CAPTURING TRENDS IN ARBOVIRAL SURVEILLANCE: COMPARING TRADITIONAL REVERSE TRANSCRIPTION PCR AND QUANTITATIVE REVERSE TRANSCRIPTION PCR ASSAYS

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ABSTRACT

West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) pose a significant public health threat in the United States. These viruses are known to adapt rapidly to new amplifying hosts and geographic environments, making effective surveillance critical for public health efforts. This study evaluated the effectiveness of traditional reverse transcription polymerase chain reaction (RT-PCR) for surveillance purposes compared to quantitative RT-PCR (RT-qPCR) in detecting WNV and SLEV in mosquito pools. Mosquito pools were collected and screened for WNV and SLEV over a 10-year period. This study found an increase in the number of flavivirus-positive yet WNV-/SLEV-negative mosquito pools during 2018 compared to previous years. Quantitative RT-PCR detected more positive WNV and SLEV pools compared to traditional RT-PCR, eliminating false negatives and identifying false positives. The findings underscore the importance of using RT-qPCR for arboviral surveillance to accurately detect circulating viruses and enable timely public health interventions. Changes in local trends in mosquito-borne viruses and vector populations have the potential to impact public health, emphasizing the need for proactive surveillance measures.

Key Words: arboviral surveillance; flavivirus; public health; RT-PCR; RT-qPCR; St. Louis encephalitis virus; vector control; West Nile virus

INTRODUCTION

West Nile virus (WNV) and St. Louis encephalitis virus (SLEV), both transmitted by the bite of an infective mosquito, are members of the *Flaviviridae* family in the Japanese encephalitis virus complex (Calisher et al. 1989, Thiel et al. 2005). Both WNV and SLEV are considered endemic in the United States and pose a significant public health threat (Madewell 2020). West Nile virus was first detected in the United States during 1999 in New York City, New York (CDC 1999, Lanciotti et al. 1999), while SLEV was first documented in St. Louis, Missouri during 1933 (May et al. 2008).

RNA viruses, such as WNV and SLEV, are known to adapt rapidly to host species in new environments (Hayes 2001), due in part to their error-prone polymerases that can result in high mutation rates (Holland et al. 1982, Duffy et al. 2008, Acevedo et al. 2014). Viruses must adapt or they can become extinct (Pesko and Ebel 2012). West Nile virus quickly adapted to local mosquito populations following its introduction into the United States, which aided in the virus's ability to establish local transmission cycles (Ebel et al. 2004, Davis et al. 2005, Jerzak et al. 2005, Huang et al. 2019). The genetic diversity of WNV is greater within mosquito populations when compared to their avian hosts, and because of this, viruses from mosquitoes may provide a greater genetic variation in nature (Jerzak et al. 2005).

Vector surveillance programs are critical for informing integrated vector management plans for local mosquito control agencies and public health departments. Integrated vector management is the decision-making process for the efficient use of vector control resources to reduce or arrest pathogen transmission. Screening of mosquito pools for arboviruses is a commonly used practice to monitor the potential threat of mosquito-borne diseases in a community. With the rising trend in human cases of vector-borne diseases in the United States and the potential for development of genetic variations in RNA arboviruses, assuring the most effective surveillance efforts are being utilized is important (Rosenberg et al. 2018).

