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Real time PCR assay for detection of all known lineages of West Nile Virus

Ana Vázquez [1,*] Laura Herrero[1], Anabel Negredo[1], Lourdes Hernández[1], María Paz Sánchez-Seco[1], Antonio Tenorio[1]

Author affiliations[1]:
All authors work at the Laboratory of Arboviruses and Imported Viral Diseases,

National Centre for Microbiology, Instituto de Salud Carlos III Ctra. Majadahonda-Pozuelo, km.2
28220 Majadahonda, Madrid, Spain

* Corresponding author:
Ana Vázquez, PhD
Ctra Majadahonda-Pozuelo, Km 2,2. 28220 Madrid (Spain) Ph: +34 91 8223952. E-mail: a.vazquez@isciii.es

Abstract

West Nile virus (WNV) is one of the most widespread arbovirus and a large variety of WNV strains and lineages have been described. The molecular methods for the diagnosis of WNV target mainly lineages 1 and 2, which have caused outbreaks in humans, equines and birds. But the last few years new and putative WNV lineages of unknown pathogenicity have been described. Here we describe a new sensitive and specific real-time PCR assay for the detection and quantification of all the WNV lineages described until now. Primers and probe were designed in the 3´-untranslated region (3'-UTR) of the WNV genome and were designed to match all sequenced WNV strains perfectly. The sensitivity of the assay ranged from 1,5 to 15 copies per reaction

depending on the WNV lineage tested. The method was validated for WNV diagnosis using different viral strains, human samples

(cerebrospinal fluid, biopsies, serum and plasma) and mosquito pools. The assay did not amplify any other phylogenetically or symptomatically related viruses. All of the above make it a very suitable tool for the diagnosis of WNV and for surveillance studies.

Key words: West Nile virus, Lineages, Flavivirus, Diagnosis, Real-time PCR, Surveillance

1. Introduction

West Nile virus (WNV) is a widespread re-emerging pathogen which is classified in the Japanese encephalitis serocomplex of the *Flavivirus* genus, family *Flaviviridae*, and is one of the most threatening flaviviruses around the world (Colpitts et al. 2012). WNV is a single-stranded RNA virus with a positive-polarity genome of approximately 11kb. Both termini of the genomic contain sequences that do not encode viral proteins (5′ and 3′ untranslated regions). The encoded polyprotein is translated and co- and post- translationally processed by viral and cellular proteases into three structural (capsid [C], premembrane [prM] or membrane [M], and envelope [E]) and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins (Lindenbach and Rice 2003).

WNV is a mosquito-borne flavivirus which is maintained in nature in an enzootic transmission cycle between avian hosts and mosquito vectors, mainly of the *Culex* genus. Spillover of WNV infection to humans and horses, which are accidental hosts, may occasionally occur through the bite of infected vectors, especially when their density is very high (Barzon, Pacenti, et al. 2015). Most WNV infections are

asymptomatic in horses and humans or are associated with an influenza-like illness (fever, weakness, and myalgia). Less than 1% of infections in humans and 10% of infections in horses evolve to acute meningitis, encephalitis, or flaccid paralysis (Beck et al. 2013).

WNV was isolated from a febrile patient in Uganda in 1937 (Smithburn, Hughes, and Burke 1940), but nowadays the virus is circulating in all continents and has become one of the most widespread vector-borne viruses causing large outbreaks in the Americas, Europe, Asia and more recently Australia (Rizzoli et al. 2015). Prior to the 1990s, the WNV threat to public health was not widely appreciated, but over the last 20 years,

several outbreaks in humans have been reported in the Mediterranean basin and southern Europe, a few with fatal cases of encephalitis (Zeller and Schuffenecker 2004). This WNV re-emergence led to an intensified surveillance for WNV infection in humans, horses, birds, and mosquitoes in several high-risk areas. This has resulted in the detection and/or isolation of many different strains of WNV, and the sequencing and phylogenetic analysis of these genomes have identified different genetic lineages (lineage 1a, 1b, 1c, 2, 3, 4a, 4b, 4c, 5, 6 and 7) (Rizzoli et al. 2015). Of these lineages, only lineages 1 and more recently lineage 2 have been definitely associated with significant outbreaks in humans. Strains classified as WNV lineage 1 are endemic in the Mediterranean basin, Eastern Europe, North America, the Caribbean and Latin America, and have caused human, equine and avian epidemic outbreaks (Donadieu et al. 2013). WNV lineage 2 originally circulated only in sub-Saharan Africa, but in 2004 emerged in central Europe, spreading from Hungary to Austria and to southern European countries (Romania, Greece, Italy, Sardinia and Serbia) where, since 2010, it has led to several human outbreaks of neuroinvasive disease (Hernández-Triana LM 2014). Recent studies indicated that the virus had evolved and diverged during its extension in Europe, leading to the emergence of novel genotypes (Barzon, Papa, et al. 2015).

