IN-HOUSE TESTING OF MOSQUITO POOLS FOR WEST NILE VIRUS USING COMMERCIALLY AVAILABLE IMMUNOASSAY AND REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION KITS

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ABSTRACT

Arbovirus surveillance methods are an integral part of integrated mosquito management programs, providing information on arboviral presence, location, and transmission potential. For many vector control agencies, surveillance entails collecting vector mosquito species from the field and testing representative mosquito pools using commercially available immunoassays or real-time reverse transcriptase polymerase chain reaction test kits. In 2016, the Collier Mosquito Control District established an in-house arbovirus surveillance program to screen mosquitoes for a variety of diseases, including the endemic arbovirus, West Nile virus (WNV). Although guidance on interpreting test results is provided by the manufacturer, end users of commercial test kits are encouraged to establish their own cut-off values signifying an arbovirus positive mosquito pool. Here we report the Collier Mosquito Control District's efforts to develop cut-off values for mosquito pools using two commercially available WNV test kits.

Key Words: West Nile virus, arbovirus, RT-PCR, immunoassay

INTRODUCTION

West Nile virus (WNV; family Flaviviridae, subfamily Flavivirus) was first introduced to the State of Florida in 2001 (Blackmore et al. 2003) and continues to be a high priority disease for vector control agencies. In 2020-2021, Collier County (FL, USA) experienced heightened activity for WNV, with 11 human cases and one equine case (FLDOH, 2022). A spatiotemporal understanding of WNV vector mosquito abundance and activity is paramount to integrate mosquito management approaches to reduce WNV risk in animal and human populations. Complementary activities include arbovirus monitoring through sentinel chicken surveillance programs and/or testing of mosquito pools for arbovirus infection, both of which serve as indicators of arbovirus presence, spatial distribution, and transmission risk.

Vector-borne disease surveillance is often contracted to state public health laboratories; however, turnaround times of 2 weeks or more are standard, causing costly delays in operational responses. Thus, many vector control agencies have implemented in-house arbovirus testing programs to screen sentinel chicken sera (Peper 2021) and/or mosquito pools for arboviruses of interest. The Collier Mosquito Control District (the District) regularly tests mosquito pools from routine trap collections of Culex nigripalpus Theobald and Cx. quinquefasciatus Say from CDC miniature light traps (John W. Hock Company, Gainesville, Florida, USA), BG Sentinel traps (Biogents AG, Regensburg, Germany) and Reiter-Cummings modified gravid traps (BioQuip Products, Rancho Dominquez, California, USA) for WNV testing. Trap collections are retrieved from field sites, brought back the the District laboratory, and immediately euthanized at -80 C for 1 hr before mosquitoes are identified by morphology and pooled for arbovirus testing. Two commercially-available assays are used: the Rapid Analyte Measurement Platform (RAMP) test (Response Biomedical Corp., Burnaby, British Columbia, Canada) and the Vector Smart™ North American East (NAM-e) kit (Co-Diagnostics Inc, Salt Lake City, UT, USA). Results obtained from these tests inform operational decision making, allowing the District to respond to mosquito-borne disease threats in a timely manner.

Often, when a vector control agency identifies positive mosquito pools in-house, the pools are sent to state laboratories for secondary confirmation testing. This is of particular importance in Florida as positive pools are not included on the Florida Department of Health arbovirus report unless they receive secondary confirmation by the Bureau of Public Health Laboratories (Tampa). The District has observed that samples testing

positive for WNV using in-house methods do not always test positive when sent to the state laboratory for confirmation, an observation previously reported by Burkhalter et al. (2014) for mosquito pools that had initially tested positive using the RAMP test. Here we report the District's efforts to establish operationally relevant cut-off values for mosquito pools testing positive for WNV when using RAMP test and the Vector Smart NAM-e kit. Determining these cut-off values increases the probability that secondary confirmation testing will be successful and provides a baseline for when a vector control agency should take operational action in response to positive pool results.