Our study evaluated the effectiveness of using traditional reverse transcription polymerase chain reaction (RT-PCR) for surveillance purposes compared to the use of quantitative RT-PCR (RT-qPCR). As part of an ongoing surveillance project, the Vector-borne Zoonoses Laboratory at Texas Tech University conducts weekly arboviral screening of mosquitoes collected in the City of Lubbock, Texas (Peper et al. 2018). Mosquitoes were collected using CO_2-baited encephalitis vector surveillance traps and were pooled by species, date, and location. Mosquito pools were initially processed by extracting RNA using the *QIAamp Viral RNA Mini Kit* (Qiagen, Cat #: 52906). Arboviral screening was accomplished using a two-stage process. First, mosquito pools were screened using flavivirus consensus primers and a traditional RT-PCR assay that targets a 220 bp region of the NS5 gene (Kuno et al. 1998). The resulting amplicons were then determined positive (i.e., band present at 220 bp) or negative (i.e., band absent at 220 bp) by electrophoresis using a 2% agarose gel. When determined positive by the flavivirus consensus primer set, pools were retested using a WNV-specific primer set that targets a 408 bp region including the C and prM genes, again using traditional RT-PCR (Lanciotti et al. 2000). Due to the high variability associated with interpreting PCR gels, in 2018 flavivirus-positive samples were rescreened using a more sensitive and reliable RT-qPCR triplex assay that detects WNV (i.e., 70 bp region of the E gene), SLEV, and western equine encephalitis virus (WEEV) (Brault et al. 2015). These primer/probe sets are commonly used by local mosquito control programs and university research laboratories throughout the state of Texas (personal communications with specific programs). A cycle threshold value (ct-value) of 37 was used as a determinate for positive samples during the RT-qPCR assays (i.e., ct-values <37 were considered positive and ct-values >37 were considered negative). Positive controls (i.e., WNV and SLEV RNA-isolates) used for all RT-PCR and RT-qPCR assays in this study were provided by the Texas Department of State Health Services. A no template control (i.e., molecular grade water) was used in all RT-PCR and RT-qPCR assays as negative controls. As WEEV is not a flavivirus, and no WEEV-positive samples were detected, WEEV results are not further discussed in this note.

From 2009 through 2018, 140 mosquito pools tested positive for flavivirus via the traditional RT-PCR assay, 55 (39.3%) of which were negative for WNV (Table 1). The percent of flavivirus-positive yet WNV-negative pools in 2018 was 82.8%, whereas the average from the previous eight years (111 pools tested) was only 27.9% (range: 17.7-40.0%).

Table 1. Traditional and real-time polymerase chain reaction results of mosquito pools tested in Lubbock, Texas for flaviviruses, West Nile virus, and St. Louis encephalitis virus from 2009 through 2018.

	Total Pools			Pools Tested by Both Methods					
	Traditional PCR			Traditional PCR			Triplex		
Year	Flavi+ (%)[a]	WNV+	Flavi+/WNV- (%)	Flavi+[b]	WNV+	Flavi+/WNV- (%)	WNV+	SLE+	Flavi+[c]/WNV-/SLEV- (%)
2018	29 (30.2)	5	24 (82.8)	29	5	24 (82.8)	7	4[d]	20 (69.0)
2017	11 (6.2)	9	2 (18.2)	11	9	2 (18.2)	9	1[e]	2 (18.2)
2016	9 (4.4)	6	3 (33.3)	9	6	3 (33.3)	3	2	4 (44.4)
2015	18 (5.3)	12	6 (33.3)	16	11	5 (31.3)	12	1[f]	4 (25.0)
2014	5 (0.7)	3	2 (40.0)	5	3	2 (40.0)	5	3[g]	0 (0)
2013	13 (2.2)	8	5 (38.5)	8	7	1 (12.5)	8	0	0 (0)
2012	8 (1.8)	5	3 (37.5)	7	4	3 (42.9)	6	0	1 (14.3)
2010	30 (3.1)	23	7 (23.3)	5	4	1 (20.0)	5	0	0 (0)
2009	17 (2.2)	14	3 (17.7)	6	4	2 (33.3)	4	0	2 (33.3)
Total	140	85	55 (39.3)	96	53	43 (44.8)	59	11	33 (34.4)

[a] Percent of all samples that tested positive (total flavivirus positive and negative samples)
[b] Mosquito pools that tested positive for flavivirus and had enough RNA extraction that could be tested again using the RT-qPCR triplex assay.
[c] Flavivirus results used from the "Flavi+" column from the Traditional PCR under the Pools Tested by Both Methods section.
[d] Two samples were both WNV- and SLEV-positive
[e] One sample was both WNV- and SLEV-positive
[f] One sample was both WNV- and SLEV-positive

Of the 140 flavivirus-positive pools, 96 had enough RNA extract to be retested using the RT-qPCR triplex assay (Table 1). When pools were retested using the RT-qPCR triplex assay, 63 pools tested positive for WNV and/or SLEV compared to the 53 pools that were WNV-positive via the traditional RT-PCR assay; i.e., 13 pools were originally identified as negative via the traditional RT-PCR assay were later identified as positive for WNV and/or SLEV (*note*: three pools collected during 2016 were originally incorrectly identified as WNV-positive as they later tested negative using the RT-qPCR triplex assay).

