Molecular Typing of West Nile Virus, Dengue, and St. Louis Encephalitis Using Multiplex Sequencing

Thuraiayah Vinayagamoorthy,* Kirk Mulatz,*
Michael Drebot,[†] and Roger Hodgkinson*

From Bio-ID Diagnostic Inc.,* Saskatoon, Saskatchewan; and the
National Microbiology Laboratory,[†] Canadian Science Centre for
Human and Animal Health, Winnipeg, Manitoba, Canada

We report the development of an assay to simultaneously identify three of the clinically important flaviviruses (West Nile Virus, Dengue, and St. Louis encephalitis). This assay is based on the nucleotide sequence variations within a 266-bp region of the non-structural protein 5. Further, based on the nucleotide variations in the same region of the non-structural protein 5, four of the present Dengue serotypes were identified. To identify some of the subtypes of WNV we have developed a second assay using multiplex sequencing technology. The format of the result of this assay is an electropherogram of two genomic segments of the WNV genome: a 48-nucleotide sequence from the anchored core protein C and a 45-nucleotide sequence coding for the non-structural proteins (proteinase and putative helicase genes). (*J Mol Diagn* 2005, 7:152–159)

West Nile virus (WNV) is an RNA virus that belongs to the Flaviviridae family of viruses that also includes the Japanese/St. Louis encephalitis, Dengue, and yellow fever viruses.¹ WNV has a range of animal hosts,^{2,3} is transmitted mainly by a mosquito vector,⁴ and in severe infections produces various neurological disorders including paralysis.⁵ Recently there have been more than 3500 cases and 200 deaths due to WNV in North America and many more in rest of the world.^{6–10} Although the common route of infection via mosquito bites is associated with low fatality,¹¹ previous studies have established that other potential routes, including infection through blood^{12,13} and organ transplants,¹⁴ often carry a worse prognosis.

Presently, the presumptive diagnosis of WNV is mainly based on clinical symptoms and a definitive diagnosis is established by serology^{5,8} Diagnostic procedures such as antigen capture (ELISA)¹⁵ have been developed for viral identification, but due to the low sensitivity of these methods, antibody detection continues to be the assay of choice when carrying out human case investigations. Nucleic acid-based assays^{14,16–18} have been used to identify the viral genome in clinical samples such as cerebrospinal fluid and blood samples.

Various studies^{19–22} have identified genetic variations among different isolates of WNV, and in some cases the presence of these variants have created difficulty in establishing identity. Hence, there is a need for an accurate assay with highest specificity to identify WNV subtypes. Furthermore, since infection with other flaviviruses (Dengue, St. Louis encephalitis) presents similar clinical symptoms, it is also necessary to be able to test for the other flaviviruses for optimal patient care.

Genome sequencing is increasingly accepted as the reference method for identification of viruses. Our assay includes generating a 266-bp amplicon that is common to WNV, Dengue, and St. Louis encephalitis. This amplicon was sequenced using the chain termination sequencing method. To distinguish between WNV serotypes, we generated two amplicons from separate regions of the WNV genome and sequenced them simultaneously.

Materials and Methods

Multiplex Sequencing Method (MultiGEN)

MultiGEN is a platform technology that allows for simultaneous determination of nucleotide sequences from multiple genomes or multiple segments from the same genome or a combination of both.²³ This technology consists of three steps: The first is preparation of total nucleic acid from the sample. This could be DNA, RNA, or both. The second step is simultaneous amplification of all target genomes. This can be carried out by any one of present target amplification methods including PCR. The third step is simultaneous sequencing short stretches (20–40 nucleotides) at the 3' end of all amplicons. The molecular weight of sequencing primer (WNVseq2) is higher than the molecular weight of the longest truncated species generated by sequencing primer (WNVseq1). The sequences generated were identified by BLAST search (Figure 1). The test protocol will vary based on the type of genomic targets to be analyzed (DNA, RNA, or both), the number of targets (2–20 are feasible), and the expected copy number of the targets in the samples.

Accepted for publication August 18, 2004.

