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Natural Vertical Transmission of WNV in Montana

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Natural Vertical Transmission of WNV in Montana

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Abstract

West Nile Virus (WNV) emerged and rapidly spread throughout the United States within several years. WNV is spread in Montana primarily by the mosquito vector *Culex tarsalis*. Horizontal transmission of WNV has been extensively studied, while little is known regarding vertical transmission. Previous research suggests vertical transmission is possible in controlled laboratory studies. This research attempts to document natural vertical transmission in Montana. Larvae were collected and analyzed for WNV via RT-PCR. Results show no presence of natural vertical transmission. Culex larvae and adult male mosquito collection methods were insufficient, fluctuations in temperatures and variant rainfall were contributing factors. Future directions should examine methods to improve collection of Culex larvae. Gravid traps infusions attracting gravid Culex *tarsalis* mosquitoes may be a promising direction to investigate.

Keywords: WNV, *Culex tarsalis*, vertical transmission

Introduction

Infectious diseases have existed since humans have roamed the earth. The emergence of agriculture and domestication allowed for an increase in population densities, leading to insanitary conditions and greater impact of infectious disease (Armelagos et al., 2005). Vector borne diseases can be transmitted to humans and are called infectious diseases. As a vector born disease WNV requires a host and a vector. A host is defined as the organism that replicates the infectious disease, while a vector is an organism that serves as the bridge by transmitting the disease to new members of a population (Sutherst, 2004). Vertebrate hosts such as birds and vectors such as insects, ticks, snails, and others are common examples (Sutherst, 2004). West Nile Virus (WNV) was non-existent in the United States until 1999, when it appeared in American Crows, Fish Crows, and Chilean Flamingos (Lanciotti et al., 1999) and by 2002 WNV had spread through the entire continental United States (Roehrig, 2013). Fifty-nine human patients were hospitalized for WNV during August and September 1999 (Nash et al., 2001). DNA analysis suggested that the WNV strain outbreak in the United States was 99.8% similar (only two nucleotides differed) to the strain that was isolated from a dead goose in Israel in 1998 (Lanciotti et al., 1999). WNV could have been introduced into the United States a number of different ways via: infected humans, infected birds, domestic animals, or infected ticks and/or mosquitoes (Lanciotti et al., 1999). WNV has been isolated from several different mosquito species, but the primary carrier is the *Culex* genus (Baqar et al., 1993). In the Western United States *Culex tarsalis* and *Culex pipiens* are dominant vectors of WNV (Goddard et al., 2002).

WNV can be transmitted in a variety of ways, including: 1) horizontal transmission from a vector to a host, eg. mosquito to bird which has been recognized as the most

prominent form of transmission allowing WNV to rapidly spread throughout the Western Hemisphere (Roehrig, 2013). Alternatively, 2) WNV can spread through vertical transmission in which WNV passes from an infected mother mosquito to the F_1 offspring (Goddard et al., 2003). If vertical transmission is above a mathematically derived threshold, WNV can be maintained uncontrollably in a region (Cruz-Pacheco et al., 2005). Vertical transmission of WNV was first observed in the laboratory (Baqar et al., 1993) and later discovered naturally in adult male *Culex univittatus* at the Kenya-Uganda boarder (Miller et al., 2000). Naturally infected *Culex erythrothorax* larvae have been observed in Moab, Utah (Phillips and Christensen 2006**)** and in Baton Rouge, Louisiana where *Aedes triseriatus* and *Culex salinarius* male mosquitoes tested positive for WNV (Unlu et al., 2010) demonstrating the presence of vertical transmission in the United States.

Diapause allows mosquitoes to slow metabolism, optimizing survival in unfavorable conditions. Diapause is a way in which mosquitoes survive during winter months, and during this time they can be carriers of WNV (Goddard et al., 2003). In Connecticut, vertically transmitted *Culex pipiens* reintroduced WNV after diapause (Anderson and Main 2006). Thus, diapause or overwintering can be an avenue for vertical transmission of WNV during the spring. Diapause further amplifies mosquito infection rates in summer months (Fetchter-Legget et al., 2012). Increased amplification is a concern for the public due to an invested interest in human infection.

