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DEVELOPMENT AND VALIDATION OF A COLORIMETRIC REVERSE TRANSCRIPTASE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR DETECTION OF EASTERN EQUINE ENCEPHALITIS VIRUS IN MOSQUITOES

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ABSTRACT. Eastern equine encephalitis virus (EEEV) is a highly pathogenic alphavirus that causes periodic outbreaks in the eastern USA. Mosquito abatement programs are faced with various challenges with surveillance and control of EEEV and other mosquito-borne illnesses. Environmental sampling of mosquito populations can be technically complex. Here we report the identification of biomarkers, development and validation of a colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of EEEV. Positive samples are easily visualized by a color change from pink to yellow. The assay was validated using EEEV from viral culture, experimentally spiked mosquito pools, and previously tested mosquito pools. The RT-LAMP assay detected viral titers down to approximately 10% of what would be present in a single infectious mosquito, based upon EEEV viral titers determined by previous competency studies. The RT-LAMP assay efficiently detected EEEV in combined aliquots from previously homogenized pools of mosquitoes, allowing up to 250 individual mosquitoes to be tested in a single reaction. No false positive results were obtained from RNA prepared from negative mosquito pools acquired from known and potential EEEV vectors. The colorimetric RT-LAMP assay is highly accurate, technically simple, and does not require sophisticated equipment, making it a cost-effective alternative to real time reverse transcriptase-polymerase chain reaction (RT-PCR) for vector surveillance.

KEY WORDS Eastern equine encephalitis virus, mosquito, reverse transcription loop-mediated isothermal amplification assay, surveillance, vector

INTRODUCTION

Eastern equine encephalitis virus (EEEV) is the most pathogenic arbovirus in the Eastern USA. It is primarily found in the Gulf Coast, mid-Atlantic, and New England regions of the USA (Armstrong and Andreadis 2010, Soghigian et al. 2018). In recent years, EEEV has expanded its range into parts of Canada (Armstrong and Andreadis 2010, Rocheleau et al. 2017). With the expansion of the virus's geographic range across the northeastern USA and Canada, EEEV risk has increased during the primary transmission season from July to October in the northern regions (Sellers 1989, Barba et al. 2019, Ludwig et al. 2019). Phylogenetic studies have demonstrated that Florida may be providing an ecological niche for sustained wintertime circulation of EEEV, allowing for year-round transmission risk and potential reintroduction into the northeastern regions in the spring (Tan et al. 2018, Heberlein-Larson et al. 2019). The EEEV infection in humans can be asymptomatic, self-limiting, or in some cases cause neurological disease with a fatality rate in humans ranging from 30% to 70% (Smith et al. 2020). Neurological sequelae also occur in other mammals, including horses, resulting in a fatality rate of 80% to 90% (Scott and Weaver 1989, Ronca et al. 2016). While EEEV vaccinations are available for horses, there are currently no approved vaccines available for humans, making surveillance and vector control top priorities to prevent infection in humans (Honnold et al. 2015).

Eastern equine encephalitis virus (EEEV) is maintained in an enzootic cycle between the ornithophilic mosquito species *Culiseta melanura* Coquillett and passerine birds (Vander Kelen et al. 2012, Bingham et al. 2014, Burkett-Cadena et al. 2015, Molaei et al. 2015, Skaff et al. 2017, Blosser et al. 2017, Soghigian et al. 2018). Due to the mainly ornithophilic nature of *Cs. melanura*, arboviral monitoring warrants the use of sentinel chickens as a means of surveillance for EEEV activity in Florida (Komar et al. 1999; Armstrong and Andreadis 2010, 2013; Burkett-Cadena et al. 2015; Tabachnick 2016). However, in most other states where EEEV is endemic, sentinel chickens are not widely deployed, and surveillance of mosquitoes is the primary method used to gauge risk of infection (Tabachnick 2016).

Currently real time reverse transcription-polymerase chain reaction (real time RT-PCR) is the gold standard for EEEV surveillance in mosquitoes (Lambert et al. 2003). Real time RT-PCR requires an expensive real time thermocycler (Wheeler et al. 2016) and highly trained laboratory staff with the ability to interpret threshold (Ct) values and amplification curves (Gonçalves et al. 2019). Therefore, this technique is not suited for surveillance programs

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where laboratory resources are limited, e.g., county health departments and mosquito control districts.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) represents an attractive, technically less complex alternative to real time RT-PCR. The RT-LAMP test has been shown to be an effective method for detection of other mosquito-borne viruses (Wheeler et al. 2016, Lamb et al. 2018, da Silva et al. 2019, Xia et al. 2019). This technique detects the presence of a target gene sequence of a virus in a single-step reaction by using primers precisely designed to recognize distinct regions on the target gene and a DNA polymerase with strand displacement activity, which enables amplification of the target at constant temperature (Calvert et al. 2017, da Silva et al. 2019). Detection of RNA targets is accomplished by the addition of a reverse transcriptase to the LAMP reaction (Parida et al. 2006, 2007; da Silva et al. 2020). When the pH sensitive dye (phenol red) is included in the reaction, a positive sample is easily detected by a color change from pink to yellow, resulting from a pH decrease due to amplification of the target (Poole et al. 2017). The RT-LAMP test can be easily performed using a basic heating device, such as a hot water bath or a heat block, making it a cost-effective alternative arboviral diagnostic tool (da Silva et al. 2020). It is rapid and can be completed in approximately 1/3rd the time that is necessary to run real time RT-PCR (Poon et al. 2006). In addition, the sensitivity of RT-LAMP can sometimes exceed that of standard RT-PCR (Calvert et al. 2017).