Address reprint requests to Thuraiayah Vinayagamoorthy, No. 1, 410 Downey Road, Innovation Place, Saskatoon, Saskatchewan, Canada S7N 4N1. E-mail: moorthy@bio-id-diagnostic.com.

Figure 1. Stages of the MultiGEN process and the basic scientific principles. These include: Multiplex amplification, Multiplex sequencing, electrophoresis and computer analyzed electropherogram that is ready for BLAST search.

Sample Preparation

All clinical isolates were propagated in Vero E6 cells. Total RNA was extracted using the RNeasy Mini Kit (Qia-gen). 100 μ l of the cell lysates containing WNV, 12,040

pfu/ μ l; SLE, 420 pfu/ μ l; Dengue 1, 840 pfu/ μ l; Dengue 2, 840 pfu/ μ l; Dengue 3, 420 pfu/ μ l, and Dengue 4, 980 pfu/ μ l were extracted separately. The reagents of the kit were prepared according to manufacturer's instructions, and 350 μ l of RLT buffer and 250 μ l of 100% ethanol were

Table 1. Primers and Thermocycle Profiles

RT-PCR primers	
WNV1	
f: 5'-GCCGGGCTGTCAATATGCTAAAA-3'	
r: 5'-GCACTGGTCAAGTCCCTAGTTC-3'	
WNV2	
f: 5'-GGAGCGGGCGTGATGGTTGAA-3'	
r: 5'-ACCCTGCACTATCGCGCTTAT-3'	
Flav1/Flav2	
f: 5'-TACAACATGATGGGAAAGAGAGAGA-3'	
r: 5'-GTGTCCAGCCAGCTGTGTC-3'	
Thermocycle profile	
RT-PCR	
50°C/15 minutes, 94°C/2 minutes, (94°C/15 seconds,	
50°C/30 seconds, 71°C/1 minute) × 35, 4°C/hold	
Sequencing primers	
WNV1 seq	
5'-D-AACATTGCCCTCTTCAGTCCAATCA-3'	
WNV2 seq	
5'-D-TCATCAGAGCGGCTCCTTTTGTGT-3'	
Thermocycle profile	
Cycle sequencing	
(96°C/10 seconds, 51°C/5 seconds, 60°C/4 minutes) ×	
25, 4°C hold	

Δ = primer modification at 5' end.

added to 100 μ l of viral lysate. The sample was transferred to the spin column and centrifuged at 8000 rcf for 30 seconds. The sample was washed on the spin column three times, once with 700 μ l of RW1 buffer and twice with

500 μ l of RPE buffer. After each wash the spin column was centrifuged at 8000 rcf for 30 seconds. After washing, the column was thoroughly dried with a 1-minute centrifugation at 8000 rcf. Finally, the sample was eluted from the column by adding 50 μ l of RNase-free water to the column and centrifuging for 1 minute at 8000 rcf. The RNA was further concentrated by re-applying the eluate to the column and centrifuging a second time at 8000 rcf for 1 minute. RNA/DNA concentrations were determined using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, UK).

RT-PCR

The SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen) was used for individual RT-PCR. The reaction volume was 50 μ l, which consisted of 25 μ l 2X Reaction mix, 200 nmol/L each primer, 1 μ l RT/Platinum mix *Taq*, and 10 μ l of RNA extract. The tubes were placed in a thermocycler, GeneAmp 2400 (Applied Biosystems) and amplified according to the thermocycling profile shown in Table 1.

Gel Electrophoresis

The amplicons were visualized by loading 2 μ l of 6X loading buffer, 5 μ l of TBE buffer, and 5 μ l of the PCR

Figure 2. Nucleotide sequences from two selected regions from the 266-bp amplicon. **A:** Signature nucleotide sequences that identify WNV and St. Louis encephalitis. **B:** Signature nucleotide sequences that identify Dengue types 1–4.

Figure 3. Electropherograms showing nucleotide sequences from six RNA extracts analyzed. **A:** WNV; **B-E:** Dengue serotypes; **F:** St. Louis encephalitis.

product onto a 2% agarose gel and applying 110 V for 50 minutes. The gel was then stained for 15 minutes in ethidium bromide (5 $\mu\text{g/ml}$ in TBE) solution and then destained in distilled water for 15 minutes.

