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Real-Time Rt-Pcr Assays To Detect West Nile Virus In Mosquito Pools

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Abstract

Introduction: West Nile virus (WNV) is a mosquito-borne flavivirus that was first reported in North America in 1999. Human infections with West Nile are often mild or asymptomatic, but some infections progress to life-threatening encephalitis. The Connecticut Agricultural Experiment Station performs viral cell culture screening on mosquito pools collected throughout the state each summer to detect viruses circulating in mosquito populations. Three different real-time RT-PCR assays are used to identify WNV in positive cell cultures. Many surveillance programs do not use cell culture and only use real-time PCR to screen pools. Although this can provide for faster analyses, some positive pools may be missed if mutations or other factors cause the PCR reaction to fail. **Objectives:** My goal was to evaluate the performance of three primer/probe sets in comparison to cell culture to evaluate the feasibility of direct PCR analysis for surveillance. **Methods**: RNA was extracted from 90 WNV positive mosquito pools and 90 controls from the 2013 surveillance season. Real-time RT-PCR was performed once on all samples for each primer and probe set. Pools known to be positive in cell culture that failed to amplify were sequenced and aligned with primer and probe sequences to identify any mutations in the primer or probe binding regions. **Results**: Of the three assays, the Tang, et al. series published in 2006 had the highest sensitivity and specificity. Only one strain that failed to amplify had mutations in the critical primer/probe binding regions, so most false negatives are likely due to other factors. **Conclusion**: The three assays performed well, with one set performing better than the other two. Surveillance using only one real-time RT-PCR assay to test samples should use the assay designed by Tang, et al (2006).

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Introduction

Objectives

West Nile virus (WNV) is a mosquito-borne flavivirus capable of causing severe neuroinvasive disease in humans. Surveillance systems throughout the United States and Europe use cell culture and/or real-time reverse transcriptase polymerase chain reaction (RT-PCR) to identify virus in collected mosquitoes[1,2,3,4]. While cell culture is robust and considered the "gold standard", PCR assays are faster and easier to use on a large scale. My objective was to evaluate three commonly used TaqMan real-time RT-PCR primer series against the "gold standard" of viral cell culture. Comparison of the two can identify strengths and weaknesses of PCR-based detection, and these results facilitate selection of the most appropriate detection method.

Background

West Nile virus (WNV) is a flavivirus in the Japanese encephalitis serotype group of the genus *Flavivirus*. As with all flaviviruses, WNV has a single strand, positive sense genome approximately 11kb long that contains a single open reading frame with 5'- and 3'-untranslated regions (UTRs). The genome codes for four structural proteins and seven non-structural proteins that are translated as a single polypeptide. While the UTRs do not code for proteins, these sequences are highly conserved, and interaction between secondary structures in these regions are critical for genome cyclization. Cyclization of the genome is required for the function of the flavivirus RNA-dependent RNA polymerase (RdRp), and certain mutations in these un-translated regions prevent transcription of viral RNA[5].

WNV has a worldwide geographic distribution and has been isolated on all continents except Antarctica. There are multiple diverging lineages with lineage 1 being the most widespread and virulent and the lineage responsible for the most human outbreaks. The virus was first identified in 1937 in a sample from a woman in the West Nile region of Uganda[6]. Outbreaks occurred throughout Africa, Europe, Asia, and Australia starting in the 1950s, but the Western hemisphere was free of WNV until very recently[2]. WNV was first detected in the Western hemisphere in New York City in 1999, with the virus isolated from birds and mosquitoes[7]. The initial strain NY99 was closely related to a sample isolated in Israel in 1998, and it has never been determined whether the virus was introduced by an infected human or an infected mosquito[6]. By 2004, the virus had been identified in all 48 contiguous states, with two new strains WN02 and WN03 replacing the original NY99 strain[8,9].

Culex species mosquitoes are the primary vector for WNV, and the primary reservoir and amplifying hosts are passerine birds. Humans and other mammals are incidental hosts that do not develop sufficient viral titers to re-infect mosquitoes. Though most humans infected have an asymptomatic infection, 20% of infected individuals develop West Nile Fever, a short, mild illness, and <1% develop potentially fatal neuroinvasive disease[10].

