



RNA isolated from Mosquito Pools Inhibits West Nile Virus Real Time RT-PCR: A Case Study Using the Smart Cycler

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INTRODUCTION

In the period between August and November 2000, we tested 440 birds and 200 mosquito pools for West Nile Virus. The virus was detected in 7 birds, but none of the mosquito pools tested positive for the virus. Since these pools were collected in the areas where positive birds were identified, the possibility exists that some positive mosquito pools may have been missed due to inhibition of the RT-PCR reaction. Preliminary experiments were launched in the spring of 2001 to answer this question. Mosquito pools were spiked with a known amount of RNA that tested positive for WNV. The Ct values reported for these reactions were compared with that from the positive RNA. Real Time RT-PCR reactions (25 μ L) were carried out using the TaqMan One Step RT-PCR kit (Roche) or the MasterAmp™ RT-PCR Kit for High Sensitivity (Epicentre), and a FAM-tagged probe. Thermal cycling was performed in the Smart Cycler (Cepheid) with fluorescence detection during the extension step.



Culex pipiens, the most common WNV mosquito vector in the northeastern part of the United States.

Our preliminary data show that the majority of mosquito pools tested (57 out of 66) exhibited some level of inhibition. Inhibition patterns could be grouped into 3 categories: minor (where Ct values increased by up to 3 cycles), significant (Ct increased by more than 5 cycles), and total (Ct = 0, i.e. signal disappeared completely). Thirty-seven pools exhibited minor inhibition, 18 showed significant inhibition and 2 pools were totally inhibited. The majority of these pools (42 out of 57) contained 1-4 mosquitoes each, 12 pools had 6-25 mosquitoes each, while 3 pools had 26-50 mosquitoes each. There was no apparent correlation between the number of mosquitoes in the pools tested and the level of inhibition observed. Furthermore, we found no clear link between mosquito genus or species and inhibition.

The cause of RT-PCR inhibition was not the focus of this study. One possible explanation however could be the presence of inhibitors in the blood mosquitoes feed on. Our results demonstrate the importance of internal controls in these tests to ensure the validity of the negative results. We are currently in the process of developing controls for WNV and related viruses.

RATIONALE

RNA from various mosquito pools was used in real time RT-PCR reactions. Each experiment contained three reactions:

Reaction	Description	Expected Results
A	2.5 μ L of RNA from a mosquito pool that has previously tested negative for WNV	Ct = 0 (no signal)
B	2.5 μ L of WNV RNA isolated from the brain of a crow (POSITIVE C)	signal with a Ct value ranging from 15 to 30
C	2.5 μ L "A" + 2.5 μ L "B"	Ct value may be unchanged, increase due to partial inhibition, or no signal will be detected (Ct = 0) due to total inhibition

Collected mosquitoes were first separated by species and species from each trap were pooled together. A total of 66 pools were used in this study, of which only 9 pools exhibited no detectable inhibition of the RT-PCR reaction. In the remaining 57 pools, the number of mosquitoes in each pool was as follows

Mosquitoes/Pool	Number of Pools
1-4	42
6-25	12
26-50	3

While each pool contained only one species, a large number of different species were tested in this study.

METHODS

In a Biological Safety Cabinet

Mosquito Preparation: Mosquito pools were homogenized in 15 mL sterile Falcon conical tubes using 2 copper BBs per tube and 2 mL of M4 medium (Micro Test, GA). The tubes were vortexed for 2 minutes, then centrifuged at 4 °C for 15 minutes. The supernatant was stored at -20 °C until ready for RNA extraction.

RNA Extraction: RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen). Typically, 140 μ L of the supernatant were mixed with the lysis buffer (provided in the kit) and vortexed for 1 minute in the BSC.

On the Bench

RNA Extraction: RNA extraction was resumed on the bench using the manufacturer's instruction without modifications.

Primers and Probes:

Designation	Sequence (5'-3')
Forward	cag acc acg cta cgg cg
Reverse	cta ggg cgg cgt ggg
Probe	6FAM-ctg cgg aga gtg cag tct gcg at-TAMRA

Reaction Mixtures: Two commercial kits were used interchangeably in this study: the MasterAmp™ RT-PCR Kit for High Sensitivity (Epicentre Technologies) and the TaqMan™ One Step PCR Master Mix Reagent Kit (Roche). Reactions contained the following components:

Epicentre Kit		Roche Kit	
Component	Final Conc.	Component	Final Conc.
RNase Free Water	To 20 µL	RNase Free Water	To 20 µL
RT-PCR Buffer	1X	2X PE READY MIX	1X
MgCl ₂	3 mM	Forward Primer	1 µM
PCR Enhancer	1X	Reverse Primer	1 µM
MnSO ₄	0.5 mM	Probe	0.5 µM
dNTP Mix	200 µM	40X ENZYME MIX	1X
Forward Primer	1 µM		
Reverse Primer	1 µM		
Probe	0.5 µM		
RetroAmp RT Polymerase	1.25 U		