The RAMP test is a common immunoassay utilized by vector control agencies to detect WNV antigen in pools of local mosquitoes. Pools of up to 50 female mosquitoes are homogenized in a manufacturer-supplied RAMP buffer. An aliquot of the processed homogenate is combined with fluorescently-bound WNV antibodies, which bind to WNV if it's present in the sample. The homogenate-antibody mixture is transferred to the RAMP WNV test cartridge. The mixture migrates along a test strip through capillary action, and WNV-bound antibodies become immobilized at the detection zone. After a 90-minute incubation period, the test cartridge is inserted into a RAMP reader, which provides a fluorescence reading ranging from 10.0 to 640.0 RAMP units.

The manufacturer indicates that readings of $\geqslant$ 30 RAMP units as the cut-off for positive WNV mosquito pools but encourages end users to set their own local cut-off values and establish "gray zones" of uncertainty (Response Biomedical 2016). Although several agencies have published their own cut-off values and interpretation guidelines (Burkhalter et al. 2006, Williges et al. 2009, Kesavaraju et al. 2012, Burkhalter et al. 2014, Coatsworth et al. 2022), the Centers for Disease Control and Prevention (CDC) suggests using a cut-off value of 50 RAMP units for positive WNV mosquito pools that do not require additional confirmatory testing (Buckhalter et al. 2014, Response Biomedical 2016). For tests that do require secondary confirmation, the CDC suggests a more conservative approach, defining a "gray zone" for readings between 50-100 RAMP units that are likely to contain some amount of virus but may not be able to be confirmed via real time reverse transcriptase polymerase chain reaction (qRT-PCR) due to the inhibitory action of the RAMP buffer (Burkhalter et al. 2014, Response Biomedical 2016).

The Vector Smart NAM-e kit is a qRT-PCR based multiplex assay that has recently become available to vector control agencies for mosquito pool testing of WNV, Saint Louis encephalitis virus, and eastern equine encephalitis virus. The District was an early adopter of the test kit and has used it as the primary mosquito pool test method since 2019. In this assay, mosquito pools of approximately 25 females are homogenized in 1x phosphate buffer saline. Total nucleic acid is extracted using the MagMAX CORE Nucleic Acid Purification kit (ThermoFisher Scientific, Waltham, MA, USA) in the KingFisher™ Duo Prime Purification System (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Total nucleic acid is then used with the Vector Smart NAM-e kit, and arbovirus detection data is generated on Applied Biosystems® QuantStudio® 5 Real-Time PCR System (Thermofisher, Carlsbad, CA) following manufacturer guidelines, which includes the use of an internal, positive and negative control. The manufacturer recommends that positive cut-off cycle threshold (Ct) values be determined through in-house validation testing.

Although WNV is endemic in Collier County, from 2017-2019 mosquito infection rates were rarely high enough to be detected by the District's arbovirus surveillance program using RAMP assay or qRT-PCR methods. Further, WNV human and equine infections were low during the same timeframe, with only one WNV equine infection reported in 2017 (FLDOH 2022). However, an unusually large number of mosquito pools tested positive for WNV in 2020 and 2021. Due to staff limitations, supply chain delays, and laboratory accessibility constraints associated with the COVID-19 pandemic, the District used either RAMP or Vector Smart NAM-e kits for testing during this period. A total of 2,286 pools were tested, 32 of 579 tested by using RAMP (cut-off: $\geqslant$ 30 RAMP units) were positive, and 23 of 1,707 tested using the Vector Smart NAM-e kit (cut-off: Ct value $\leqslant$ 40) were positive. Processed homogenate (in PBS or RAMP buffer) of positive pools were sent to the state laboratory for confirmation testing.