Other genetic lineages described more recently, and not associated so far with human or animal disease, showed 20%–30% genetic divergence from lineages 1 and 2. WNV lineage 3 (Rabensburg virus) was first isolated from *Cx. pipiens* mosquitoes in Czech Republic in 1997 (Bakonyi et al. 2005). Lineage 4a (or lineage 4) consists of the LEIVKrnd88-190 strain isolated from *Dermacentor marginatus* ticks in Russia in 1998 and later in mosquitoes and frogs (Lvov et al. 2004). Lineage 4b was identified in 2006 in *Cx. pipiens* mosquitoes from southern Spain (Vazquez et al. 2010). Lineage 4c was detected in *Uranotaenia unguiculata* mosquitoes in Austria in 2013 (Pachler et al. 2014). The WNV lineages 5, 6 and 7 include the Sarawak viruses found in Malaysia in 1996 from *Cx. neavei* mosquitoes, the Koutango virus found in ticks and rodents from Senegal, and a new virus isolated in Kedougou, southeastern Senegal, in 1992 (Fall et al. 2014).

Laboratory diagnosis of WNV infection is based on the detection of WNV RNA or virus isolation in cell culture from serum, CSF or urine samples

(direct diagnosis) and on the demonstration of a specific immune response against the virus (indirect diagnosis) (Sambri et al. 2013). Nucleic acid based techniques such us RT-PCR have the advantage of sensitivity, specificity and rapidity in the RNA detection of WNV in biological specimens and represents a rapid method to unambiguously prove acute infection. A major disadvantage of all PCR techniques is that new emerging WNV strains may acquire mutations in the PCR-primer binding sites which render them undetectable to currently existing assays (De Filette M 2012). Therefore, it is essential that current PCR methods are constantly being checked and updated for their sensitivity and suitability to detect newly emerging WNV strains to cope with the observed and predicted genetic variability of WNV strains and lineages (Rizzoli et al. 2015). The molecular assays developed until now to detect this virus are focused only on the detection of the WNV lineages 1 and 2 and these methods do not cover all the genetic diversity demonstrated for this virus. Therefore new molecular methods able to

detect not only the widespread lineage 1 and 2 but also the other lineages described should be designed. For this propose, in this study, we developed the first real time PCR assay capable to detect all the WNV lineages described until now.

2. Material and methods

2.1. Primers and probe design

For primers and probe design, 79 sequences from different WNV linages were obtained from NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) and aligned using the Clustal W program (MEGA 5 software). Primers were designed to target a highly conserved region of the 3'NC region of the WNV genome. In this method, two different TaqMan probes with minor groove binder (MGB) were designed, one specific for WNV detection and the other as internal reaction control, labeled with different fluorophores at the 5´end, and both with NFQ (non-fluorescent quencher) at the 3´ end. The primers and probes sequence set were evaluated using the Primers Express software v2.0 (Applied Biosystems, Foster City, CA, USA).

2.2. Construction of in vitro DNA standards and internal control for viral quantification and evaluation of qPCR sensitivity

Six different strains corresponding to different lineages were used to optimize the real- time PCR method (Table 1). For quantitation and evaluation of the test system, plasmids containing the PCR target region were constructed. To obtain DNA standards, RNA of WNV lineages 1, 2, 3 and 6, available in our laboratory, were extracted from a cell culture of these viruses on Vero E6 cells, using QIAamp Viral RNA extraction kit (QIAGEN GmbH, Heiden, Germany) followed by a reverse transcription using Superscript III Reverse transcriptase kit (Invitrogen, CA92008) and random hexamers (Random primers Roche) according to Manufacturer's instructions.

94 bp fragments were amplified using the real-time PCR primers WNRT-F and WNRT- Re. The PCR products were purified and cloned into a TA cloning vector (TOPO TA Cloning Systems, Invitrogen) and the sequences of the inserts were verified by sequencing. The plasmid concentration was determined spectrophotometrically, and 10-fold serial dilutions of the plasmid were prepared in RNase-free H_2O. Due to the lack of available virus in our laboratory, DNA standards of WNV lineages 4 and 5 and of internal control (IC) were artificially constructed. Respectively, a 94 (in the case of WNV lineage 4 and 5) and 65 bp (in the case of the IC) DNA fragment was obtained by an end-point PCR carried out with overlapping primers (Table 2).