Reactions:

	1	2	3
Master Mixture	20.0	20.0	20.0
RNA from mosquito pool	2.5	0.0	2.5
RNA from WNV-positive crow (POSITIVE C)	0.0	2.5	2.5
Water	2.5	2.5	0.0

RT-PCR Conditions: Real time RT-PCR was performed in a Smart Cycler (Cepheid) using the following program:

Stage	Program	Epicentre Kit		Roche Kit		
		Temp, °C	Time, Sec.	Program	Temp, °C	Time, Sec.
1	HOLD	60	1200	HOLD	50	1800
2	HOLD	94	60	HOLD	95	600
3	30 cycles	94	10	45 cycles	95	15
		60	10		60	60
		68	30			

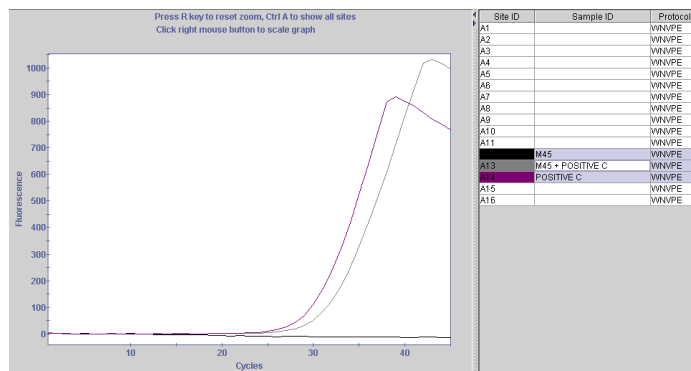


Figure 1. Minor Inhibition. Thirty-seven of the 66 mosquito pools tested in this study showed a pattern similar to the one shown here. The Ct value increased on the average by 3 cycles. *Reaction conditions: Roche kit.*

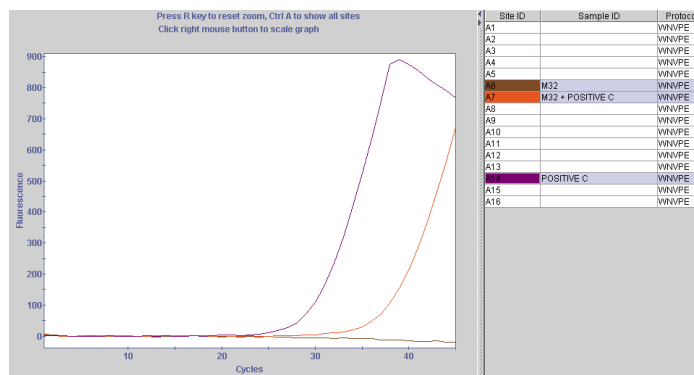


Figure 2. Significant Inhibition. Eighteen mosquito pools showed significant inhibition of the RT-PCR reaction, as demonstrated by a 5-cycle increase in their Ct value. *Reaction conditions: Roche kit.*

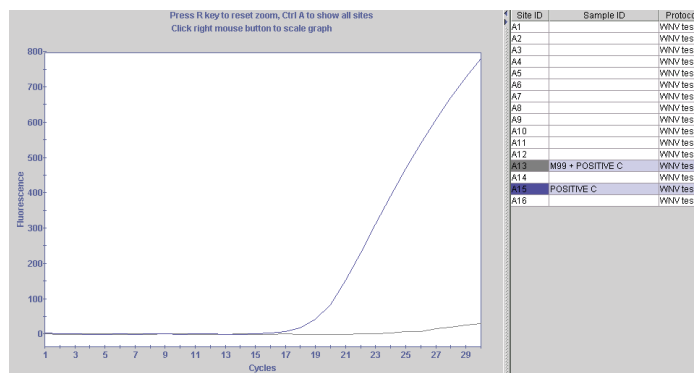


Figure 3. Total Inhibition. This is one of the 2 mosquito pools where the RT-PCR was totally inhibited in this study. Pool M99 was negative for WNV when tested without spiking (not shown). *Reaction conditions: Epicentre kit.*

INTERPRETATION OF RESULTS

Our results showed inhibition of the RT-PCR reactions in 57 out of the 66 mosquito pools tested. Inhibition was classified onto 4 categories as described in the following table.