Here we report a series of experiments describing the development and validation of a RT-LAMP assay for the detection of EEEV RNA in mosquitoes. The results indicate that RT-LAMP is a technically simple and cost-effective alternative to real time RT-PCR for the detection of EEEV in pools of vector mosquitoes. The method can be easily deployed by mosquito control programs, without the need to obtain expensive specialized equipment or technical expertise.

MATERIALS AND METHODS

Viral culture

All experiments involving EEEV and EEEV genomic material were carried out in a select agent certified biosafety level-3 (BSL-3) facility by select agent certified personnel at the University of South Florida. This facility has been approved to conduct experiments with EEEV under registration No. 20171201-1988 from the US Centers for Disease Control and Prevention (CDC). The EEEV strain M05-316 was used for the research; this isolate originated from a pool of *Cs. melanura* collected in Florida in 2005. The virus was cultured in ATCC® CCL-81 Monkey Kidney Vero cells (American Type Culture Collection, Manassas, VA) as previously described (Bingham et al. 2014). The viral titer was determined via plaque assay as previously described (Honnold et al. 2015). Molecular diagnostic assays involving the use of EEEV or EEEV genomic material were performed in BSL-3 conditions.

RNA extraction

Mosquito pools and individual experimentally infected mosquitoes (infected by feeding on EEEV inoculated baby chicks [Bingham et al. 2016]) were chosen from the Unnasch lab BSL-3 sample archive. Prior to storage, pools were mechanically homogenized in 1 ml biological field diluent (BFD; 90% minimum essential medium with Hank's salts, 10% fetal bovine serum, with antibiotics 200 U/ml penicillin, 200 µ/ml streptomycin, 2.5 µg/ml amphotericin B) using a Qiagen® TissueLyser II (Qiagen, Hilden, Germany) at 25 Hz for 4 min. Mosquito homogenates were subjected to centrifugation 14,000 $\times g$ for 1 min at room temperature. A total of 140 µl of the supernatant was used for RNA extraction, and RNA was purified using a Qiagen QIAamp® viral RNA mini kit, according to manufacturer's instructions. RNA was eluted into a final volume of 60 µl of elution buffer, and RNA samples were stored at –80°C.

Synthetic RNA synthesis

Synthetic EEEV RNA positive controls for the EEEV RT-LAMP assay were generated via in vitro transcription. Briefly, gBlock DNA fragments containing a T7 promoter sequence followed by a distinct region of the EEEV genome were synthesized (Integrated DNA Technologies™, Coralville, IA) and amplified using Q5® high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) following manufacturer's instructions. In vitro transcription of the gBlock PCR product to produce synthetic RNA template was performed using the NEB HiScribe™ T7 quick high yield RNA synthesis kit (NEB E2050) following the manufacturer's protocol for standard RNA synthesis. Synthesized RNA was purified using the NEB Monarch® RNA cleanup kit (T2040) and quantified using a Nanodrop. High concentration stock solutions were then aliquoted and stored at –80°C to prevent multiple freeze–thaws. Each of the synthetic RNAs were serially diluted in nuclease free water or in 1 ng/µl of Hela RNA when concentration of the RNA template was lower than 1 ng/µl.

LAMP primer design

The EEEV genome is highly conserved with an average nucleotide similarity of 99.2% (Yi et al. 2018). Three genes (6K, E1, and nsP3) specific for EEEV were selected based on either their previous use in real time RT-PCR or due to their subgenomic high copy number. Due to biosafety constraints, synthetic RNAs corresponding to each of the 3 target genes were generated and used for assay develop-

ment and optimization. The LAMP primer sets targeting the genes encoding the structural proteins 6K and E1 and the nonstructural protein nsP3 were designed using the PrimerExplorer V5 (http://primerexplorer.jp/e/) software or the NEB Primer Design Tool (https://lamp.neb.com/). Each primer set includes an outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward primer (LF), and loop backward primer (LB). Primers were synthesized by Integrated DNA Technologies.

Colorimetric RT-LAMP assay

In the final optimized assay, each 20 µl reaction contained 10 µl NEB WarmStart® colorimetric LAMP 2× master mix with uracil-DNA glycosylase, 2 µl 10× primer mix (F3/B3 2 µM each; FIP/BIP 16 µM each; LF/LB 4 µM each), 2 µl 10× guanidine hydrochloride (400 mM), 2–4 µl synthetic RNA (6K, E1, or nsP3) or RNA sample, and DNase/RNase free water. Reactions were assembled on ice followed by an incubation at 65°C for 30 min using either a thermocycler or a Fisher Scientific™ IsoTemp® Digital Dry Bath (Fisher Scientific International, Inc., Waltham, MA). Samples were considered positive for the presence of EEEV if a color change from pink to yellow was observed, while negative samples remained pink. During assay development and optimization, 1 µM of SYTO™ 9 green fluorescent nucleic acid stain (Invitrogen, Waltham, MA) was also included in the LAMP reaction to enable reaction dynamics to be monitored in real time using a qPCR machine (CFX-96 Touch Thermal Cycler, Bio-Rad Laboratories, Hercules, CA). This will generate a Ct value and provide a quantitative measure. Each Ct unit is equivalent to 22 sec of incubation time and can be used to evaluate the speed of the amplification reaction. The lower Ct value corresponds to a faster amplification reaction. Experiments were performed using at least 2 replicates (examples provided in supplemental materials).