Vertical transmission rates have been tested in naturally infected adult mosquitoes. In California 50% of egg rafts and 40% first instar larvae tested positive for WNV in *Culex pipiens* (Fetchter-Legget et al., 2012). WNV infections of *Culex pipiens* are lost during

larval development to adults (Nelms et al., 2013). During development WNV is lost, suggesting different rates of infection of egg rafts, larvae, and adult F_1 mosquitoes.

In Montana, *Culex tarsalis* is the prominent WNV mosquito vector (Johnson 2005) and has the shortest extrinsic incubation period of WNV through vertical transmission (Anderson et al., 2012). Natural vertical transmission research in *Culex pipiens* is extensive, but *Culex tarsalis* has no research regarding natural vertical transmission. Montana as a state has no evidence of vertical transmission.

Regions containing greater *Typha* (cattails) stem radius and density are good indicators of *Culex tarsalis* larvae presence (Goudarz Molaei et. al. 1990). In my research I gathered mosquito larvae by dipping methods throughout north central Montana, particularly at sites that contained *Typha*. CO_2 traps were good indicators in determining the presence of *Culex* mosquitoes in a region. Samples were brought back for analysis via RT-PCR for detection of WNV. First, I hypothesized naturally infected *Culex* larvae would be observed in Montana. Secondly, *Culex* adult males found in CO_2 baited traps would test positive for WNV. Thirdly, *Culex* species would display an overall loss of infection through development (e.g. 4^{th} instar vs. emerging adults).

Materials and Methods

Step 1: *Sampling for Culex mosquito larvae in Montana.*

Sample sites were chosen based on a record of *Culex* mosquitoes presence in Montana ((Hokit et al, submitted). Ponds and wetlands containing *Typha* in a sample site were dipped for *Culex* larvae. A wooden stick connected to a plastic bowl was used to capture larvae (Figure 1). Once a site was identified to have larvae, larvae were placed into

bigger plastic containers to collect as many larvae as possible over a 30-minute period. Once collection was complete larvae were transferred to 50-mL Falcon tubes under ice to be transported to the laboratory where they were stored in a refrigerator at 4 ^{0}C. A sub-set of the sample was kept alive and placed into a growth container to be reared into adults. Larval samples to be stored were strained through a strainer having a mesh gap of 0.34 mm. Larvae were concentrated, then killed using a 100% ethanol solution and stored in 50-mL Falcon tubes at 4 ^{0}C.

Step 2: *Identification of Culex larvae*

Ethanol killed larvae were placed onto empty petri dishes to be examined under the microscope. Patches of hairs located on siphons of the larvae allowed for identification of *Culex* larvae. Plastic Pasteur pipettes were used to transfer mosquito larvae from the petri dish into appropriate Falcon tubes labeled "non-*Culex*' and '*Culex*'. Once they were identified as *Culex* larvae they were placed into the -80 ^{0}C freezer for later testing.

Step 3: RNA extraction

RNA was extracted from individual larvae in 1.5 mL centrifuge tubes to which 300 µL of buffer RLT and 600 µL of RNAlater (Qiagen) were added. A 1/8 metric bead was placed into each centrifuge tube to homogenize samples in a TissueLyser LT (Qiagen) for 30 seconds or until larval parts were well into solution at maximum speeds. A total of 300 µL of homogenate was pipetted into a separate centrifuge tube labeled for later testing and placed into -80 ^{0}C freezer. The remaining 600 µL was placed into a QIACUBE. (QIAGEN miniprep kit for fibrous tissue Protocol 372). After RNA extraction samples were prepped for Real Time Polymerase Chain Reaction (RT-PCR).