In all cases, PCR was carried out using the AMpliTaq DNA Polimerasa with Buffer II (Applied Biosystems, Spain) in a PCT-200, Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). PCR was performed in a final volume of 50 µl of a reaction mix containing: 5 µl buffer II 10X, 2.5 mM $MgCl_2$, 2.5 U of enzyme, 0.5 µM of dNTPs (GE Healthcare, Spain), 1 µM of each primer IC-F/IC-Re, 4WNF/4WNRe and 5WNF/5WNRe (Table 3) and RNAse-free water. PCR conditions were 5 min 95 °C, 30 cycles of 30 seconds 94oC, 30 seconds 60oC, 1 min 72oC and 10 min 72oC. The obtained product was cloned in pCR®4-TOPO® (Life Technologies, Spain) according to manufacturer's instructions, and sequenced with standard primers M13F and M13Re to confirm the absence of mutations. DNA concentration was determined by spectrophotometry using a NanoDrop instrument (Thermo Scientific, Spain). The plasmids were linearized using the *Sna* BI and *Alf*II restriction enzymes for internal control and positive DNA standards respectively.

2.3. Evaluation of sensitivity and specificity of the assay

To determine the sensitivity of the real-time PCR assay, a standard curve was constructed using serial 10-fold dilutions of all plasmid standards, from 10^8 to 0.1 copies /µl. Four replicas were tested in a same assay, and the limit of detection was

calculated for each standard. The specificity was evaluated using RNA viral from a panel of different flaviviruses and other encephalitic viruses belonging to several viral families (Table 5). To determinate the applicability of the assay, clinical human samples and insect samples were tested.

2.4. Real time PCR assay

Viral RNAs were extracted using the QIAamp RNA Viral Kit (Qiagen GmbH, Heiden, Germany) according to the manufacturer's recommendations. cDNA was synthesized in presence of RNase inhibitor (Invitrogen) using the SuperScriptTM III Reverse Transciptase kit (ThermoFisher, Spain) following the manufacturer instructions.

The qPCR was carried out using a commercial kit (TaqMan ® Universal PCR Master Mix, no AmpErase ® UNG, Applied Biosystems). For the assay, 5 µl of sample cDNA was mixed with a 45 µl reaction mix containing: 25 µl of buffer 2X, 0.8 µM of each primer, 0.4 µM of WNV specific probe, 0.2 µM of internal control probe, 100 copies of internal control plasmid and nuclease-free water. Amplification conditions consisted of an initial DNA denaturalization of 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C, and 1 minute at 60 °C for annealing and extension. qPCR was carried out in a 7000 Sequence Detection System from Applied Biosystems. Fluorescence for FAM and NED was measured during the 60 oC step. Cycle threshold (Ct) values were measured as the point at which the sample fluorescence signal crossed a predetermined threshold value (the background level).

3. Results

3.1. Design of primers and probes

Primers to target a highly conserved fragment of the 3'NC region of the WNV genome were designed with degenerated nucleotides in variable

positions. A short sequence of 94bp was amplified. The assay uses two different TaqMan probes, a specific WNV detection probe labeled with FAM and a reaction internal control probe labeled with NED (Table 3). The IC probe was synthesized to complement the probe binding sequence of the DNA IC.

3.2. Real time PCR sensitivity and specificity

The detection limit of the assay was evaluated using serial dilutions of quantified DNA plasmid standards. The calculated limit of detection of the real-time PCR assay at which 50% of four replicates amplified with a crossing point value under 40 varied between 1.5-15 copies/reaction depending on the WNV lineage tested, the mean of sensitivity being 5 copies/reaction (Table 4). Thus, the method here developed showed a quantitative detection of all WNV lineages at similar sensitivity. The standard curves and mean C_t values obtained in different assays for the different lineages indicated that the assay is highly robust and reproducible (Figure 1). The amount of IC included in each reaction was fixed at 100 copies because it reliably yielded crossing points between 35 and 38, without affecting the amplification of different amounts of WNV.