Real time RT-PCR

Real time RT-PCR was performed using iTaq™ universal probes one-step kit (Bio-Rad®) with EEEV set A primers and probes as previously described (Lambert et al. 2003). At the start of each assay, a master mix was prepared that contained all of the ingredients except the RNA. Sufficient master mix was prepared to perform the number of reactions for the entire experiment, plus 10% extra to compensate for loss during pipetting (each 20 µl reaction contained 5.25 µl H_2O, 10 µl 2× real time reaction mix, 0.5 µl enzyme mix, 0.075 µl 100 µM forward primer, 0.075 µl 100 µM reverse primer, 0.1 µl 25 µM probe). A total of 16 µl of this master mix was then aliquoted into each individual reaction tube and the sample RNA added (4 µl RNA sample). Real time RT-PCR was performed on a Qiagen Rotor-Gene Q™ monitoring SYBR Green fluorescence on the Fluorescein amidites channel. Cycling conditions consisted of a reverse transcription step at 50°C for 10 min, initial denaturation at 95°C for 5 min, and then 45 cycles of 95°C for 15 sec and 60°C for 30 sec. All samples were run in a confirmatory real time RT-PCR using the EEEV primer/probe set B as previously described (Lambert et al. 2003). Results were reported as Ct values with a cut off at 35 cycles. Experiments were performed using at least 2 replicates.

RESULTS

Identification of biomarkers and RT-LAMP assay development

Three genes (6K, E1, and nsP3) specific for EEEV were selected, and LAMP primers were designed corresponding to each biomarker (Fig. 1A–C). To optimize the RT-LAMP assays, different reaction temperatures as well as the impact of adding guanidine hydrochloride (GuHCl) were evaluated using 1 pg of in vitro transcribed RNA fragment as template. GuHCl has been shown to improve both the speed and sensitivity of a SARS-CoV-2 RT-LAMP assay (Zhang et al. 2020). Each LAMP reaction containing its corresponding RNA template showed a color change from pink (before amplification) to yellow (after the amplification), while the non-template control remained pink (supplemental Fig. 1).

Analytical sensitivity of RT-LAMP

To evaluate the sensitivity of RT-LAMP using each biomarker, synthetic RNA fragments corresponding to biomarkers 6K, nsP3, and E1 were serially diluted in Hela RNA (1 ng/µl) and tested from 0.1 pg/µl down to 0.1 ag/µl. Positive results were evident by a color change from pink to yellow, and Ct values correlated with the amount of template RNA in each reaction. All 3 assays consistently showed a high level of sensitivity and 100% agreement in results obtained from colorimetric and Ct values (supplemental Fig. 2).

Comparison of colorimetric RT-LAMP and real time RT-PCR assays for detection of eastern equine encephalitis virus

To evaluate and validate the performance of each RT-LAMP assay for detection of EEEV, RNA extracted from serially diluted viral stocks with titers ranging from 0.1 PFU/ml to 100,000 PFU/ml were tested in both RT-LAMP and real time RT-PCR. A standard curve of PFU versus Ct value demonstrated that the real time RT-PCR assay was able to detect EEEV down to 1 PFU/ml of media with a cutoff of 35 cycles (Fig. 2). In the colorimetric RT-LAMP, the E1 LAMP primer set also detected EEEV at 1 PFU/ml, the lowest concentration tested using RT-qPCR

A

PCR Primer	Sequence (5' – 3')
6K_gBlock_F	AAGTACTAATACGACTCAC
6K_gBlock_R	TGTGTTCGTACGCTGCGGCG

Primer	Sequence (5' – 3')
6K_F3	GGCTCCTTTGCAGGACTC
6K_B3	CGATAAGCGCTGCAAGTGG
6K_FIP (F1c-F2)	ACGCCAGGAGTATTGGGACTTG-GCAATCTTTGCATAACCCCG
6K_BIP (B1c-B2)	GAGGGCAGACGACACCTTGC-GATAAGCGTCTGCATCCAGA
6K_LF	AGCGTTCGGGGCTAGTTTAT
6K_LB	GTGCTGAATTATCTGTGGAACAACA

B

PCR Primer	Sequence (5' – 3')
NSP3_gBlock_F	TTCCAGGTAATACGACTCAC
NSP3_gBlock_R	CACCTGGCATTTACGCGGCTTT

LAMP Primer	Sequence (5' – 3')
NSP3_F3	CGACAATGGTTGTCGAAGCA
NSP3_B3	TGCAACGCTGTCAAACGT
NSP3_FIP (F1c-F2)	CACGGGTTTCAGATCCGGTGAT-GAGATTCACGCCAGTCAAGG
NSP3_BIP (B1c-B2)	CTCCGTCACCTCCAAGTCAGGA-CACGGACGTGTGTGAACC
NSP3_LF	CTGGGTATGCTCCACAGTGAT
NSP3_LB	CCTTCCACCCCATCTGCA

C

PCR Primer	Sequence (5' – 3')
E1_gBlock_F	AACCTAATACGACTCACTATAG
E1_gBlock_R	ACCTGTAGTGTAATTGTATATAC

Primer	Sequence (5' – 3')
E1_F3	CTGTCTTACTAAACACTAAATTCAC
E1_B3	CCTGTAGTGTAATTGTATATACGG
E1_FIP (F1c-F2)	GCAGCCTTATGCACTGCATCCCTAGTTCGATGTACTTCCG
E1_BIP (B1c-B2)	ACCAAATTATAACACTAAGGGCAGTTTATGCCTGATTATAAAGTGTGT
E1_LF	GCACCACCGTCACCATA
E1_LB	GCATAATGCTGCTCCTAAGTAA

Fig. 1. Primer sets targeting the 6K, nsP3, and E1 genes of the eastern equine encephalitis virus. (A) 6K, (B) nsP3, and (C) E1.

assay (Fig. 3). The nsP3 primer set detected 10 PFU/ml, whereas the 6K primer set detected a viral concentration of 1,000 PFU/ml consistently and 100 PFU/ml in 50% of the samples (Fig. 3).