Step 4: RT-PCR

Primers, as used by Lanciotti, and probes were first diluted from stock solutions to appropriate concentrations. Master mix (Qiagen) was then made using stock solutions of probe, forward primer, reverse primer, RNase free water, and 4x TaqMan. Primers and probes were made for both the 3' end and ENV genes found in the WNV, this allowed for a broader identification of the presence of WNV. Each well on the PCR plates contained: 2.5 µL of sample, 9.5 µL of RNase free water, 1 µL working probe, 1 µL working forward primer, 1 µL working reverse primer, and 5 µL 4x TaqMan (Lanciotti et al., 2012). Positive (previous samples testing positive for WNV) and negative controls were also placed on PCR plates. A plate consisted of repeats of each sample (including positive and negative samples) for both the 3' and ENV (Table 1). Once PCR plates were prepared, adhesive PCR plate seals were placed over the PCR plates and the apparatus was inserted into the RT-PCR. Results were then displayed on the BIO-RAD IQ5 software showing the presence of WNV in the appropriate well locations.

Results

A total of 17 sample sites were surveyed for *Culex* larvae. Four sample sites out of seventeen resulted in successfully captured *Culex* larvae all within Fergus, Petroleum, and Phillips counties (Figure. 2). A total of 78 fourth instar larvae were captured from the four sample sites. Twenty-five of these fourth instar larvae were *Culex* larvae, zero tested positive for WNV (Table 2). Larvae that were too small to be identified were reared to adults for identification. A total of 87 adults were reared from first instar larvae. Only two *Culex* reared adult mosquitoes were present, zero tested positive for WNV (Table 3). Two

male *Culex tarsalis* mosquitoes were identified in CO_2 traps, zero tested positive for WNV (Table 4).

Discussion

This study looked to expand the understanding of natural vertical transmission of WNV. Results suggest that either natural vertical transmission is non-existent in Montana, thus not supporting the previously stated hypotheses, or vertical transmission does exist but was not detected. There may be several contributing factors for observing such results. First, the overall sample sizes for *Culex* larvae and *Culex* adult males were low. These low numbers can be attributed to both the collecting methods and locations surveyed in this research. Hours of surveying using the dipping method produced few *C. tarsalis* larvae. Although several sites yielded hundreds of larvae, they were often non-*Culex* larvae. CO2 baited traps proved to be an ineffective tool in collection of adult *Culex* males (Table 4). Second, the incidence of WNV in the state of Montana were low in summers of 2015 and 2016. A low incidence of WNV suggests a lower frequency of WNV in mosquito populations, thus limiting the ability of WNV to spread through vertical transmission. Limited WNV can be partially attributed to the fluctuating temperatures and frequent/variant rainfall during the summer months in Montana. Culex *tarsalis* populations and WNV thrive best in warm and dry temperatures (Hokit et al, 2017)

Future research should look to alternative methods in capturing *C. tarsalis* larvae. Gravid traps in particular are useful in capturing bloodfed mosquitoes and egg rafts (Popko & Walton 2016). Gravid traps employ a water source with different infusions that attract different species of mosquitoes (Popko & Walton 2016). Currently little is known regarding infusions that attract bloodfed *C. tarsalis,* although bulrush infusions may

suggest promise (Du & Millar 1999). Future research may also look to obtain samples from locations that have a high incident of WNV.

In summary, this research demonstrated the difficulty of collecting *Culex tarsalis* larvae. Methods used in this research were not effective and future research should look to improve upon these methods. Natural vertical transmission may be occurring in *C. tarsalis* population, but without adequate methods of collection it is difficult to document. Gravid traps with infusions attracting *C. tarsalis* is a direction that should be considered for larvae collection in the future.

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Figures and Tables

Figure 1.

Sampling for *Culex* larvae.