The specificity of this novel method was verified by testing the genomes of several strains of WNV belonging to several lineages, and with other members of the *Flavivirus* genus and other encephalitic pathogens belonging to other viral families. All WNV strains tested were efficiently amplified by the real-time PCR assay, whereas no amplification could be seen with the other viruses tested, which indicated a high specificity of the assay (Table 5). The presence of genome of the non-WNV viruses was confirmed with specific PCR protocols (data not shown).

3.3 Evaluation and validation of the assay with human serum samples and positive mosquito pools.

The real-time PCR assay was validated with clinical specimens and with insect samples. During the outbreaks of WNV lineage 2 disease occurring in Greece and Romania in 2010 (Papa et al. 2011; Sirbu et al. 2011), we were able to diagnose and confirm the infection due to WNV in both serum and CSF human samples from Romania and in *Culex sp*. mosquitoes from Greece with this assay. Moreover, the method has been validated in insect samples (mosquitoes, sandflies and ticks) from several

studies carried out in the last few years in insects from Spain and Italy to monitor the presence of WNV and/or other flaviviruses (Vazquez et al. 2010; Vázquez et al. 2011; Grisenti M 2015). In some of these works from Spain, WNV lineage 1 and a new putative lineage were detected and described.

4. Discussion

West Nile virus (WNV) is continuously spreading across Europe and other continents, showing a high genetic diversity with remarkable phenotypic variation. In recent years, WNV infection has become a public health concern in Europe due to the virus introduction into new areas and the increasing number of human outbreaks with severe neurological consequences and mortality (Rizzoli et al. 2015). Recent studies from different European countries, using phylogenetic analysis of field-collected strains, demonstrated a wide diversity of WNV lineages and clades, although less information is available about several important aspects of virus ecology, biology, and pathogenicity (Di Sabatino et al. 2014). Therefore, the increasing expansion of WNV worldwide and the description of new strains and lineages showed the need to develop updated rapid diagnostic assays and specific monitoring of the virus. A rapid detection of the virus in field-collected specimens can accelerate appropriate mosquito control

measures that could prevent transmission and disease among equine and human populations, which is very important due to the lack of safe vaccines and specific therapeutic treatments, especially for humans. Moreover, viral load levels reached during human WNV infection are low, therefore the availability of a high-sensitivity test is critical to achieve efficient detection for diagnosis (Barzon, Pacenti, et al. 2015). For these purposes, we developed a rapid, sensitive and specific real time PCR for the detection of all WNV lineages described until now. This assay will be a very useful tool to be integrated in the WNV surveillance system programs of human and equine cases and vectors, with the objective of increasing knowledge of WNV strains and lineage circulation and thus to reduce the probability of virus transmission via blood, tissue and organ donation. The method presented here has the advantage of being updated with the sequence of all the latest isolates compared to the previously published assays which have been designed to detect the 1 and 2 lineages

(Faggioni et al. 2014; Del Amo et al. 2013; Jiménez-Clavero, Agüero, and Rojo 2006; Eiden et al. 2010). It can be readily seen that the sensitivity obtained for 1 and 2 lineages is similar to that reported with other methods (Del Amo et al. 2013; Lim et al. 2013 ; Eiden et al. 2010; Linke et al. 2007). This method is a generic assay so it does not able to identify the WNV lineage, therefore additional sequencing studies or diagnostic methods are needed to characterize the WNV strain present in the sample. The assay includes an internal control to assure the absence of false negative results. Its specificity has been proven to be useful in the differential diagnosis of neuroinvasive viral infectious diseases. In addition, our method has been validated and used to study WNV infection in clinical and insect samples. Actually the assay is used for the WNV diagnosis in the Laboratory of Arboviruses from the National Center of Microbiology (ISCIII).

In conclusion, our real-time PCR provides a rapid and sensitive tool for WNV detection and is, as far as we know, the first to detect all WNV lineages. Its high specificity and sensitivity makes it a useful tool for clinical and entomologic investigation into WNV infection.