Detection of eastern equine encephalitis virus in mosquitoes using RT-LAMP

To investigate the limit of detection of RT-LAMP when handling mosquito pools for EEEV surveillance, each of 9 real time RT-PCR negative sample homogenates of *Cs. melanura* were individually spiked with 10-fold serial dilutions of EEEV from culture and extracted RNA were tested in RT-LAMP. The E1 primer set detected EEEV at viral loads down to 100 PFU/ml in 9/9 (100%) of the biological replicates and detected a concentration of 10 PFU/ml in 3/9 (33%) of the replicates (Fig. 4). A similar trend was observed using the nsP3 primer set, with a small number of samples scoring positive at 1 PFU/ml. These results indicated that both E1 and nsP3 primer sets appeared to demonstrate a higher level of analytical sensitivity when used to detect EEEV in mosquito homogenates than the 6K primer set. The 6K-based test detected EEEV in the homogenates at 1,000 PFU/ml in 9/9 (100%) of the trials, with the

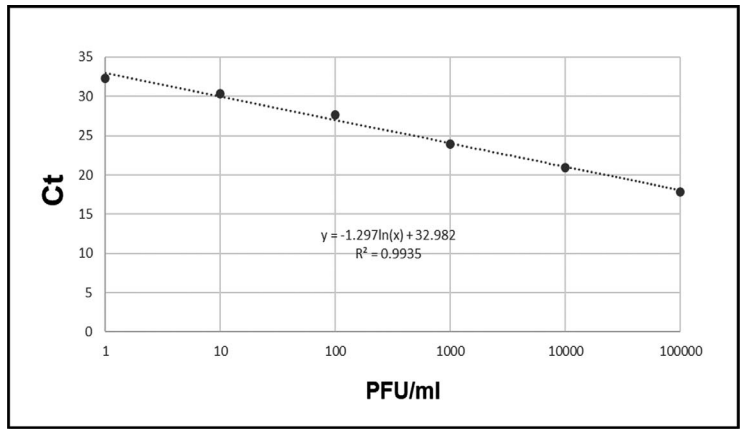

Fig. 2. Analytical sensitivity of real time RT-PCR for EEEV. EEEV was cultured, titered and RNA extracted from serial dilutions as described in the "Materials and Methods" section. Real time RT-PCR was carried out on the resulting RNA preparations as described by Lambert et al. (2003).

sensitivity decreasing at lower concentrations (Fig. 4).

The sensitivity of EEEV RT-LAMP was also evaluated using a collection of real time RT-PCR positive homogenates from field acquired and experimentally infected mosquito pools (Table 1); 1 EEEV positive field-caught mosquito pool of the major vector *Cs. melanura* ($n = 4$); and 10 experimentally infected *Culex erraticus* Dyar and Knab, a less competent vector for EEEV (Bingham et al. 2016). In general, EEEV RT-LAMP detected EEEV in the real time RT-PCR positive mosquitoes when the Ct values were below 30. The only exception to this was a single instance where the nsP3 primer set was able to detect a positive pool with a Ct value of 34.38 (Table 1).

To evaluate the analytical limit of detection in a larger collection of mosquitoes, 30 µl of a homogenate prepared from a *Cs. melanura* pool (pool H-442 containing 4 individuals) that was previously found to be EEEV positive via real time RT-PCR was diluted 1:5 in BFD. The diluted sample was combined with 30 µl from each of 4 EEEV negative pooled homogenates prepared from pools of *Cs. melanura*. The final homogenate mixture consisted of aliquots from 5 pools of mosquitoes, which together contained a total of 178 mosquitoes. Similarly, 30 µl aliquots of homogenates from 4 EEEV negative pools of *Cx. erraticus* (a potential bridge vector) each containing 50 individuals were combined with a 30 µl aliquot of EEEV positive *Cs. melanura* pool H-442. The combination of the 5 aliquots thus represented portions of 204 individual mosquitoes. Finally, 30 µl aliquots of homogenates from 4 EEEV negative *Cx. erraticus* pools ($n = 50$ in each pool) were combined with 30 µl of a homogenate prepared from a single experimentally infected EEEV positive *Cx. erraticus* that had a real time RT-PCR Ct value similar to that of *Cs. melanura* pool H-442. This mix thus contained aliquots of homogenates from 201 individual mosquitoes in the final pooled sample. RNA was extracted from the homogenate mixtures and tested in EEEV RT-LAMP (Table 2). Two mixtures containing an aliquot of the mixture of the homogenates from the positive field isolate pool H-442 of *Cs. melanura* and the homogenates from 4 pools of *Cs. melanura* that were negative for EEEV by real time RT-PCR were positive in all of the EEEV RT-LAMP reactions. Similarly, the mixture of the positive *Cs. melanura* field isolate pool H-442 homogenate and homogenates from 4 pools of *Cx. erraticus* found to be negative by real time RT-PCR were positive in all EEEV RT-LAMP reactions. Finally, the mixture containing the homogenate from the single experimentally infected *Cx. erraticus* combined with homogenates from 4 *Cx. erraticus* negative pools was also positive in all RT-LAMP assays. Taken together, these results indicate that EEEV RT-LAMP is highly sensitive and can be used for accurate pathogen detection in individual and pools of highly competent and less competent vector species.