Of the RAMP positive pools, 21 were sent to the state laboratory for confirmation testing. Five had readings over 100 RAMP units, six fell within the gray zone (50-100 RAMP units) defined by Burkhalter et al. (2014) and the remainder had readings of less than 50 RAMP units but exceeded the manufacturer's recommended cut-off of 30 RAMP units (Table 1). Only three of the 21 pools were confirmed positive using RT-PCR (FLDOH, 2022), and the readings for all three exceeded 130 RAMP units. Based on a conservative interpretation of these results, the District has defined readings between 30-100 RAMP units as our gray zone and categorizes pools with readings falling in this range as "marginally positive," with the expectation that these pools are unlikely to test positive if sent for RT-PCR based confirmation testing (Burkhalter et al. 2014,

Table 1: Mosquito pools tested in-house via RAMP assay. Trap types include: CDC miniture light traps (CDC), BG-Sentinel traps (BGS) and and Reiter-Cummings modified gravid trap (GRV). Red highlight signifies confirmed positive samples, grey highlight signifies mariginally positive samples, and green highlight signifies negative samples.

Collection Date	Trap Type	Species	Number Mosquitoes	RAMP Units	Confirmation
9/1/20	CDC	Cx. nigripalpus	25	640	Detected
9/15/20	CDC	Cx. nigripalpus	25	269.9	Detected
9/15/20	CDC	Cx. nigripalpus	25	133.1	Detected
10/14/20	CDC	Cx. nigripalpus	25	116.2	Not detected
9/1/20	CDC	Cx. nigripalpus	25	112.1	Not detected
9/25/20	CDC	Cx. nigripalpus	25	98.5	Not detected
10/6/20	CDC	Cx. nigripalpus	25	84.5	Not detected
9/15/20	CDC	Cx. nigripalpus	25	79.2	Not detected
9/20/20	CDC	Cx. nigripalpus	25	71.9	Not detected
10/6/20	CDC	Cx. nigripalpus	25	60	Not detected
9/18/20	CDC	Cx. nigripalpus	25	53.9	Not detected
11/17/20	CDC	Cx. nigripalpus	25	*53.5*	*Not submitted*
11/9/21	CDC	Cx. nigripalpus	25	*48.3*	*Not submitted*
7/27/21	CDC	Cx. nigripalpus	25	*46.5*	*Not submitted*
10/6/20	CDC	Cx. nigripalpus	25	46.1	Not detected
12/4/20	CDC	Cx. nigripalpus	19	*43.5*	*Not submitted*
12/14/20	CDC	Cx. nigripalpus	25	*42.4*	*Not submitted*
10/6/20	CDC	Cx. nigripalpus	25	39.3	Not detected
10/8/20	GRV	Cx. quinquefasciatus	1	39.2	Not detected
11/17/20	CDC	Cx. nigripalpus	25	*36.7*	*Not submitted*
2/17/20	CDC	Cx. nigripalpus	25	*35.8*	*Not submitted*
2/17/20	CDC	Cx. nigripalpus	25	*35.8*	*Not submitted*
9/23/20	CDC	Cx. nigripalpus	25	35.6	Not detected
10/6/20	CDC	Cx. nigripalpus	25	32.2	Not detected
9/1/20	CDC	Cx. nigripalpus	25	32.1	Not detected
10/6/20	BGS	Cx. nigripalpus	21	31.8	Not detected
7/27/21	CDC	Cx. nigripalpus	25	*31.5*	*Not submitted*
10/6/20	CDC	Cx. nigripalpus	25	31.3	Not detected
7/16/21	GRV	Cx. nigripalpus	4	31	Not detected
11/17/20	CDC	Cx. nigripalpus	25	*30.6*	*Not submitted*
11/2/21	BGS	Cx. nigripalpus	25	*30.6*	*Not submitted*
10/8/20	BGS	Cx. nigripalpus	2	30	Not detected

Response Biomedical 2016). Marginally positive mosquito pools are taken into consideration by the District when making operational decisions but do not fully dictate treatment decisions. Pools with readings exceeding 100 RAMP units are considered positive for WNV, sent to the state laboratory for testing, and used to make operational treatment decisions.