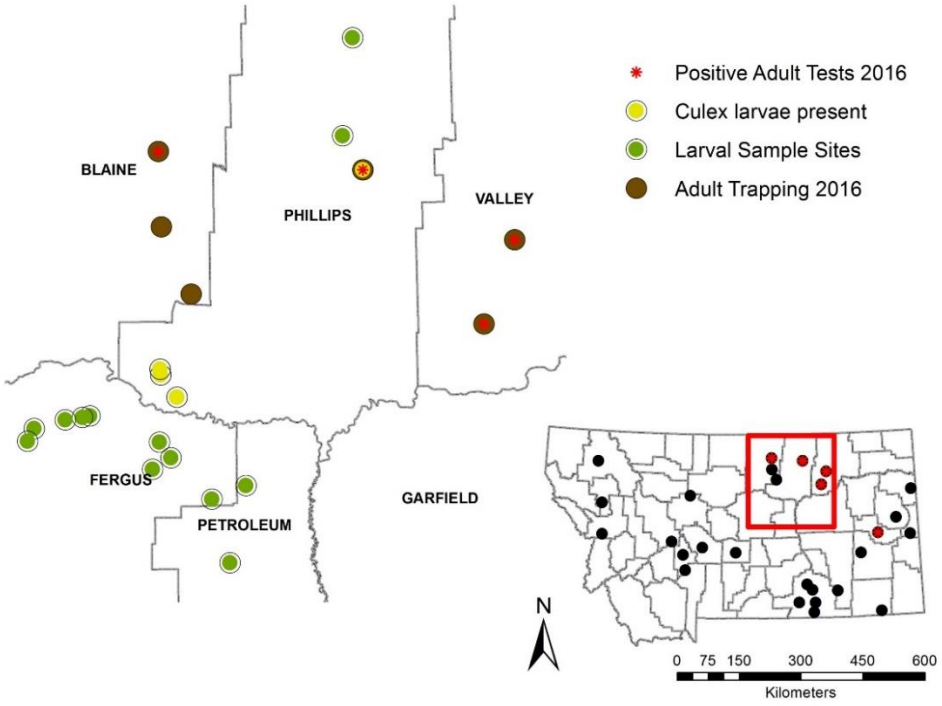

Figure 2.

Sites sampled for *Culex tarsalis*

Table 1.

PCR plate setup example of 3’ and ENV primer. (+) positive control well locations, (-) negative control well locations, A3, A4, and etc. well locations for different sites.

	3' Primer/Probe set			ENV Primer/Probe set		
	1	2	3	4	5	6
A	+	3	7	+	3	7
B	+	3	7	+	3	7
C	-	4	8	-	4	8
D	-	4	8	-	4	8
E	1	5	9	1	5	9
F	1	5	9	1	5	9
G	2	6	10	2	6	10
H	2	6	10	2	6	10

Table 2.

Culex larvae found at sample sites and tested for WNV.

County	Location	Date Sampled	Total Larvae	Culex Larvae	% Culex larvae	(+) WNV
Phillips	Auto Trail #1	7/14/2016	1	1	100.00%	0.00%
Phillips	Auto Trail #2	7/14/2016	3	3	100.00%	0.00%
Phillips	Highway 191 CMR	7/14/2016	7	7	100.00%	0.00%
Bowdoin	Bowdoin	7/27/2016	67	14	20.90%	0.00%

Table 3.

1st instar larvae reared to adults to determine species and tested for WNV.

County	Location	Date Sampled	Total Larvae	Total Reared Adults	Reared Culex Tarsalis	% Culex Tarsalis	(+) WNV
Mix	2015 trails	summer 2015	100+	55	1	1.80%	0.00%
Phillips	16-001 Dry Fork	7/14/2016	100+	23	0	0.00%	0.00%
Phillips	16-002 Dry Fork	7/14/2016	100+	8	0	0.00%	0.00%
Phillips	Valentine 27.3	7/26/2016	5	1	1	100.00%	0.00%

Table 4.

Adult male *Culex tarsalis* found in CO_2 traps, tested for WNV.

County	Location	Date Sampled	Male Culex Tarsalis	(+) WNV
Bowdoin	Bowdoin	8/8/2016	1	0.00%
Hill	Havre	8/8/2016	1	0.00%