Culiseta melanura is considered the major enzootic vector for EEEV; however, several other mosquito species can serve as alternate or bridge vectors for the virus (Armstrong and Andreadis 2010, Bingham et al. 2014, Burkett-Cadena et al. 2015, Bingham et al. 2016, Oliver et al. 2018). While no false positive results were obtained when using *Cs. melanura* or *Cx. erraticus* lysates, it was of interest to determine whether homogenates from other mosquito species might generate false positive results in the EEEV RT-LAMP assays. The RNA was purified from homogenates of real time RT-PCR negative pools comprising of 1–50 field-caught mosquitoes and tested in RT-LAMP. None of the pools of the other EEEV real time RT-PCR negative species tested were found to be positive in the LAMP assays (Table 3).

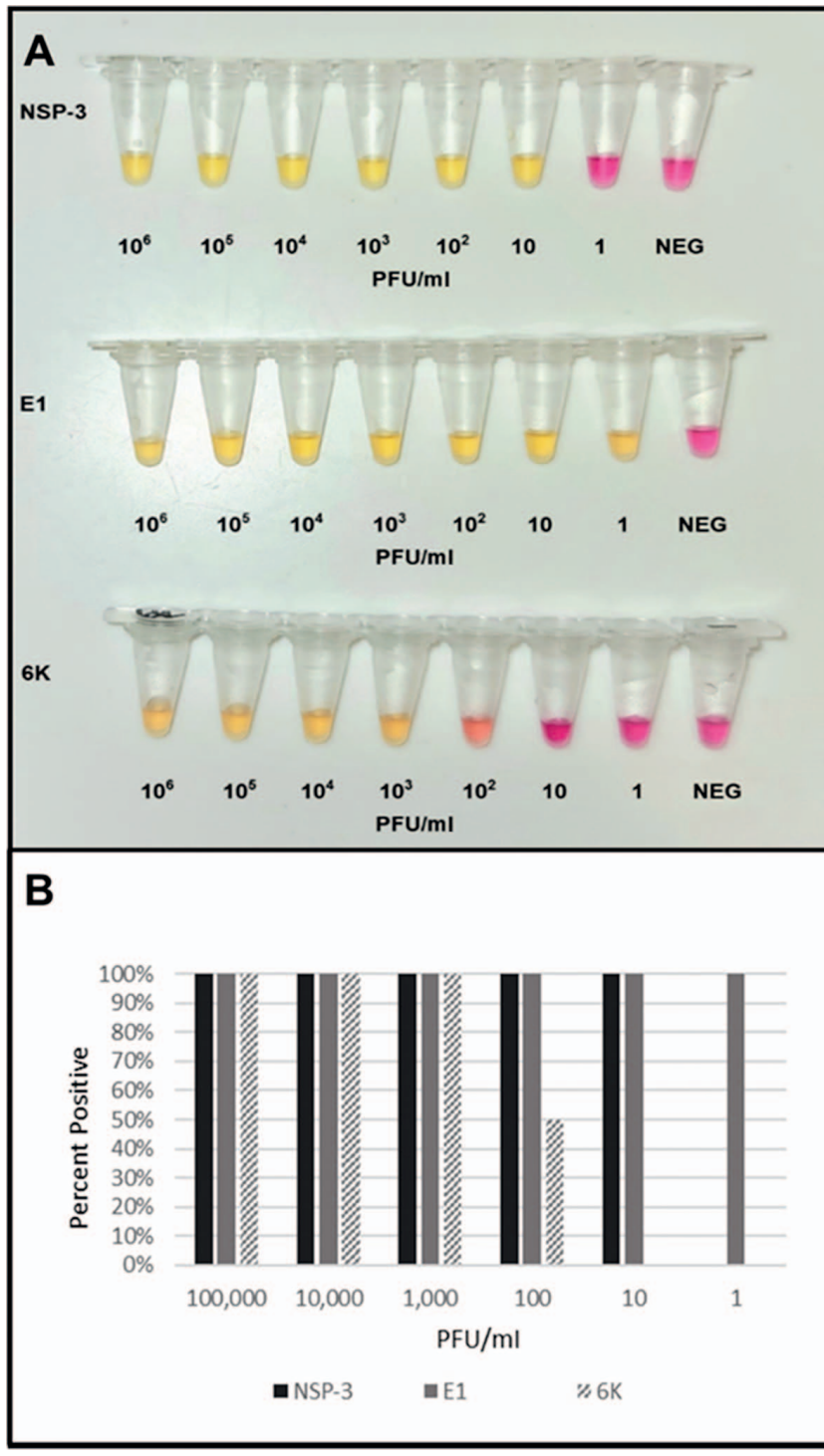

Fig. 3. Analytical sensitivity of RT-LAMP for EEEV. (A) Colorimetric RT-LAMP assays targeting nsP3, E1, or 6K, were performed using serially diluted viral RNA (PFU/ml) as a template. A color change from pink to yellow indicates a positive result. (B) The percentage of samples which tested positive at each serial dilution (PFU/ml) of viral RNA using LAMP primer sets for nsP3 (black bar), E1 (gray bar), or 6K (hatched bar) are shown (calculation based on biological replicates of each PFU concentration run in duplicate with each primer set).