There are multiple ways to screen mosquitoes for WNV infection. Cell culture is used to screen for the presence of cytopathic effect (CPE), a particular type of cell death that is a marker of viral infection. Collected mosquitoes are sorted by sex and species, and pools of up to 50 mosquitoes per species per site are assembled. These mosquitoes are ground and inoculated into Vero cell cultures. Cultures are checked days 3-7 post-inoculation for signs of CPE. After

identifying potentially positive cultures, molecular assays such as real-time and conventional RT-PCR are used to identify the virus present. This process requires maintaining a cold chain and biosafety level 3 containment to grow virus in cell culture, making cell culture logistically challenging[11]. The development of real-time reverse transcription polymerase chain reaction (RT-PCR) assays to detect WNV has allowed for rapid identification of WNV RNA in birds, mosquitoes, humans, and other mammals[2,4]. These real-time RT-PCR reactions are often used independently of cell culture by surveillance programs to test mosquitoes of interest (*Culex* species, primarily). However, PCR and other molecular assays may fail to detect the RNA of viruses that contain mutations in the primer and/or probe binding regions, leading to false negative results[12]. Cell culture is a robust and accurate method to determine the presence of virus in pools because a positive cell culture can drive additional testing if the first primer set does not give a positive result. This subsequent PCR testing can potentially identify strains carrying mutations that interfered with the first test. Antigen-detecting tests like VecTest can also be used for West Nile virus surveillance, though they are more commonly used to test for the presence of virus in dead birds. VecTest has also been shown to have a lower sensitivity than RNA-detecting tests like PCR[13]. As the geographic distribution of WNV spreads and overlaps with other flaviviruses, the focus is beginning to shift toward the development of multiplex assays capable of identifying multiple WNV lineages[4,14,15] and additional, closely related ,viruses in a single test[16,17,18].

Surveillance programs to detect WNV in mosquitoes and humans were established in several European countries in the late 1990s and early 2000s[1]. Many programs focus on the identification of WNV in mosquitoes because the number of positive mosquitoes in an area is

predictive of the risk of human disease, but collection and testing of dead birds is also a component of many programs[19]. When WNV emerged in New York in 1999, the state of Connecticut expanded its mosquito trapping program, then focused on identifying mosquitoes infected with Eastern Equine Encephalitis Virus, to screen mosquitoes in lower Fairfield and New Haven counties for WNV. WNV was successfully isolated in mosquitoes by the Connecticut program in 1999 using cell culture and detected by conventional RT-PCR[7]. New York began its own screening program in the summer of 2000 using the two real-time RT-PCR assays developed and published by Lanciotti et al in 2000[20]. Since then, the Centers for Disease Control and Prevention (CDC) established agreements with all fifty states under the Epidemiology Laboratory Capacity. This provided funding for detection and response to WNV and also created ArboNET, a national surveillance platform. All states established surveillance and control programs by 2005, but funding has declined substantially since then. In 2012, the CDC identified 45 control jurisdictions that continued to maintain mosquito surveillance capacity, though 70% of these sites were decreasing trapping and testing of mosquitoes. Currently, there are concerns regarding whether the United States can continue enough intensive surveillance to provide prevention and early detection services[3].

Research Design

Virus Strains

All WNV strains were isolated and identified by the Connecticut Agricultural Experiment Station in mosquito pools during the 2013 surveillance season using the methods described in Armstrong et al, 2011.

Real-time PCR

RNA was isolated from frozen mosquito pools for use in real-time RT-PCR analysis using the QIAmp Viral RNA Mini Kit (Qiagen, Valencia, CA). RNA was also isolated from an equal number of negative samples matched by species and time of collection to serve as controls. Amplification and data collection were performed using the BioRad MyiQ2 real-time polymerase chain reaction (PCR) detection system on 25µl TaqMan reactions for 50 cycles. Each 25µl reaction contained 8.8µl RNAse-free water, 12.5µl TaqMan ready-mix, 100µM of each primer, 33µM probe, and 0.5µl of PE kit enzyme. Analysis of real-time RT-PCR results was performed using BioRad iQ5 software. Cycles 5-10 were used for obtaining background fluorescence, and the threshold was set at 300 relative fluorescence units (RFU). Samples were considered positive if the cycle threshold (Ct) value was less than 37. Analyses were also performed for Ct cutoffs of 40 or 42, as these are sometimes used as described in the literature[12]. Calculation of sensitivity, specificity, Cohen's kappa statistic, and positive and negative predictive values was performed in Microsoft Excel for Mac 2011 (Microsoft, Redmond, WA, USA). Sensitivity and specificity provide important insights into how well a test can discriminate between positive and negative samples, and predictive values evaluate the

reliability of positive and negative test results. Cohen's kappa statistic provides some information regarding the agreement between two tests.

