-AmpRT WNV assay shows promise as a suitable virus screening tool for vector surveillance programs. The Pro-AmpRT WNV assay indicates substantial agreement (79.4%) between real-time RT-PCR and the Pro-AmpRT WNV assay in a single kit. However, as the RT-LAMP assay output is qualitative, other methods would have to be employed if quantifiable results are desired. It should be noted that a reliable method of RNA extraction must be established, and that precise sample handling and best laboratory practices conducted by skilled operators are prescribed for this assay as they are for other nucleic acid amplification techniques such as RT-PCR.

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