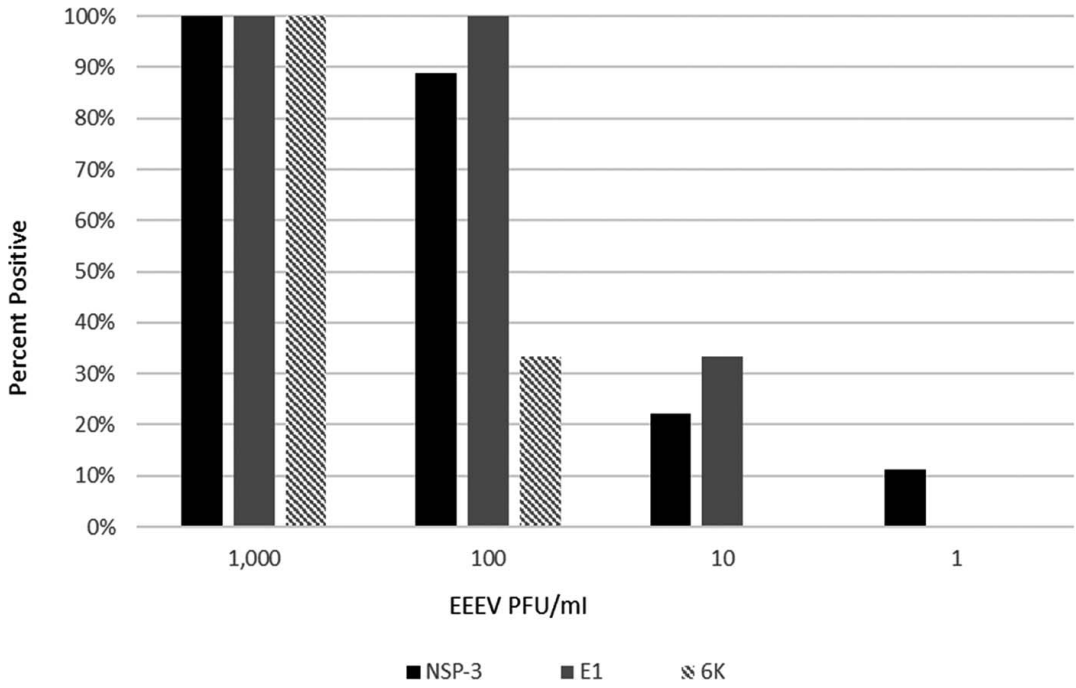

Fig. 4. Performance of RT-LAMP assays using RNA from EEEV-spiked mosquito homogenates. The 3 primer sets (nsP-3, E1, and 6K) tested against RNA prepared from mosquito pools spiked with EEEV. Bars summarize the results of 9 biological replicates for each PFU concentration, tested in duplicate with each primer set.

DISCUSSION

Real time RT-PCR is currently the gold standard for screening mosquito pools for the presence of EEEV (Lambert et al. 2003, Oliver et al. 2018). However, EEEV real time RT-PCR presents some obstacles for routine implementation by a mosquito control district. First, real time RT-PCR is a difficult technique to implement outside of a well-equipped laboratory. The method requires relatively sophisticated equipment (a real time thermocycler) and substantial technical skill. Another limitation of the test until recent years has been the need to use EEEV RNA as a positive control. Although synthetic EEEV RNA is commercially available through ATCC (ATCC 2021), it runs approximately $10 per test (per plate of reactions run in real time RT-PCR) (ATCC 2021). Under BSL-3 conditions, both EEEV and its genomic RNA can be produced in a cost-effective manner, but they are tightly regulated as select agents and require BSL3 laboratory containment according to current US government regulations. The RT-LAMP method described overcomes these obstacles: the reaction can be performed using simple equipment such as a water bath and synthetic RNA that is economical to produce, which is not

Table 1. Reverse transcription-LAMP assay for the detection of eastern equine encephalitis virus in field acquired and experimentally infected mosquitoes.

Sample ID	Species (pool size)	Real time RT-PCR (Ct)	NSP-3 LAMP	E1 LAMP	6K LAMP
H442 (field specimen)	*Culiseta melanura* (4)	21.45	**Positive**[1]	**Positive**	**Positive**
MOS42 (exp. infected)	*Cx. erraticus* (1)	27.20	**Positive**	**Positive**	**Positive**
MOS43 (exp. infected)	*Cx. erraticus* (1)	29.76	**Positive**	**Positive**	**Positive**
MOS44 (exp. infected)	*Cx. erraticus* (1)	35.13	Negative	Negative	Negative
MOS45 (exp. infected)	*Cx. erraticus* (1)	32.62	Negative	Negative	Negative
MOS46 (exp. infected)	*Cx. erraticus* (1)	26.36	**Positive**	**Positive**	**Positive**
MOS47 (exp. infected)	*Cx. erraticus* (1)	35.05	Negative	Negative	Negative
MOS48 (exp. infected)	*Cx. erraticus* (1)	33.63	Negative	Negative	Negative
MOS49 (exp. infected)	*Cx. erraticus* (1)	35.22	Negative	Negative	Negative
MOS50 (exp. infected)	*Cx. erraticus* (1)	34.38	**Positive**	Negative	Negative
MOS51 (exp. infected)	*Cx. erraticus* (1)	22.77	**Positive**	**Positive**	**Positive**

[1] Boldface indicates EEEV LAMP positive samples (all samples were RT-PCR positive for EEEV).

Table 2. LAMP assay for eastern equine encephalitis virus in combined pools of *Culiseta melanura*.

EXP	Negative Sample 1	Negative sample 2	Negative sample 3	Negative sample 4	Positive sample	Real time RT-PCR Ct	NSP-3	E1	6K
1	H329 *Cs. melanura* ($n = 6$)	H257 *Cs. melanura* ($n = 13$)	H229 *Cs. melanura* ($n = 10$)	H410 *Cs. melanura* ($n = 17$)	*Cs. melanura* ($n = 4$) Ct = 21.45	23.59	Positive	Positive	Positive
2	SW167 *Cs. melanura* ($n = 50$)	SW169 *Cs. melanura* ($n = 40$)	SW170 *Cs. melanura* ($n = 50$)	SW171 *Cs. melanura* ($n = 34$)	*Cs. melanura* ($n = 4$) Ct = 21.45	23.91	Positive	Positive	Positive
3	H19503 *Cx. erraticus* ($n = 50$)	H19504 *Cx. erraticus* ($n = 50$)	H19523 *Cx. erraticus* ($n = 50$)	H19535 *Cx. erraticus* ($n = 50$)	*Cs. melanura* ($n = 4$) Ct = 21.45	24.12	Positive	Positive	Positive
4	H19503 *Cx. erraticus* ($n = 50$)	H19504 *Cx. erraticus* ($n = 50$)	H19523 *Cx. erraticus* ($n = 50$)	H19535 *Cx. erraticus* ($n = 50$)	*Cx. erraticus* ($n = 1$) Ct = 22.77	25.03	Positive	Positive	Positive

subject to select agent regulations and can be generated at scale to serve as a low-cost positive control. Thus, EEEV RT-LAMP may be an attractive and more accessible alternative to real time RT-PCR for use in mosquito control districts for EEEV surveillance.