Cycle Sequencing

Amplicons were sequenced by cycle sequencing using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on a GeneAmp 2400 thermocycler (PE Applied Biosystems) using the thermocycler profile in Table 1. Unincorporated dye terminators were removed using Centricon chromatography columns (Princeton, NJ). The samples were then dried and re-suspended in 20 μl of ABI PRISM Template Suppression Reagent. Samples were analyzed by capillary electro-

phoresis using the ABI PRISM Genetic Analyzer 310. The 47 cm $\times$ 50 μm uncoated capillary was filled with a Performance Optimized Polymer 6 (acrylamide/urea polymer) and heated to 50°C. 20 μl of the sequencing mixture was pipetted into a 0.2-ml microfuge tube provided by the manufacturer (Applied Biosystems). Samples were drawn into the capillary by electrokinetic injection at 2 kV for 50 to 200 seconds. The electrophoresis was carried out at 15 kV for 20 minutes.

Results

Selection of Target Regions and Primer Design

West Nile virus, Dengue, and St. Louis encephalitis genomic nucleotide sequences were obtained from Gen-

Figure 4. A: Region of the WNV genome from which two amplicons were generated, positioning of the sequencing primers, and the electropherograms produced from two samples. **B:** Photograph of an ethidium bromide stained 2% agarose gel showing the DNA bands of the two amplicons.

Bank (NIH) and aligned using GeneDoc version 3.1 (<http://www.psc.edu/biomed/genedoc>) and GenBank BLAST. Based on this alignment, a segment common to all three flaviviral genomes was selected. This segment carried a core region showing nucleotide variations among WNV, Dengue, and St. Louis encephalitis, and sequences that could distinguish between various serotypes of Dengue and conserved flanking regions. The primers were designed using Oligo 6 Software version, 6.65 (Molecular Biology Insights) within conserved regions flanking the core region. Based on the nucleotide sequences within the amplicon WNV, Dengue and St. Louis encephalitis could be identified (Figure 2). Furthermore, comparing the equivalent nucleotide sequences, the four Dengue serotypes could be identified (Figure 2). The same approach was used to design two sets of PCR primers within the WNV genome to generate two ampli-

cons (250 bp from the anchored core protein region and 396 bp from the proteinase and putative helicase regions). To sequence the 3' end of these two amplicons, two modified (Bio-ID Diagnostic Inc) sequencing primers (Table 1) were designed for simultaneous sequencing.

Detection of WNV, Dengue, and St. Louis Encephalitis

Total RNA was prepared from six viral isolates 10 μ l of these RNA extracts were amplified separately using one step RT-PCR with Flav1/Flav2 primer set (Table 1), with all six generating an amplicon of 266 bp from the non-structural protein 5 region. These amplicons were sequenced separately using Flav1 primer. The nucleotide sequences generated are shown in Figure 3. Various

Table 2. PCR and Sequencing Results

Samples	WNV identification		Flavivirus identification	
	PCR	Sequencing	PCR	Sequencing
Sample 1	Positive	WNV	Positive	WNV
Sample 2	Positive	neg.	Positive	Dengue type 1
Sample 3	Negative	n/a	Positive	Dengue type 2
Sample 4	Negative	n/a	Positive	Dengue type 3
Sample 5	Negative	n/a	Positive	Dengue type 4
Sample 6	Negative	n/a	Positive	St Louis encephalitis
Negative control	Negative	Negative	Negative	Negative

regions (highlighted in yellow) on each electropherogram (Figure 3) were subjected to BLAST search. Based on the alignments from BLAST searches they were identified as follows: Figure 3. WNV (A), Dengue subtypes (B-E), St. Louis encephalitis (F). The black boxes highlighting nucleotide sequences (Figure 3, electropherogram regions of samples B to E) denote regions that are conserved among the serotypes of Dengue and can be used to distinguish between the different serotypes. All of the sequences were confirmed by BLAST searches.