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Table 1: WNV strains used to develop DNA standards

Strain	Lineage	Eg101	1a
B956	2		

Rabensburg 97-103 3

LEIV-Krnd88-190 4

804994 5

KUN MP502–66 6

Accession number AF260968

AY532665

AY765264

AY277251

DQ256376

GU047874,

Geographic origin
Egypt 1951

Uganda 1937

Czech Republic 1997

Russia 1998

India 1980

Malaysia 1966

Year of isolation

HQ840762

Table 2: Primers used for plasmid (4WNV, 5WNV and IC) constructions

Name Sequence and labeling (5’ →3’)
Primers
4WN-F
GCCGCCACCGGAAGTTGGGTATACGGTGCTGCCTGTGACCCAACCCCAGGAGGACT
GGGAT 4WN-Re
TTCCGAAACGGTATTGAGGGCTTACGTGGATCGCTCCATGGCTTTGATATCCCAGTCC
TCCTGG

GGT
5WN-F
TCCGCCACCGGATGTTGAGTAGACGGTGCTGCCTGCGTCTCAACCCCAGGAGGACT
GGGTG 5WN-Re
CTTCCGAGGCGGTTCTGAGGGCTTACATGGATCGCTCCGCAGCTTTGTTCACCCAGT
CCTCCT

GGGGTT
IC-F CGGAAGTYGRGTAKACGGTGCTG**CCAGCACACATGTGTCTACT** IC-Re
CGAGACGGTWYTGAGGGCTTAC**AGTAGACACATGTGTGCTGG**

Underlined sequences represent complementary sequences within primers designed to construct the 4WNV and 5WNV quimeras
The sequences in bold represent complementary sequences (corresponding to the sequence of the IC probe) within primers designed to construct the IC

Table 3: Primers and probe used for WNV detection

Name	Primers	WNRT-F	WNRT-Re
Probes	WNV	ICNED	
Sequence and labeling (5' →3')	Nucleotide positions		
CGGAAGTYGRGTAKACGGTGCTG	10530-10552	CGGTWYTGAGGGCTTACRTGG	10602-10622
FAM-WCCCCAGGWGGACTG –MGB- NFQ	10564-10578	NED-CCAGCACACATGTGTCTACT-MGB-NFQ	

Positions based on the complete sequence from WNV Eg101 (GenBank acc.no. AF260968) Degenerate nucleotides: Y= T or C; R = A or G, K = G or T; W = A or T.

Fluorophores: FAM and NED. Quenchers: MGB (Minor groove binder) and NFQ (non- fluorescent quencher).

Table 4: Limit detection and sensitivity of the real time PCR for the different WNV lineages used as standards. The genome equivalents detected in 50% of the cases was calculated using the Reed-Muench Method.

LINEAGE	0,1 copies/ microlitre nb positive/nb tested	1 copies/ microlitre nb positive/nb tested	10 copies/ microlitre nb positive/nb tested	Sensitivity copies/ reaction
1WNV	0/4_ 0%	2/4_ 50%	4/4_ 100%	5
2WNV	0/4_ 0%	3/4_ 75%	4/4_ 100%	2,3
3WNV	0/4_ 0%	2/4_ 50%	4/4_ 100%	5
4WNV	0/4_ 0%	0/4_ 0%	4/4_ 100%	15,8
5WNV	0/4_ 0%	3/4_ 75%	4/4_ 100%	2,3
6WNV	0/4_ 0%	4/4_ 100%	4/4_ 100%	1,5

Figure 1: Sensitivity of the real time PCR assay. Representation of the standard curve obtained with 10-fold serial dilutions for the plasmid of WNV lineage 1. C_t values obtained are plotted against the log of the copies per reaction.