Most viral LAMP assays employ primer sets that target at least 2 distinct regions of the pathogen's genome, with the goal of improving the specificity and sensitivity of the assay (Liu et al. 2012, Calvert et al. 2017, Lopez-Jimena et al. 2018). This also offers assurance and continuity in assay performance if a mutation occurs in a genomic region corresponding to where 1 particular primer set is targeted. In the present study, 3 highly conserved EEEV biomarkers targeting different regions of the genome were used. Primer sets were designed, and assay conditions were optimized for each biomarker using the appropriate synthetic RNA template. During assay development and optimization, both real time detection, which generates a quantitative (Ct) value, and an end point color change (from pink to yellow) were used as a readout. All 3 primer sets consistently showed a high level of sensitivity, with a limit of detection of 0.02 fg of RNA for the 6K primer set, while nsP3 and E1 based RT-LAMP detected as low as 2 ag target RNA in a 20 µl reaction. There was 100% agreement in results obtained from real time detection and a color change visible by eye in the EEEV RT-LAMP assays, regardless of the primer set/target used. Although real time monitoring of LAMP reactions using turbidity or fluorescent dyes is common and provides a semiquantitative result, a simple visual colorimetric readout is more suited to field studies or low resource settings (Poole et al. 2012, 2015, 2019).

When using EEEV RNA purified from cultures of the virus, the E1 primer set exhibited an analytical limit of detection equivalent to real time RT-PCR (1 PFU/ml), while nsP3 and 6K reactions were 1 and 2 logs lower (10 PFU/ml and 100 PFU/ml, respectively). Not surprisingly, some loss in sensitivity was observed using mosquito homogenates spiked with virus. Substances present in biological samples are known to interfere with nucleic acid amplification; however, in general, LAMP has a greater tolerance compared with PCR to polymerase inhibitors, including those present in insects (Alhassan et al. 2014). Increasing the volume of RNA in each RT-LAMP reaction by 2- or 2.5-fold, while keeping the concentrations of all the reagents the same, resulted in a minor improvement in signal detection when using the 6K LAMP primer set but not for the nsP3 or E1 primer sets (data not shown). Future efforts to optimize these assays may help to improve the sensitivity of this reaction.

The analytical sensitivity of the EEEV RT-LAMP method is more than sufficient to detect mosquitoes infected with and capable of transmitting EEEV. One EEEV infectious *Cs. melanura* contains approximately a million virus particles on average (Scott and Weaver 1989, Komar et al. 1999). As mosquito pools

Table 3. Specificity of EEEV-LAMP assays evaluated against various mosquito species.

Sample ID	Species (pool size)	Real time RT-PCR Ct	nsP-3 LAMP	E1 LAMP	6K LAMP
H 264 RNA	*Anopheles crucians* (1)	Negative	Negative	Negative	Negative
H 279 RNA	*An. crucians* (2)	Negative	Negative	Negative	Negative
H 251 RNA	*An. crucians* (4)	Negative	Negative	Negative	Negative
H 242 RNA	*An. inundatus* (1)	Negative	Negative	Negative	Negative
H 263 RNA	*An. inundatus* (7)	Negative	Negative	Negative	Negative
H 230 RNA	*An. perplexens* (1)	Negative	Negative	Negative	Negative
H 270 RNA	*Culiseta melanura* (6)	Negative	Negative	Negative	Negative
H 238 RNA	*Cs. melanura* (8)	Negative	Negative	Negative	Negative
H 302 RNA	*Cs. melanura* (8)	Negative	Negative	Negative	Negative
H 232 RNA	*Culex erraticus* (1)	Negative	Negative	Negative	Negative
H 239 RNA	*Cx. erraticus* (1)	Negative	Negative	Negative	Negative
H 281 RNA	*Cx. erraticus* (4)	Negative	Negative	Negative	Negative
H 304 RNA	*Cx. erraticus* (5)	Negative	Negative	Negative	Negative
H 250 RNA	*Cx. erraticus* (8)	Negative	Negative	Negative	Negative
H 233 RNA	*Cx. nigripalpus* (1)	Negative	Negative	Negative	Negative
H 303 RNA	*Cx. nigripalpus* (1)	Negative	Negative	Negative	Negative
H 299 RNA	*Cx. quinquefasciatus* (1)	Negative	Negative	Negative	Negative
H 260-B RNA	*Cx. quinquefasciatus* (11)	Negative	Negative	Negative	Negative
H 260-A RNA	*Cx. quinquefasciatus* (50)	Negative	Negative	Negative	Negative
H 276 RNA	*Cx. salinarius* (3)	Negative	Negative	Negative	Negative
H 289 RNA	*Cx. salinarius* (5)	Negative	Negative	Negative	Negative
H 291 RNA	*Cx. salinarius* (14)	Negative	Negative	Negative	Negative