For samples that were tested by both RT-PCR and RT-qPCR during 2009 through 2017, pools were flavivirus-positive yet WNV-negative 28.4% (19/67) of the time (range: 12.5% - 42.9%) using the traditional RT-PCR assay. However, 24 pools (82.8%) collected during 2018 were flavivirus-positive yet WNV-negative. This represents a 2.9-fold increase during 2018 in the number of flavivirus-positive yet WNV-negative pools from the previous years' average. After retesting these pools using the RT-qPCR triplex assay, mosquitoes collected during 2009 through 2017 had pools that were flavivirus-positive yet WNV-/SLEV-negative 19.4% (13/67) of the time (range: 0% - 44.4%). Meanwhile, mosquitoes collected during 2018 had 20 (69.0%) flavivirus-positive yet WNV-/SLEV-negative pools after retesting. This represents a 3.6-fold increase from the previous years' average in the number of flavivirus-positive yet WNV-/SLEV-negative pools after retesting.

The use of the RT-qPCR triplex assay enabled the detection of more WNV- and/or SLEV-positive mosquito pools compared to the traditional RT-PCR assay, most likely due to human error associated with interpreting the lack or presence of bands during the gel electrophoresis process. When comparing the two assay methods, additional positive pools were identified in seven of the nine years in this study (Table 1). Thus, we recognize the benefits of using the RT-qPCR assay for arboviral surveillance as it is capable of eliminating false negative results, and in three instances was able to identify false positives. Another benefit to the RT-qPCR triplex assay was the ability to rapidly identify positive SLEV pools in addition to the WNV pools without having to run additional/secondary assays, which typically happens with traditional PCR assays. The RT-qPCR triplex assay has a positive impact on the public health ramifications of a community as abatement efforts are able to be implemented sooner.

On the other hand, the use of traditional RT-PCR in this study was able to capture the dramatic increase in flavivirus-positive yet WNV-negative pools during 2018. This trend may not have been noticed if mosquito pools were not first being screened using the traditional RT-PCR flavivirus consensus primer set. As a potential flaw to this evaluation, all of the RT-qPCR assays were conducted in 2018. This provides the potential for RNA degradation to occur in older pools. However, this is not likely an issue because, with the exception of 2016, none of the previous years had a reduction in positive pool results after retesting via the RT-qPCR triplex assay – only gaining positives – and all samples were stored in the same location and under the same conditions (-80°C).

Next steps after identifying changes in local trends will be to identify what has caused these trends to occur. Is there a genetic mutation in the locally circulating arboviruses that make them more or less detectable through certain surveillance techniques, or allows them to more readily infect certain vector species? The authors recognize the increase in flavivirus-positive yet WNV/SLEV-negative pools during 2018 may have resulted from an increase in insect-specific flaviviruses and not a potential genetic change, however, this still demonstrates a change in local trends. The ability of surveillance efforts to detect current trends and the genetic composition of circulating strains of pathogens is critical. As an example, a single cytosine (C) to thymine (T) mutation in the probe-binding region reduced assay sensitivity for the same WNV RT-qPCR assay used for screening mosquito pools (Brault et al. 2012).

The 3.6-fold increase in flavivirus-positive yet WNV-/SLEV-negative mosquito pools is concerning as it could potentially lead to a public health crisis due to the potential undetected circulating arboviruses in the local vector populations – especially depending on what testing assay is being used. The change in local trends has the potential to reduce or eliminate the would-be 'call to action' by local vector control programs to manage emerging vector populations and recognize specific arboviral infections in humans. Observing the change in trends over the years, such as was accomplished by this study or through spot checking the genetic sequences in the probe binding regions of currently circulating viral strains, can potentially help identify changes in locally circulating arboviruses and vector species populations, and implement proactive measures to prevent a potential public health crisis.

