Comparison of RNA Extraction Methods for Detection of Virus in Mosquitoes

Eric Haas-Stapleton[1], Dereje Alemayehu[1], Warren Macdonald[2], and Nayer Zahiri[2]

[1]*Alameda County Mosquito Abatement District, 23187 Connecticut Street, Hayward, CA 94545*
[2]*San Mateo County Mosquito and Vector Control District, 1351 Rollins Road, Burlingame, CA 94010*
Please direct correspondences to Eric.Haas@mosquitoes.org; (510) 783-7744

ABSTRACT: The isolation of viral RNA from mosquitoes and its analysis using quantitative reverse transcription polymerase chain reaction (qRT-PCR) is becoming increasingly important for arbovirus surveillance and evaluating risk reduction practices. We evaluated the quantity and quality of West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), and Western equine encephalitis virus (WEEV) RNA that was isolated in the presence of adult mosquitoes using two commercial kits (RNeasy Mini Kit columns (Qiagen) and MagMAX Viral RNA Isolation Kit (Thermo Fisher Scientific)). The RNA that was eluted from the MagMAX Kit contained a brown-colored precipitate that increased with the number of mosquitoes that were included in the sample, as measured by the absorbance at 525 nm. Clarification of RNA eluted with the MagMAX Kit using centrifugation reduced the absorbance of the RNA solution relative to the unclarified samples, with the greatest reductions of 5- and 8-fold for samples containing 25 or 50 mosquitoes, respectively. However, there was no significant difference in the concentration of RNA that was detected before and after clarification. The clarified samples produced significantly lower cycle threshold (Ct) values when the RNA was assayed for WNV, SLEV and WEEV RNA using qRT-PCR. The Ct values generated from samples isolated using the MagMAX Kit were significantly lower than those of samples isolated with RNeasy Mini Kit columns (Ct difference of 1.01 +/- 0.28, range of 0.542 – 1.882), suggesting that MagMAX method may be more sensitive for use in arbovirus surveillance by vector control agencies.

INTRODUCTION

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) is employed to analyze low quantities viral RNA that is isolated from mosquitoes for estimating the prevalence of arboviruses and intensity of infection in mosquitoes (Brault et al. 2015). Two solid phase extraction technologies predominate for isolating RNA: silica gel membranes (GM) or silica conjugated to magnetic particles (MP). Both technologies employ relatively simple methods that rapidly yield nucleic acid for use in qRT-PCR assays. Comparison of these technologies showed that when RNA was isolated from West Nile virus (WNV)-infected mosquitoes using MP and analyzed using qRT-PCR, lower cycle threshold (Ct) values were produced relative to duplicate samples isolated using GM (Fang et al. 2010), demonstrating that MP is more sensitive than GM. However, the effect of mosquito number in a sample, and the relative quantity or quality of the isolated RNA has not been evaluated in a study that compares GM- and MP-based methods. Herein, we compared arbovirus RNA isolated in the presence of multiple numbers of adult mosquitoes per sample (0, 1, 5, 10, 25, or 50) using GM from the RNeasy Mini Kit (Qiagen) to the RNA eluted from MP in the MagMAX Viral RNA Isolation Kit (Thermo Fisher Scientific). The quantity and purity of the isolated RNA was evaluated, as was its suitability for use in multiplex TaqMan-based qRT-PCR assays that detect arboviruses.

METHODS

Adult mosquitoes were collected using CDC EVS CO_2 traps, *Culex erythrothorax* separated on a chill table (BioQuip Products, Compton, CA) using a dissection microscope, and frozen at -80 ^oC until use. *Cx. erythrothorax* mosquitoes, inactivated virus (WNV, SLEV and WEEV; provided by the Davis Arbovirus Research and Training Lab, Davis CA) and a glass bead were added to lysis buffer included with the RNeasy Mini Kit (Qiagen, Hilden, Germany) or MagMAX Viral RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA). Tubes containing 0, 1, 5, 10, 25 or 50 mosquitoes were homogenized with each lysis buffer using a 'bead beater' (45 s; Biospec Products, Bartlesville, OK) to assess the impact of RNA isolation method and number of mosquitoes in a tube (n = 3 replicates per treatment). Samples were subsequently centrifuged (21,000 x g, 1 m) and the RNA isolated from the supernatant using RNeasy columns with a vacuum manifold (Qiagen) followed by centrifugation for the final wash and RNA elution, or using the MagMAX Kit with a MagMAX Express instrument (Applied Biosystems, Foster City, CA), as described by the manufacturers. Identical sample and RNA elution volumes were used for each sample. Half of each eluted RNA sample was subsequently clarified with centrifugation to remove residual precipitates from the isolated RNA (21,000 x g, 4 m). The optical density at 525 nm, the ratio of absorbance at 260 and 280 nm, and RNA concentration of the samples were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific), before and after clarifying. Triplex TaqMan qRT-PCR was used to assess the relative quantity of WNV, SLEV and WEEV in each sample. Briefly, 2 µl or 10 µl of each elution was added to TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific; final volume of 20 µl), with the TaqMan primers and probes described in Brault et al. (2015); primer and probe concentrations were for WNV: 600 nM primers, 150 nM probe (FAM-QSY); SLEV: 800 nM primers, 200 nM probe (VIC-QSY); WEEV: 900 nM primers, 250 nM probe (ABY-QSY)), and analyzed using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) using the following amplification protocol: 48.0 ^oC for 15 m, 95 ^oC for 30 s, and 40 cycles of 95 ^oC for 3 s with 60.0 ^oC for 30 s.

