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Molecular detection and characterization of West Nile virus associated with multifocal retinitis in patients from southern India

Jyoti Shukla [a], Divyasha Saxena [a], Sivakumar Rathinam [b], Prajna Lalitha [b], Cornelia Reena Joseph [b], Shashi Sharma [a], Manisha Soni [a], P.V.L. Rao [a], Manmohan Parida [a,*]

[a] Division of Virology, Defence Research and Development Establishment, Jhansi Road, Gwalior 474002, Madhya Pradesh, India
[b] Department of Microbiology, Aravind Eye Hospital, Madurai, Tamil Nadu, India

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SUMMARY

Background: In late 2009/early 2010, approximately 2000 people were affected by a mysterious viral outbreak in a southern district of Tamil Nadu; this particularly affected those living in coastal areas. Blood samples from affected patients were sent for clinical analysis to determine the actual cause of the illness, but reports were inconclusive.

Methods: The present study describes the clinical observations and laboratory investigations involving molecular methods performed on 170 of the 2000 clinically suspected cases. These were patients who were admitted to Aravind Eye Hospital, Madurai, Tamil Nadu with ocular complications. Conventional reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, and reverse transcription loop-mediated isothermal gene amplification (RT-LAMP) assays were used to detect West Nile virus (WNV) infection. Further investigation of the genetic diversity of the WNV implicated in ocular complications was undertaken by sequence phylogeny.

Results: Out of 170 samples, 25 (15%) were positive for chikungunya IgM antibody, 10 (6%) for chikungunya antigen, and 30 (18%) were positive for dengue IgM antibody. The remaining 105 seronegative samples were further processed for WNV detection by IgM capture ELISA and molecular methods. Out of the 105 samples, 35 (33%) were positive for WNV IgM antibody, 15 (14%) were positive for WNV by RT-PCR, and 27 (26%) were found to be positive for WNV by both real-time RT-PCR and RT-LAMP assays. Comparative evaluation with acute-phase patient serum samples revealed 100% concordance between the real-time RT-PCR and RT-LAMP assays. These assays had an overall higher sensitivity than the conventional RT-PCR as they picked up 12 additional samples with a low copy number of template. Further genotyping through sequence phylogeny revealed that all the WNV isolates were grouped in lineage I.

Conclusions: The association of West Nile virus with ocular infection in South India during an epidemic of mysterious fever in the first half of 2010 was clearly established through molecular approaches employing envelope gene-specific real-time RT-PCR and RT-LAMP assays followed by nucleotide sequencing.

1. Introduction

West Nile virus (WNV) is an arthropod-borne virus that is taxonomically classified within the family *Flaviviridae*, genus *Flavivirus*, and is a member of the Japanese encephalitis (JE) virus serocomplex. WNV circulates in natural transmission cycles involving primarily *Culex* species mosquitoes and birds, and humans are incidental hosts.[1] The clinical features of severe WNV infection vary and include severe headache, ocular manifestations, muscle weakness, cognitive impairment, tremors, and a poliomyelitis-like flaccid paralysis.[2,3] Historically, WNV has circulated primarily in Africa, the Middle East, southern Europe, Australia, Russia, India, and Indonesia, causing epidemics from time to time.[4–8] However, the recent outbreak of WNV in North America is of global public health concern.

Routine laboratory diagnosis of WNV infection is primarily based on serodiagnosis, followed by virus isolation and identification. Serologically, WNV infection can be inferred by immunoglobulin M (IgM) and immunoglobulin G (IgG) capture ELISA. Recently, several investigators have reported PCR-based detection systems for the rapid detection of WNV infection in clinical specimens that are negative for virus isolation, suggesting that nucleic acid-based assays hold great promise for the detection of WNV infection.[9] In addition, other PCR-based methods, such as the

* Corresponding author. Tel.: +91 751 2233495; fax: +91 751 2351148.
E-mail address: paridamm@rediffmail.com (M. Parida).