	Sequence 5' to 3'
WNV 10668 series	
WNV 10668	CAGACCACGCTACGGCG
WNV 11770	CTAGGGCCGCGTGGG
WNV 10692	[6~FAM]TCTGCGGAGAGTGCAGTCTGCGAT[TAMRA~6~FAM]
WNV 1160 series	
WNV 1160	TCAGCGATCTCTCCACCAAAG
WNV 1229C	GGGTCAGCACGTTTGTCATTG
WNV 1186	[6~FAM]TGCCCGACCATGGGAGAAGCTC[TAMRA~6~FAM]
WNV 10533 series	
WNV 10533	AAGTTGAGTAGACGGTGCTG
WNV 10625	AGACGGTTCTGAGGGCTTAC
WNV 10560	[6~FAM]CTCAACCCCAGGAGGACTGG[BHQ1a~6~FAM]

Table 1: Primers and probes for TaqMan WNV assays

Sequencing

Samples positive for WNV in vero cell culture and negative by one or more real-time PCR reactions were sequenced to determine whether mutations were present in the primer and/or probe binding regions. This information provides important guidance on potential reasons for real-time RT-PCR failure and whether accumulation of mutations is a problem that may be impacting results in surveillance settings. RNA was isolated from frozen vero cell culture using the QIAmp Viral RNA Mini Kit (Qiagen, Valencia, CA). Conventional RT-PCR using the Titan One Tube RT-PCR system (Roche, Indianapolis, IN) was performed to amplify the region of interest, either the envelope region or the 3' un-translated region (3'UTR). Reactions mixtures were 25µl containing 5µl 5X RT-PCR buffer, 2µl 25mM/each dNTPs, 1.25µl 100mM DTT solution, 0.5µl Titan enzyme mix, 0.25µl 20U/µl RNAse inhibitor, 0.5µl each 20µM primer, and 2µl RNA.

Samples were amplified in a BioRad MyiQ2 thermocycler for 35 cycles. Electrophoresis of the PCR product was performed on a 1.5% agarose gel stained with ethidium bromide to ensure the successful amplification of PCR product, and PCR product was purified for sequencing using the QIAquick PCR Purification Kit (Qiagen). For sequencing, 18µl reactions contained 12µl nuclease free water, 2µl 4µM primer, and 4µl template. Sequencing was performed by the Keck Biotechnology Resource Laboratory at Yale University. Due to unsuccessful sequencing reactions, four samples were re-submitted to the Yale DNA Analysis Facility on Science Hill. These 10µl reactions contained 7µl nuclease free water, 1µl 4µM primer, and 2µl template. Completed sequences were compiled using Chromas Pro (Technelysium, http://technelysium.com.au/?page_id=27) and aligned with the NY99 prototype strain using MEGA 4 (Molecular Evolutionary Genetics Analysis, http://www.megasoftware.net/mega4/mega.html).

	Sequence 5' to 3'
3'UTR	
WNV 10367	ACACAACTTTGGTTGAGGACAC
WNV 10916c	TTGTGGTGTTTTGTGGCACG
env	
WNV 1101	GATGAATATGGAGGCGGCCA
WNV 1816A	CCGACGTCAACTTGACAGTG

Table 2: Sequencing primers for the envelope gene and the 3'UTR

Presentation and Analysis of Findings

Sensitivity and Specificity

Three real-time RT-PCR primer sets were evaluated for sensitivity and specificity compared to viral cell culture results.