Change in Ct Value	Interpretation	Example
Increased by up to 3 cycles	Minor inhibition	Figure 1
Increased by more than 5 cycles	Significant inhibition	Figure 2
Ct = 0 (no signal)	Total inhibition	Figure 3
No change	No inhibition	Figure 4

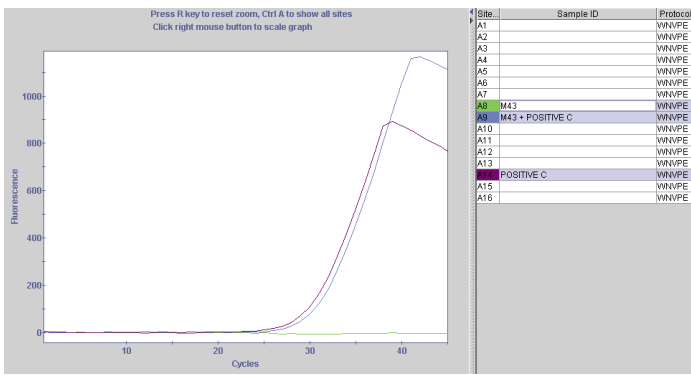


Figure 4. No Inhibition. Nine mosquito pools showed no evidence of RT-PCR inhibition. The Ct value of the positive control did not change in the presence of RNA from mosquito pools. *Reaction conditions: Roche kit.*

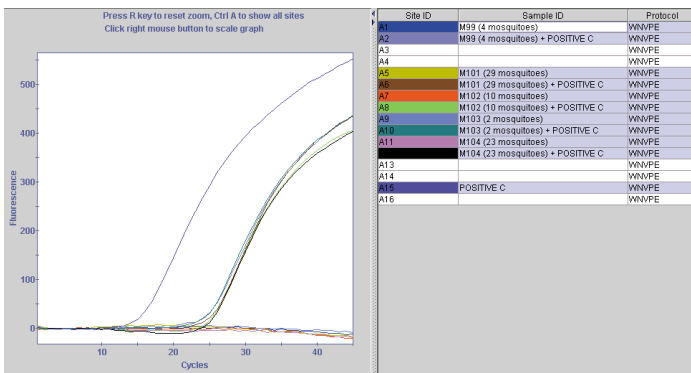


Figure 5. Effect of the Number of Mosquitoes in Each Pool. The inhibitory effect of some mosquito RNA preparations on RT-PCR does not appear to be related to the number of mosquitoes present in each pool. In this study, this number ranged from 1–50. The extracted RNA was eluted consistently with the same volume of elution buffer. *Reaction conditions: Roche kit.*

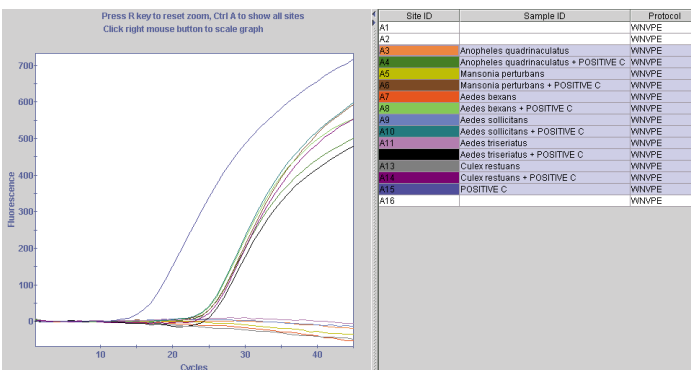


Figure 6. Effect of Genus and Species on Inhibition. Numerous mosquito species were used in this study. Representative results from some of these are shown here. It is clear that inhibition is seen regardless of the genus or the species. Moreover, some RNA preparations from a given species may show inhibitory effects, while other preparations from the same species may not (not shown). This suggests that it is probably the feeding patterns of the mosquitoes rather than the genus or species that are responsible for these observations. *Reaction conditions: Roche kit.*

CONCLUSIONS

- ◆ RNA extracted from homogenized mosquito pools often contains inhibitors to the PCR reaction (and possibly the reverse transcription reaction as well).
- ◆ The level of inhibition varies from one pool to the other, and ranges from none to total.
- ◆ Inhibition was observed in reactions containing RNA from mosquito pools ranging in size from 1 to 50 mosquitoes, with no apparent correlation between the number of mosquitoes in the pool and the level of inhibition.
- ◆ Inhibition does not appear to be linked to a specific genus or species.
- ◆ The cause of RT-PCR inhibition is not yet understood, but it is likely to be linked to the blood these mosquitoes feed on rather than the mosquitoes themselves.
- ◆ These observations demonstrate the importance of including internal controls in such tests in order to ensure the validity of the negative results.
- ◆ We are currently in the process of developing such controls for West Nile and related viruses.

ACKNOWLEDGEMENT

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