Detection of WNV

Of the six samples tested, two produced amplicons (Figure 4B) using WNV1 and WNV2 PCR primer sets (Table 1); the other four and the negative control did not (Table 2). The amplicons produced from these two samples were sequenced (separately) at their respective 3' end (Figure 4A) using two modified (Bio-ID Diagnostic Inc) sequencing primers WNV1seq, WNV2seq (Table 1). The truncated molecular species from these two targets were then separated in a single capillary on an ABI 310 Genetic Analyzer. Of the two samples sequenced, only one produced an electropherogram. The nucleotide sequence from the electropherogram produced (Figure 4C) were BLAST searched and the sequences were found to be in 100% alignment with West Nile virus sequence subtype NY99 (Table 3). The experiment was repeated with the same sample of WNV total RNA extracts (15 ng) spiked with 15 ng of human genomic DNA, and the MultiGEN analysis produced the expected WNV nucleotide sequences (data not shown). As an extension of this assay, the expected amplicons were generated using

500 pg of the original WNV extract corresponding to 1852 viral particles.

Discussion

The ability of MultiGEN technology to simultaneously generate nucleotide sequences from multiple viral targets raises the parameters of identification to its highest standard. Reverse transcriptase lacks 3'-5' proofreading and hence are prone to error in replication.²⁴ This inherent ability to create progeny variation gives the virus an enhanced opportunity to survive the host immune response from previous infections.²⁵ WNV RNA binding proteins have been identified²⁶ and these may be associated with WNV replication. Using infected C3H/RV mouse cells, it has been shown²⁷ that mutations at two unique spots in WNV RNA affects the efficiency of the production of virus progeny, and suggests that the WNV genome carries a conserved 3'-terminal secondary structure that is a polymerase recognition and binding site.²⁸ Furthermore, truncation of the 2'-5' oligoadenylate synthetase L1 isoform point mutation in mouse has been correlated with the restriction of WNV replication.²⁹ Such events create two challenges for effective management of epidemics. 1) Genetic changes in the RNA recognition motif (RRM) could affect the pathogenicity of the virus. Determination of nucleotide sequences in the relevant motif will indicate the change in pathogenicity. 2) If the mutational changes are within annealing regions of the target amplification primers (with PCR or sequencing) this will alter the melting temperature (T_m) of primer/target binding and hence affect target amplification. As the assay in this report

Table 3. Nucleotide Variation among WNV Isolates

WNV strain	Accession number	Region 1 (locus 4720–4766) WNV2 primer set
R097-50	AF260969	GTGCCAAAGAGTGTGGAAAACGCCTTCAACCATCAGCCCGCTCC
Assembled genome	NC_001563	*****T**T*****C**C**C**T*****T**G*****
Eg101	AF260968	A*****G*****C*****T*****
NY99-flamingo-99	AF186835	A*****G*****A*****T*****
WNV strain	Accession number	Region 2 (locus 128–176) WNV1 primer set
R097-50	AF260969	AGGACAACACGCGGGGCATTCGCGCTTTTAGCATATTGACAGCCCCGGC
Assembled genome	NC_001563	*****T**C*****A*****T*****
Eg101	AF260968	*****T*****A*****T*****
NY99-flamingo-99	AF186835	*****T*****A*****T*****

Table 4. Sequence Variations among Flaviviruses

Name: West Nile virus	CTTAGGACTGCAGAAGCTCGGGTACATCTTGAAGGAAGT-GGAACAAAGCCTGGAGGAAAGGTT
Accession: NC_001563	
Locus: 9204-9266	
Name: yellow fever virus	*A*T*CT*A*AT*C**A**A**TG*GA*C*GA**CC*G-CTG***T*GAT**T*TGGAT*C
Accession: NC_002031	
Locus: 9166-9229	
Name: Japanese encephalitis	**C***CG*C**A*****G**A*****C*CCGT**CA*-*C*GG*****AA*****G**AA*G
Accession: NC_001437	
Locus: 9209-9272	
Name: Dengue virus	AGA***G*****T*****A**T*****A*GA**G**GA*C**G****-A*****GCAA*G
Accession: NC_001474	
Locus: 9087-9150	

targets two genomic regions, this increases the chance of obtaining a positive result even if such mutations should occur. Even though sample number 2 generated an amplicon with only WNV PCR primers, it failed to produce an electropherogram with the WNV sequencing primer. However, the same sample when tested with PCR primers for WNV, Dengue and St. Louis encephalitis was found to generate an electropherogram that definitively identified sample 2 as Dengue. Hence use of MultiGEN technology was able to prevent the potentially false identification, had PCR been used alone.