Of the pools that tested positive using the Vector Smart NAM-e kit, 22 were sent to the state laboratory for confirmation testing. Eight of these were confirmed positive using the state's method of RT-PCR, and an additional three were considered "equivocal" (FLDOH, 2022). Equivocal results were due to the RT-PCR detecting WNV using one primer set but not the other (A. Morrison, personal communication; August 10, 2021). There was some overlap in the Ct values for the samples that were confirmed at the state level; confirmed samples had Ct values ranging from 21-36, and unconfirmed samples had

Table 2: Mosquito pools tested in-house via Vector Smart NAM-e kit. Trap types include: CDC miniture light traps (CDC), BG-Sentinel traps (BGS) and and Reiter-Cummings modified gravid trap (GRV). Red highlight signifies confirmed positive samples, grey highlight signifies mariginally positive samples, and green highlight signifies negative samples.

Collection Date	Trap Type	Species	Number Mosquitoes	Ct Value	Confirmation
8/24/21	BGS	Cx. nigripalpus	25	20.9	Detected
8/27/21	GRV	Cx. quinquefasciatus	14	21.4	Detected
8/20/21	GRV	Cx. quinquefasciatus	25	22.2	Detected
8/6/21	GRV	Cx. quinquefasciatus	4	24.3	Detected
8/5/20	BGS	Cx. nigripalpus	17	24.7	Detected
8/20/21	GRV	Cx. quinquefasciatus	25	27.5	Detected
7/13/21	CDC	Cx. nigripalpus	25	28.3	Detected*
7/13/21	CDC	Cx. nigripalpus	25	29.3	Equivocal*
7/13/21	CDC	Cx. nigripalpus	25	30.7	Equivocal*
7/13/21	CDC	Cx. nigripalpus	25	33.9	Equivocal*
7/13/21	CDC	Cx. nigripalpus	25	34.2	Not detected*
8/24/21	BGS	Cx. nigripalpus	25	34.4	Not detected
8/5/20	BGS	Cx. nigripalpus	25	36.2	Not detected
6/22/21	CDC	Cx. nigripalpus	16	36.4	Not detected
8/10/21	CDC	Cx. nigripalpus	25	36.4	Not detected
8/27/21	GRV	Cx. nigripalpus	11	36.5	Detected
6/25/21	GRV	Cx. quinquefasciatus	25	37.5	Not detected
7/13/21	CDC	Cx. nigripalpus	25	37.8	Not detected*
7/28/21	GRV	Cx. nigripalpus	14	38.4	*Not submitted*
8/20/21	GRV	Cx. nigripalpus	1	38.5	Not detected
8/27/21	GRV	Cx. quinquefasciatus	10	38.7	Not detected
8/24/21	GRV	Cx. nigripalpus	25	38.9	Not detected
8/10/21	BGS	Cx. nigripalpus	17	39.2	Not detected

* Shipping issues may have had impact on confirmatory testing

values ranging from 29-39 (Table 2). Due to shipping issues, pools with a collection date of July 13, 2021 are noted in Table 2; however, WNV positive homogenate has been shown to be stable at ambient temperatures (Erandio et al. 2020). Based on these results, the District has defined samples with Ct values between 31-37 as marginally positive and pools with Ct values ⩽ 30 as positive. As with the RAMP test, marginally positive mosquito pools are considered operationally but do not dictate actions, while positive mosquito pools strongly influence treatment decisions. All pools testing either marginally positive or positive using the Vector Smart NAM-e kit are sent to the state laboratory for testing.

As of 2017, almost all independent mosquito control agencies in Florida reported in-house arbovirus testing of mosquito pools, whereas more than half of dependent mosquito control agencies depended exclusively on state-level testing (Moise et al. 2020). The District typically has a 24-48 hr turnaround time between when mosquitoes are captured and when pool results are available to operational decisionmakers, while state testing takes 1-2 weeks at a minimum. In-house testing therefore minimizes the delay between sample acquisition and operational decision-making and represents a significant improvement in the District's ability to respond quickly to emergent disease threats. The cut-off values outlined here are appropriate for District uses, but variations in the arbovirus being tested, mosquito species, local populations, and laboratory protocols can influence pathogen detection measurements (Kesavaraju et al. 2012, Burkhalter et al. 2014, Response Biomedical 2016). For this reason, agencies should develop cut-off values tailored to their particular testing conditions in order to increase confidence in arboviral test results and help optimize organizational responses to mosquito-borne disease threats.

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