RESULTS

Visual inspection of the eluted RNA solutions from the MagMAX system showed a brown precipitate that was absent from the samples eluted from the RNeasy columns (not shown). To evaluate the extent of brown precipitate in the eluted RNA samples, the absorbance at λ_{max} (525 nm) was measured for each, before and after clarifying the samples. The absorbance of the 25 and 50 mosquitoes per tube samples were significantly greater for those isolated with MagMAX than the RNeasy column samples (0.507 +/- 0.030 for MagMAX samples and 0.01667 +/- 0.0025 for RNeasy column samples containing 50 mosquitoes; Paired t tests, $P < 0.011$). There was no significant difference in the absorbance of eluted RNA for samples that contained 0, 1, 5 or 10 mosquitoes (Unpaired t tests, $P > 0.05$; not shown). Using centrifugation to clarify the RNA solutions that were eluted using the MagMAX Kit significantly reduced their absorbance by an average of 0.327 +/- 0.056 for samples with 25 mosquitoes, and 0.413 +/- 0.020 for those containing 50 mosquitoes (8.39-fold and 5.37-fold reductions, respectively; Two-way ANOVA, $P < 0.0001$). We next sought to determine whether the presence of the brown precipitate affected RNA concentration or quality. There was no significant difference in RNA concentration before and after clarification of the same sample (Two-way ANOVA, RNeasy $P = 0.826$, MagMAX $P = 0.879$; not shown). For samples containing equivalent numbers of mosquitoes and isolated using RNeasy columns or MagMAX, there was no significant difference in the concentration of eluted RNA when the samples contained 0 to 10 mosquitoes (Multiple paired t tests, $P > 0.1$; not shown). However, when there were 25 or 50 mosquitoes in a sample, there was significantly less RNA in samples isolated using RNeasy columns relative to those from MagMAX (Unpaired t test, $P < 0.01$), with 45 % less RNA in the elutions from samples that contained 50 mosquitoes (not shown). RNA quality, as measured by the ratio of absorbance at 260 and 280 nm, was high for all samples (260/280 = 2.15 ± 0.147; not shown). The amplification efficiency of WNV, SLEV and WEEV in each sample, before and after clarification, was subsequently evaluated using triplex qRT-PCR (2 µl and 10 µl of each RNA sample was analyzed). Because differences in the Ct values among treatments were similar for WNV, SLEV, and WEEV Taqman assays, only results for WNV are described. The Ct values from TaqMan assays with 10 µl of eluted RNA were significantly lower relative to assays with 2 µl of RNA (Two-way ANOVA, $P < 0.001$; Figure 1). Similarly, the Ct values for samples assayed after clarification were significantly lower compared to those analyzed with TaqMan before clarification (Two-way ANOVA, $P < 0.001$; Figure 1). Within an isolation method (e.g. clarified MagMAX samples), the Ct values of samples containing no mosquitoes were always higher than samples that contained mosquitoes (Figure 1). Moreover, the Ct values from samples that did not contain mosquitoes were significant outliers of the others within an isolation method (Grubb's test, $P < 0.05$), with the exception of samples isolated using MagMAX and not clarified (Grubb's test, $P > 0.05$). Within each isolation method, there was no significant difference in the Ct values for samples containing 1, 5, 10, 25 or 50 mosquitoes (Multiple t tests, $P > 0.1$). The Ct values from samples isolated using MagMAX were significantly lower than those from RNA isolated using RNeasy columns, even when samples that did not contain mosquitoes were excluded from the analysis (Two-way ANOVA, $P < 0.0001$; Figure 1).

DISCUSSION

Clarification of the eluted RNA using centrifugation did not affect RNA concentration, but did reduce the quantity of brown precipitate in the eluted RNA samples, and improved the detection of arbovirus RNA using qRT-PCR. Increasing the quantity of eluted RNA in the qRT-PCR assay from 2 µl to 10 µl improved the sensitivity for detecting arbovirus RNA. Inclusion of 1 mosquito in the sample improved arbovirus detection, suggesting that cellular biomolecules such as mRNA may interact with arbovirus RNA to enhance its isolation or detection with qRT-PCR. Increasing the number of mosquitoes in a sample tube (*i.e.* mosquito pool) from 1 to 50 did not significantly affect the amplification of WNV, SLEV or WEEV in the qRT-PCR assay. Because the Ct values from RNA samples isolated using RNeasy columns were higher than the Ct values for samples isolated using MagMAX magnetic beads, use of the later method may increase the sensitivity of arbovirus detection in mosquitoes.