		Ct < 37	Ct < 40	Ct < 42
10533 series	Sensitivity	0.933	0.956	0.978
	Specificity	1.000	1.000	1.000
1160 series	Sensitivity	0.900	0.933	0.933
	Specificity	1.000	0.989	0.989
10668 series	Sensitivity	0.867	0.933	0.933
	Specificity	1.000	0.989	0.989

Table 3: Sensitivity and specificity for all three assays at three Ct cutoff values

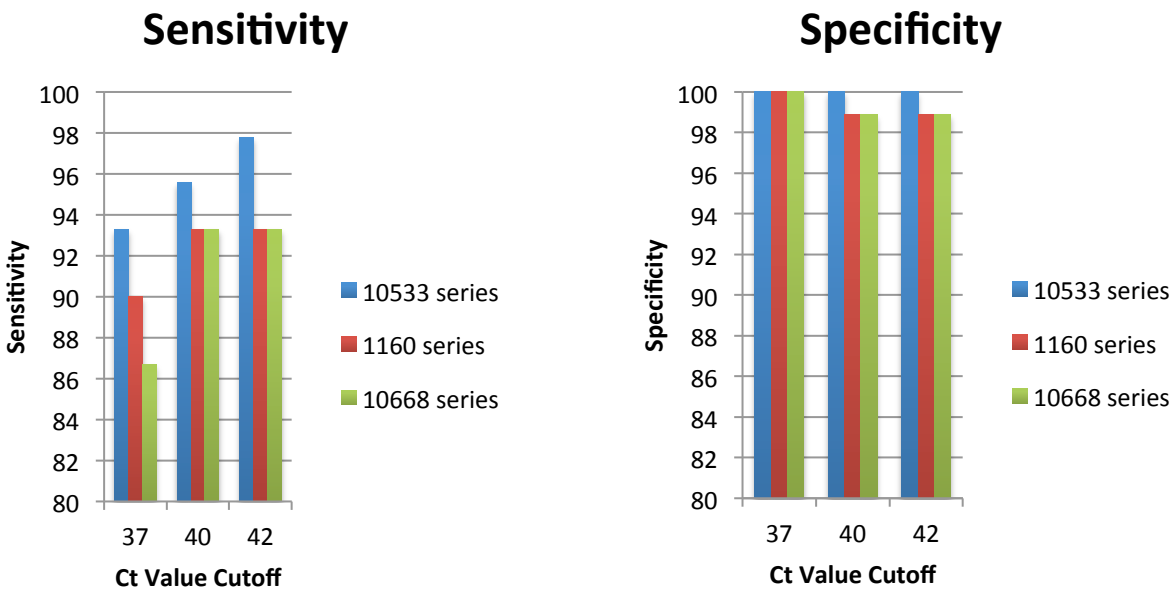


Figure 1: Sensitivity and specificity for each assay at different Ct cutoffs

The 10533 series was most sensitive for all Ct cutoff values and maintained 100% specificity. The 1160 series and the 10668 series had equivalent sensitivity and specificity at higher cutoff values, though the 1160 series was more sensitive when a Ct value of 37 was used.

Predictive Values

Positive and negative predictive values were calculated for all three assays. During the 2013 season, the CAES tested a total of 13,601 mosquito pools of which 90 were positive for WNV. The majority of virus-positive mosquito pools (n=88) were from four mosquito species: *Culex pipiens, Cx. restuans, Cx. salinarius, and Culiseta melanura*. The number of pools of these species tested in 2013 was 3,758. Both values for prevalence (0.7% of all mosquitoes and 2.2% of selected species) were used to calculate positive and negative predictive values for low and high prevalence settings respectively.

A.

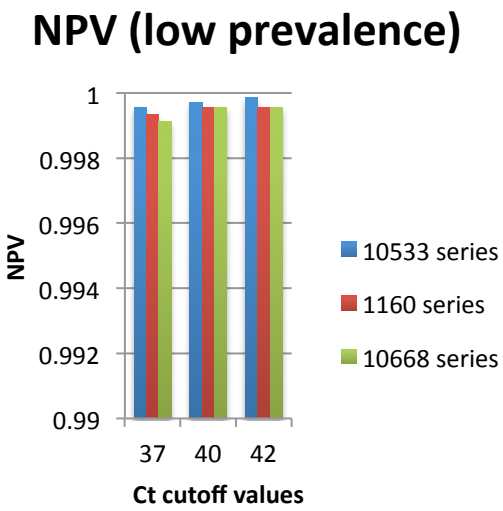
Prevalence 0.7%		Ct < 37	Ct < 40	Ct < 42
10533 series	PPV	1.0000	1.0000	1.000
	NPV	0.9995	0.9997	0.9998
1160 series	PPV	1.0000	0.3610	0.3610
	NPV	0.9993	0.9995	0.9995
10668 series	PPV	1.0000	0.3610	0.3610
	NPV	0.9991	0.9995	0.9983

B.