In summary, the reality of present international travel greatly facilitates the rapid global spread of the gene pool of microbial pathogens. Such challenge to global public health calls for the most accurate method for both rapid pathogen identification and monitoring the spread of the epidemic. MultiGEN technology removes practical limitations that are associated with conventional sequencing method being used for viral identifications.

References

- Solomon T, Vaughn DW: Clinical features and pathophysiology of Japanese encephalitis and West Nile virus infections. *Current Topics in Microbiology and Immunology: Japanese Encephalitis and West Nile Virus Infection*. Mackenzie JS, Barret AD, Deubel V, eds. Berlin, Springer-Verlag, 2002, pp 171-194
- Komar N, Langevin S, Hinton S, Nemeth N, Edwards E, Hettler D, Davis B, Bowen R, Bunning M: Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis* 2003, 9:311-322
- Porter MB, Long MT, Getman LM, Giguere S, MacKay RJ, Lester GD, Alleman AR, Wamsley HL, Franklin RP, Jacks S, Buergeit CD, De-trisac CJ: West Nile virus encephalomyelitis in horses: 46 cases. *J Am Vet Med Assoc* 222:1241-1247, 2003
- Drebot MA, Linder R, Baker IK, Buck BA, Fearon M, Hunter F, Sockett P, Arstob H: West Nile virus surveillance and diagnostics: a Canadian perspective. *Can J Infect Dis* 2003, 14:105-114
- Martin DA, Biggerstaff BJ, Allen B, Johnson AJ, Lanciotti RS, Roehrig JT: Use of immunoglobulin m cross-reactions in differential diagnosis of human flaviviral encephalitis infections in the United States. *Clin Diagn Lab Immunol* 2002, 9:544-549
- Chowers MY, Lang R, Nassar F, Ben-David D, Giladi M, Rubinshtein E, Itzhaki A, Mishal J, Siegmán-Igra Y, Kitzes R, Pick, Landau Z, Wolf D, Bin H, Mendelson E, Pitlik SD, Weinberger M: Clinical characteristics of the West Nile fever outbreak, Israel, 2000. *Emerg Infect Dis* 2001, 7:675-678
- Platonov AE, Shipulin GA, Shipulina OY, Tyutyunnik EN, Frolochkina TI, Lanciotti RS, Yazzyshina S, Platonova OV, Obukhov IL, Zhukov AN, Vengerov YY, Pokrovskii VI: Outbreak of West Nile virus infection, Volgograd Region, Russia, 1999. *Emerg Infect Dis* 2001, 7:128-132
- Solomon T, Ooi MH, Beasley DW, Mallewa M: West Nile encephalitis. *Br Med J* 2003, 7394:865-869
- Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI: West Nile encephalitis epidemic in southeastern Romania. *Lancet* 1998, 9130:767-771
- Thakare JP, Rao TL, Padbidri VS: Prevalence of West Nile virus infection in India. *South East Asian J Trop Med Public Health* 2002, 33:801-805
- Mostashari F, Bunning ML, Kitsutani PT, Singer DA, Nash D, Cooper MJ, Katz N, Liljebjelke KA, Biggerstaff BJ, Fine AD, Layton MC, Mullin SM, Johnson AJ, Martin DA, Hayes EB, Campbell GL: Epidemic West Nile encephalitis, New York, 1999: results of a household-based seroepidemiological survey. *Lancet* 2001, 358:261-264
- Biggerstaff BJ, Petersen LR: Estimated risk of West Nile virus transmission through blood transfusion during an epidemic in Queens, New York City. *Transfusion* 2002, 42:1019-1026
- Lefrere JJ, Allain JP, Prati D, Saulea S, Reesink H: West Nile virus and blood donors. *Lancet* 2003, 361:2083-2084