ACKNOWLEDGEMENTS

We thank Dr. Jan Washburn (Alameda County Mosquito Abatement District) for discussions of the project design and data analysis, and Dr. Gregory White (Coachella Valley Mosquito and Vector Control District) for guidance on the primer and probe concentrations used for the qRT-PCR assays.

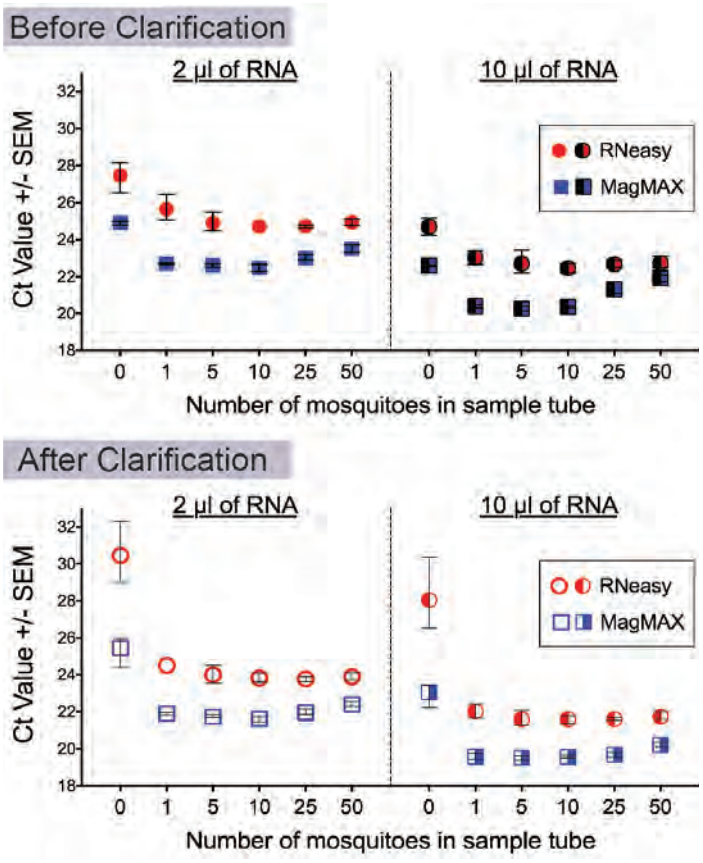

Figure 1. Triplex qRT-PCR (TaqMan) amplification of WNV using 2 µl or 10 µl of eluted RNA that was isolated using MagMAX or RNeasy columns, before and after clarification using centrifugation (top and bottom, respectively). A similar distribution of Ct values was observed for amplification of WEEV and SLEV (not shown). When the RNA samples were clarified using centrifugation, there was a significant reduction in Ct values from the TaqMan assays (Two-way ANOVA, $P < 0.001$), suggesting that clarifying extracted RNA samples with centrifugation may increase the sensitivity of the TaqMan assay for arboviruses isolated from mosquitoes. TaqMan assays with 10 µl of RNA had significantly lower Ct values relative to those with 2 µl of RNA (Two-way ANOVA, $P < 0.001$), suggesting that use of the highest volume of eluted RNA in the TaqMan assay may enhance detection of arboviruses. When the number of mosquitoes in a sample was increased from 1 to 50, there was no significant difference in Ct values within each isolation method (Multiple t tests, $P > 0.1$), suggesting that isolating arbovirus RNA from up to 50 mosquitoes in a single tube does not negatively affect the TaqMan assay. RNA isolated using MagMAX produced significantly lower Ct values in the arbovirus TaqMan assays relative to RNA isolated using RNeasy columns (Two-way ANOVA, $P < 0.0001$), suggesting that use of MagMAX may be more sensitive in screening mosquitoes for arbovirus infection.

REFERENCE LIST

Brault, A. C., Y. Fang, and W. K. Reisen. 2015. Multiplex qRT-PCR for the Detection of Western Equine Encephalomyelitis, St. Louis Encephalitis, and West Nile Viral RNA in Mosquito Pools (Diptera: Culicidae). J Med Entomol 52: 491-499.

Fang, Y., M. Dannen, H. Garcia, and W. K. Reisen. 2010. Enhanced sensitivity of RNA extraction: What it Means for Wild Bird and Mosquito Surveillance. Proc Mosq Vector Control Assoc Calif 78: 23-26.