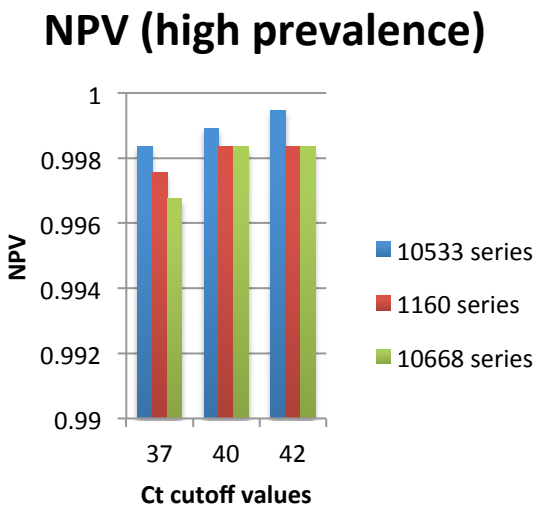
Prevalence 2.2%		Ct<37	Ct<40	Ct<42
10533 series	PPV	1.0000	1.0000	1.0000
	NPV	0.9983	0.9969	0.9994
1160 series	PPV	1.0000	0.6754	0.6754
	NPV	0.9975	0.9983	0.9983
10668 series	PPV	1.0000	0.6754	0.6754
	NPV	1.0000	0.6754	0.6754

Table 4: Positive and negative predictive values for each assay at three different Ct cutoffs. A: Predictive values for surveillance that includes testing all mosquitoes tested. B: Predictive values for surveillance that only tests specific mosquito species of interest.

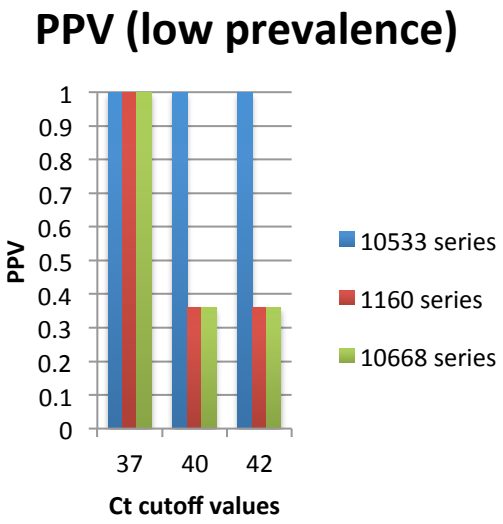
A



B



C



D

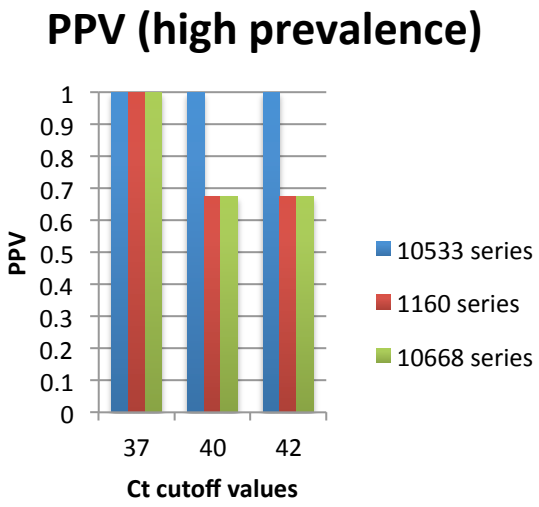


Figure 2: A: Negative predictive values for low prevalence testing B: Negative predictive values for high prevalence testing C: Positive predictive values for low prevalence testing D: Positive predictive values for high prevalence testing.

Cohen's kappa Statistic

In order to assess agreement between cell culture methods and real-time RT-PCR assays, Cohen's kappa statistic was used. Generally, values close to 1 indicate very good agreement between two tests[21]. All combinations of assay and Ct cutoff had very high kappa statistics (excellent agreement), indicating a strong concordance between the two diagnostic techniques.