- Iwamoto M, Jernigan DB, Guasch A, Trepka MJ, Blackmore CG, Hellinger WC, Pham SM, Zaki S, Lanciotti RS, Lance-Parker SE, DiazGranados CA, Winquist AG, Perlino CA, Wiersma S, Hillyer KL, Goodman JL, Marfin AA, Chamberland ME, Petersen LR: West Nile Virus in Transplant Recipients Investigation Team. Transmission of West Nile virus from an organ donor to four transplant recipients. *N Engl J Med* 2003, 348:2196-2203
- Hunt AR, Hall RA, Kerst AJ, Nasci RS, Savage HM, Panella NA, Gottfried KL, Burkhalter KL, Roehrig JT: Detection of West Nile virus antigen in mosquitoes and avian tissues by a monoclonal antibody-based capture enzyme immunoassay. *J Clin Microbiol* 2002, 40:2023-2030
- Nasci RS, Gottfried KL, Burkhalter KL, Kulasekera VL, Lambert AJ, Lanciotti RS, Hunt AR, Ryan JR: Comparison of vero cell plaque assay, TaqMan reverse transcriptase polymerase chain reaction RNA assay, and VecTest antigen assay for detection of West Nile virus in field-collected mosquitoes. *J Am Mosquito Control Assoc* 2002, 18:294-300
- Shi PY, Kramer KD: Molecular detection of West Nile virus RNA. *Expert Rev Mol Diagn* 2003, 3:357-366
- Vaughn CP, Elenitoba-Johnson KS: Hybridization-induced quenching of fluorescein-labeled oligonucleotides: a novel strategy for PCR detection and genotyping. *Am J Pathol* 2003, 163:29-35
- Hall RA, Scherret JH, Mackenzie JS: Kunjin virus: an Australian variant of West Nile? *Ann NY Acad Sci* 2001, 951:153-160
- Prilipov AG, Kinney RM, Samokhvalov EI, Savage HM, Al'khovskii SV, Tsuchiya KR, Gromashevskii VL, Sadykova GK, Shatalov AG, Vyshemirskii OI, Usachev EV, Mokhonov VV, Voronina AG, Butenko AM, Larichev VF, Zhukov AN, Kovtunov AI, Gubler DJ, L'vov DK: Analysis of new variants of West Nile fever virus. *Vopr Virusol* 2002, 47:36-41
- Scherret JH, Poidinger M, Mackenzie JS, Broom AK, Deubel V, Lipkin WI, Briese T, Gould EA, Hall RA: The relationships between West Nile and Kunjin viruses. *Emerg Infect Dis* 7:697-705, 2001
- Scherret JH, Mackenzie JS, Hall RA, Deubel V, Gould EA: Phylogeny and molecular epidemiology of West Nile and Kunjin viruses. *Curr Top Microbiol Immunol* 2002, 267:373-90
- Vinayagamoorthy T, Mulatz, K: Hodkinson R: Nucleotide sequence based multi-target identification. *J Clin Microbiol* 2003, 41:3284-3292

24. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: Laboratory Manual*. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1989, pp 5–52
25. Van Maanen C, Van Essen J, Minke J, Daly JM, Yates PJ: Diagnostic methods applied to analysis of an outbreak of quinine influenza in a riding school in which vaccine failure occurred. *Vet Microbiol* 2003, 93:291–306
26. Li W, Li Y, Kedersha N, Anderson P, Emara M, Swiderek KM, Moreno GT, Brinton MA: Cell proteins TIA-1 and TIAR interact with the 3' stem-loop of the West Nile virus complementary minus-strand RNA and facilitate virus replication. *J Virol* 2002, 76:11989–12000
27. Brinton MA, Fernandez AV: A replication-efficient mutant of West Nile virus is insensitive to DI particle interference. *Virology* 1983, 129:107–115
28. Brinton MA, Fernandez AV, Dispoto JH: The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* 1989, 153:113–121
29. Mashimo T, Lucas M, Simon-Chazottes D, Frenkiel MP, Montagutelli X, Ceccaldi PE, Deubel V, Guenet JL, Despres P: A nonsense mutation in the gene encoding 2'-5'-oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. *Proc Natl Acad Sci USA* 2002, 20:11311–11316