for both real time RT-PCR and RT-LAMP analyses are homogenized in 1 ml of buffer, a single infectious *Cs. melanura* mosquito would therefore produce a homogenate with a viral concentration of 10^6 PFU/ml. All 3 RT-LAMP assays targeting E1, nsP-3, or 6K detected virus in mosquito homogenates spiked with 100 PFU/ml and were capable of detecting the naturally infected *Cs. melanura* pool. Thus, the RT-LAMP should detect infected mosquitoes capable of transmitting EEEV. In the situation where a mosquito had recently taken an infected blood meal and the virus has not had time to disseminate and replicate, the sample may not test positive. The EEEV is infectious to feeding mosquitoes at a concentration of 10^5 PFU/ml in the blood of an infectious host (Komar et al. 1999). Assuming a typical blood meal volume is 3–5 µl (Komar et al. 1999), this would correspond to a total viral load in the blood meal of 300–500 PFU. Given that the E1 and nsP3 assays can detect EEEV at 100 PFU/ml (Fig. 4), mosquitoes with an infected bloodmeal may give a positive result in the LAMP assays, even if the virus does not disseminate and multiply in the mosquito. In this situation, an EEEV positive result would provide evidence that would indicate EEEV activity in the area, without necessarily indicating active transmission.

The RT-LAMP also detected EEEV infection in a subset of experimentally infected *Cx. erraticus* (Bingham et al. 2016). This species is not a highly competent vector for EEEV, with only 10% of the experimentally infected *Cx. erraticus* developing viral titers similar to those seen in *Cs. melanura* (Bingham et al. 2016). However, the LAMP assay was capable of detecting EEEV in any individual experimentally infected *Cx. erraticus* that produced a Ct value of less than 30 in the real time RT-PCR assay. This suggests that the EEEV LAMP assay will detect virus in bridge vectors in which the virus has been able to replicate. Given that the RT-LAMP assays did not give false positive results in many different mosquito species known or suspected to be bridge vectors of EEEV, these results when taken together, suggest that the EEEV LAMP assay will be able to detect EEEV activity in bridge vectors as well as in *Cs. melanura*, the major enzootic vector.

Surveillance of pooled mosquitoes for EEEV via real time RT-PCR is generally performed on pools containing a maximum of 50 mosquitoes per sample. Initially the RT-LAMP assays were also performed by limiting the pool size to 50 individuals for this reason. However, since RT-LAMP consistently detected samples that were EEEV positive in real time RT-PCR with Ct values of 30 or less, we explored combining aliquots of homogenates of pools as a way to increase the number of mosquitoes that could be screened in a single test. These "pools of pools" remained consistently positive when a homogenate from 1 positive pool was combined with homogenates from 4 negative pools. Therefore, combining aliquots of homogenates from up to 5 pools (representing 200 individual mosquitoes or more) would be an effective way for mosquito control districts to screen large numbers of mosquitoes. From a practical standpoint, combining aliquots from homogenates of pools of a maximum of 50 individuals was found to be easier than preparing homogenates of pools containing larger numbers of individual mosquitoes, due to the difficulty in completely homogenizing all the material in pools with a large number of mosquitoes. Furthermore, by

combining aliquots of individual homogenates, it will be possible to return to the reserved individual homogenates and screen them separately, if a more granular picture of EEEV transmission is desired.

Though there is accessibility to synthetic EEEV controls for use in real time RT-PCR, RT-LAMP has shown clear advantages due to its operational simplicity, rapid results, and cost. This RT-LAMP uses a basic heating instrument (e.g., a hot block or a water bath) and employs easy-to-use reagents and basic workflow. In contrast, the real time RT-PCR requires a real time thermocycler, which is considerably more expensive than a hot block or water bath. Furthermore, positive results in the RT-LAMP assay are demonstrated by a color change that is easily detected by eye, requiring no instrumentation. The colorimetric RT-LAMP should produce a clear color change from pink to yellow to indicate an EEEV positive, with any shade of pink denoting a negative result. These experiments did demonstrate a few instances where the color did not change completely to yellow in the 6K assay within 30 min. We would recommend that a sample be scored as negative unless there is a clear change to yellow. Therefore, nsP3 or E1 assays which provided a solid color change and the highest levels of sensitivity can be used as a primary screening assay. It should then be possible to use another one of the primer sets in an independent confirmatory assay, as each of the primer sets targets different portions of the EEEV genome. Such sequential use of the 2 assays would result in the most efficient, cost-effective and accurate approach to routine screening of mosquito pools for EEEV.

The reagent costs of the EEEV LAMP and EEEV real time RT-PCR are roughly comparable. However, the major cost for both assays resides in the cost of the kits needed to produce purified RNA for these assays. It is possible that this step could be eliminated, since colorimetric RT-LAMP has been used to detect Zika virus in crude mosquito homogenates (Bhadra et al. 2018, da Silva et al. 2019). Alternatively, it may be possible to use technically simpler and less expensive methods to purify RNA for use in the EEEV LAMP assays. For example, paramagnetic bead purification is relatively straightforward, inexpensive, can be performed without the use of toxic organic solvents and may be designed to specifically purify viral RNA (Tavares et al. 2011).

Colorimetric RT-LAMP does not confer the quantitative accuracy of real time RT-PCR, and the LAMP assay cannot replace real time RT-PCR in all circumstances. However, the data presented above suggest that the colorimetric EEEV RT-LAMP tests are rapid and appear highly accurate and simple to perform. They do not require specialized equipment or extensive technical expertise and may represent an attractive alternative to real time RT-PCR for the detection of EEEV that can be used by mosquito control programs that are not equipped to perform real time RT-PCR on a routine basis.

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