Cohen's kappa	Ct < 37	Ct < 40	Ct < 42
10533 series	0.93	0.96	0.98
1160 series	0.90	0.92	0.92
10668 series	0.87	0.92	0.92

Table 5: Calculated Cohen's kappa statistics for each assay at different Ct cutoffs

Sequencing

Sequencing was performed on all samples that had false negative results for one or more primer sets to identify any mutations that may have impacted the performance of the assay. Four WNV strains had a nucleotide substitution in primer or probe binding regions of the genome. One strain (7643-13) contained mutations in both the primer and probe binding regions of *env*, and three strains (13028-13, 8656-13, 8624-13) contained a mutation in a primer binding region in the 3'UTR. All other sequences were identical to the NY99 strain in the critical primer and probe binding regions.

Strain	**Mutations**
7645-13	1170 (C ➔ T) 1191 (G ➔ A) 1227 (C ➔ T)
13028-13	10544 (A ➔ G)
8656-13	10690 (T ➔ C)
8624-13	10690 (T ➔ C)

Table 6: WNV strains and mutations in the relevant primer or probe binding regions

Discussion

West Nile virus has now been endemic in the United States for 15 years. Two of the three commonly used primer sets assessed were designed in 2000, shortly after the introduction of WNV[2]. As an RNA virus, WNV mutates rapidly, and viruses circulating in 2014 have accumulated mutations in the process of adapting to indigenous mosquitoes in North America that may interfere with the performance of these primers. The more recently designed primer set was designed using two lineage 1 strains (one from New York and another from Europe) and one lineage 2 strain to specifically identify highly conserved regions of the genome. Lineage 2 is primarily found in sub-Saharan Africa but is becoming a greater problem in Southern and Eastern Europe[4]. This newer primer set performed better than the older primer sets in terms of both sensitivity and specificity at all three Ct cutoff values and retained higher positive and negative predictive values. It also had the greatest agreement between the cell culture and real-time RT-PCR methods. The sensitivity and specificity of the 10533 series when compared to other assays is consistent with published literature[22,23].

Because of the risk of critical mutations, use of more than one assay has been recommended in the literature[12]. The *env* gene sequence contains enough variability to be used in phylogenetic analyses, and geographic and temporal clusters of WNV can be identified by sequencing this region of the genome[24,25]. The nucleotide variability that allows for effective phylogenetic analysis may be indicative of high rates of mutation that may lead to point mutations in the primer or probe binding regions. Point mutations in these areas may compromise the sensitivity of the real-time PCR assay targeting this region (1160 series) and reduce accuracy in estimation

of viral burden in areas under surveillance. Surprisingly, our sequencing analysis of *env* showed mutations of the primer and probe binding regions in only one strain, indicating that mutations were not the most common reason for PCR failure. While the 3'UTR does not code for proteins, the sequence and secondary structures play vital roles in cyclizing the genome to permit transcription of viral RNA. Mutations in the 3'UTR can attenuate or completely inhibit viral replication, so these regions should be well conserved[5,26].

Conclusions

All assays performed well in comparison to cell culture, and mutations are not likely to be interfering with a large number of test results. One real-time RT-PCR assay outperformed the others in sensitivity, specificity, agreement with cell culture, and predictive values. For surveillance programs only running one assay on samples, the assay designed by Tang et al (2006) is recommended. Few strains contained mutations in the primer or probe binding regions of the genome, so mutation was not a factor in most of the real-time RT-PCR false negatives.

Limitations

The BioRad MyiQ2 real-time PCR system and the iQ5 software used in the real-time PCR analysis did not give clean amplification curves on many samples tested, and unstable baselines resulted in higher Ct values in some samples and lower Ct values in others. The number of baseline cycles and the threshold were adjusted in an attempt to separate the amplified signals (positive samples) from the linear signals (negative samples), and a baseline from cycles 5-10 and a threshold of 300 RFU was selected. These adjustments are by their very nature subjective. Multiple techniques can be used to select baseline and threshold values, but there is no one method that works best in all situations[27].

Mosquito pools yielding positive cell cultures are assumed to be positive for virus and may be tested repeatedly until an identification is made. However, it is possible that strains of WNV with significant mutations in multiple primer and probe binding regions may have still failed to identify these strains as WNV. Re-testing of these samples using other real-time PCR assays

should, in theory, identify strains with mutations in only one primer/probe binding region. However, two of the 90 strains tested here were missed by all three primer sets, and the prevalence of mutations that impact the sensitivity of these assays is likely underestimated.

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