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REFERENCES CITED

Acevedo A, Brodsky L, Andino R. 2014. Mutational and fitness landscapes of an RNA virus revealed through population sequencing. *Nature* 505:686–690.

Brault AC, Fang Y, Dannen M, Anishchenko M, Reisen WK. 2012. A naturally occurring mutation within the probe-binding region compromises a molecular-based West Nile virus surveillance assay for mosquito pools (Diptera: Culicidae). *J Med Entomol* 49:939–941.

Brault AC, Fang Y, Reisen WK. 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.

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE. 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 70:37–43.

CDC [Centers for Disease Control and Prevention]. 1999. West Nile encephalitis – New York. *MMWR* 48:944–946.

Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, Lambert A, Parsons RE, Beasley DW, Novak RJ, Elizondo-Quiroga D, Green EN, Young DS, Stark LM, Drebot MA, Artsob H, Tesh RB, Kramer LD, Barrett AD. 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: evidence for the emergence of a dominant genotype. *Virol* 342:252–265.

Duffy S, Shackleton LA, Holmes EC. 2008. Rates of evolutionary changes in viruses: patterns and determinants. *Nat Rev Genet* 9:267–276.

Ebel GD, Carricaburru J, Young D, Bernard KA, Kramer LD. 2004. Genetic and phenotypic variation of West Nile virus in New York, 2000-2003. *Am J Trop Med Hyg* 71:493–500.

Hayes CG. 2001. West Nile virus: Uganda, 1937, to New York City, 1999. *Ann N Y Acad Sci* 951:25–37.

Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S. 1982. Rapid evolution of RNA genomes. *Science* 215:1577–1585.

Huang YJS, Higgs S, Vanlandingham DL. 2019. Emergence and re-emergence of mosquito-borne arboviruses. *Curr Opin Virol* 34:104–109.

Jerzak G, Bernard KA, Kramer LD, Ebel GD. 2005. Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. *J Gen Virol* 86:2175–2183.

Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. 1998. Phylogeny of the genus *Flavivirus*. *J Virol* 72:73–83.

Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol* 38:4066–4071.

Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, MacKenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T, Gubler DJ. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 286:2333–2337.

Madewell ZJ. 2020. Arboviruses and their vectors. *The Southern Medical Journal* 113: 520–523.

May FJ, Li L, Zhang S, Guzman H, Beasley DWC, Tesh RB, Higgs S, Raj P, Bueno Jr. R, Randle Y, Chandler L, Barrett ADT. 2008. Genetic variations of St. Louis encephalitis virus. *J Gen Virol* 89:1901–1910.

Peper ST, Dawson DE, Dacko N, Athanasiou K, Hunter J, Loko F, Almas S, Sorensen GE, Urban KN, Wil-son-Fallon AN, Haydett KM, Greenberg HS, Gibson AG, Presley SM. 2018. Predictive modeling for West Nile virus and mosquito surveillance in Lubbock, Texas. *J Am Mosq Control Assoc* 34:18–24.

Pesko KN, Ebel GD. 2012. West Nile virus population genetics and evolution. *Infect Genet Evol* 12:181–190.

Rosenberg R, Lindsey NP, Fischer M, Gregory CJ, Hinckley AF, Mead PS, Paz-Bailey G, Waterman SH, Drexler NA, Kersh GJ, Hooks H, Partridge SK, Visser SN, Beard CB, Petersen LR. 2018. Vital Signs: Trends in reported vector-borne disease cases - United States and Territories, 2004–2016. *MMWR* 67:496–501.

Thiel HJ, Collett MS, Gould EA, Heinz FX, Meyers G, Purcell RH, Rice CM, Houghton M. 2005. Flaviviridae. In *Virus Taxonomy, Eighth Report of the International Committee for the Taxonomy of Viruses*; Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, Eds.; Academic Press, San Diego, California, USA, pp. 981–998.

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