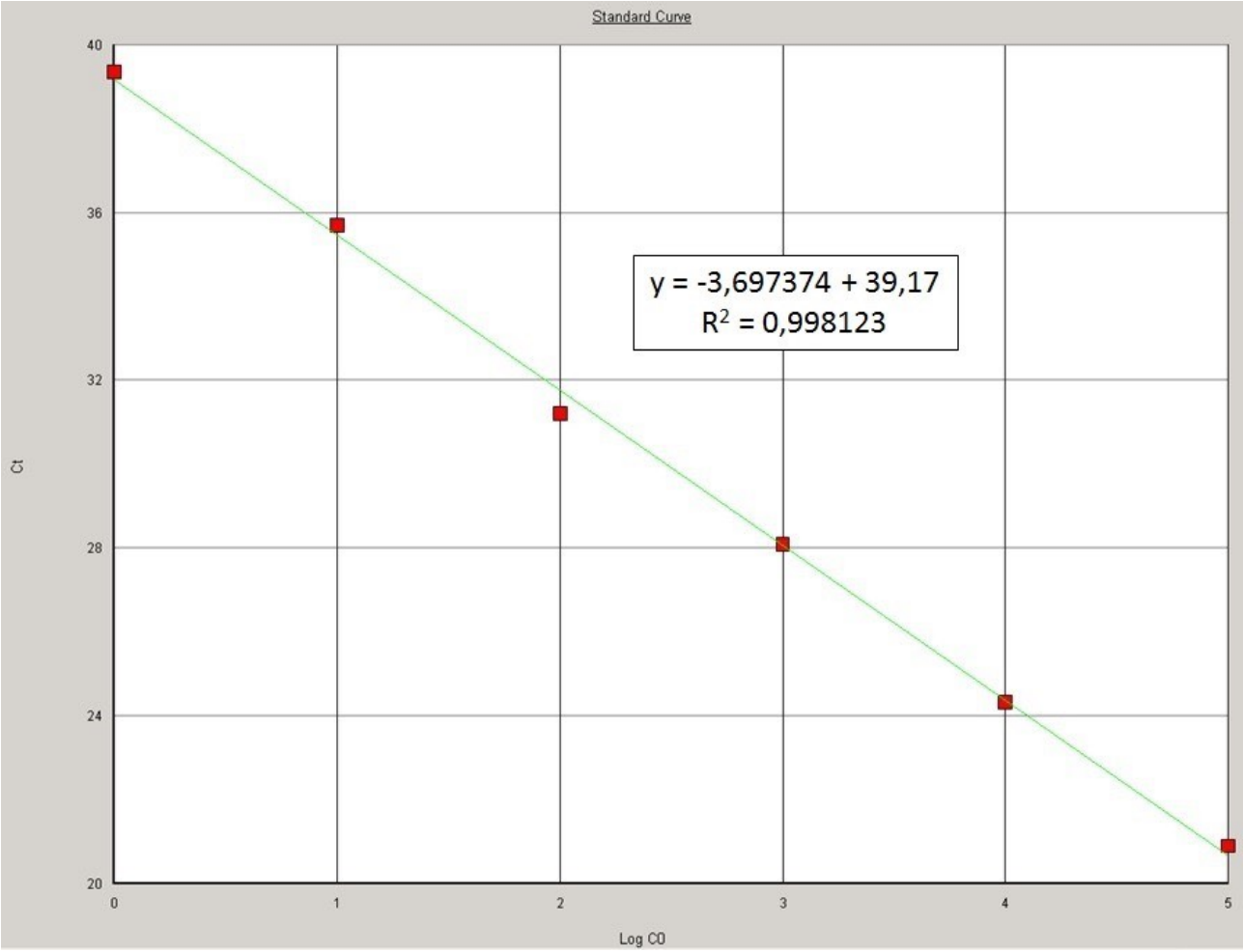

Table 5: Results of the viral strains used in this study to test the sensitivity and specificity of the assay.

Virus Family				
WNV *Flaviviridae*	WNV *Flaviviridae*	WNV *Flaviviridae*	WNV *Flaviviridae*	WNV *Flaviviridae*
WNV *Flaviviridae*	WNV *Flaviviridae*	WNV *Flaviviridae*	WNV *Flaviviridae*	WNV *Flaviviridae*
WNV *Flaviviridae*	Usutu *Flaviviridae*	MVEV *Flaviviridae*	JEV *Flaviviridae*	TBEV *Flaviviridae*
SLEV *Flaviviridae*				
Strain Lineage				
Eg101 1a	B956 2	Rabensburg 97-103 3	LEIV-Krnd88-190 4a	804994 5
HU2925/06 4b	Africa new	Koutango new	Kedougou new	Romania 2
Grecia 2	SAAR 1776			
MV/1/1951 Nakayama				
Neudörfl				
78V6507, CbaAr 4005				

Accession Number

qPCR Results

+ + + + + + + + + + + + - - - -

Dengue 1 Dengue 2 Dengue 3 Dengue 4
Yellow fever Louping Ill
Zika
Toscana
Herpes Simplex 1 Herpes Simplex 2 Rubella

Varicella zoster Parvovirus B19 Rabia Echovirus Enterovirus 68 Enterovirus 71

Flaviviridae Flaviviridae Flaviviridae Flaviviridae Flaviviridae Flaviviridae Flaviviridae Bunyaviridae Herpesviridae Herpesviridae T ogaviridae Herpesviridae Parvoviridae Rhabdoviridae Picornaviridae Picornaviridae Picornaviridae

TC 974 - New Guinea - H87 - H 241 - 17D - NCPV 212 - MR766 - ISS Phl. 3 -

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WNV: West Nile virus, MVEV: Murray Valley encephalitis virus, JEV: Japanese encephalitis virus, TBEV: Tick-borne encephalitis virus, SLEV: Saint Louis encephalitis virus

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