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reverse transcription loop-mediated isothermal gene amplification (RT-LAMP) assay, have been developed for the detection of WNV RNA.[10]

In late 2009/early 2010, a mysterious viral outbreak in a southern district of Tamil Nadu affected about 2000 people, particularly those living in coastal areas, as well as in Chennai, Tiruvallur, Dharmapuri, Madurai, Theni, and Tirunelveli. The affected people suffered from high fever and body pain, particularly acute pain in the joints, which are symptoms of chikungunya. However, many of them tested negative for chikungunya virus (CHIKV). Blood samples from the affected patients were sent for clinical analysis to determine the actual cause of the illness, but reports were inconclusive due to the identification of different etiologies by different agencies; these included dengue virus (DENV), CHIKV, and Ross River virus.

The present study describes the clinical observations and laboratory investigations involving molecular methods performed on 170 of the 2000 clinically suspected cases. These were cases who were admitted to Aravind Eye Hospital, Madurai, Tamil Nadu with ocular complications. Conventional reverse transcription PCR (RT-PCR) and real-time RT-PCR assays were used to detect WNV infection. In addition, RT-LAMP was also performed to determine the feasibility of using this method as an alternative cost-effective tool to the real-time RT-PCR. Further investigation of the genetic diversity of the WNV implicated in ocular complications was undertaken by sequence phylogeny. Data on the clinical picture of WNV-associated ocular manifestations, along with the sensitivity and specificity of the different gene amplification assays and genotyping is discussed.

2. Materials and methods

2.1. Cells and virus strain

The virus strains used in the present study were WNV strain Eg101, DENV type 2 (DENV-2) strain ThNH7/93, JE virus strain JaOArS982, St Louis encephalitis (SLE) virus strain Parton, and yellow fever virus. The viruses were propagated by regular passaging in *Aedes albopictus* clone C6/36 cells and titrated by plaque assay in Vero cells in accordance with the standard protocol.[11,12]

2.2. Clinical samples

All patients presenting to the Uveitis Clinic at Aravind Eye Hospital, Madurai, with signs of acute posterior uveitis and a recent onset of febrile illness during December 2009 to May 2010 were enrolled in the study. Demographic details including age, gender, place of residence, complaints before the onset of eye problems, and symptoms of ocular illness were recorded. Ophthalmological examination included visual acuity measurement, slit lamp biomicroscopy of the anterior and posterior chambers, and indirect ophthalmoscopic examination.[13] Clinical features and factors potentially contributing to final vision loss were also recorded. In addition, panels of 20 serum samples obtained from apparently healthy individuals who had not had any fever were included as negative controls. Informed oral consent was obtained from all patients. Serum and plasma were collected to perform all the tests.

2.3. Serology

A total of 170 acute phase human patient serum samples were tested for the presence of CHIKV and DENV using commercial kits. After ruling out DENV and CHIKV, the remaining samples were further analyzed by in-house IgM ELISA for WNV.

2.4. IgM ELISA

A recombinant envelope (env) protein (r-WNV-env)-based indirect antibody capture IgM ELISA was optimized for the detection of WNV-specific IgM antibodies. Briefly, 96-well microtiter plates were coated with 300 ng of purified r-WNV-env protein in 0.1 M carbonate buffer, pH 9.6. The coated wells were washed once with 1× phosphate-buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) in 1 × PBS overnight at 4 °C. The wells were washed once again with 1 × PBS and then incubated with a 1:100 dilution of patient serum sample in 1 × PBS for 1 h at 37 °C. Wells were washed three times using 1 × PBS–Tween and incubated with anti-human IgM–horseradish peroxidase (HRP) conjugate (1:3000 dilutions in 5% BSA). The wells were washed once again as above and incubated with 100 μl tetramethylbenzidine (TMB) substrate for 5 min at room temperature. The reaction was then stopped with 100 μl of 1 N H_2SO_4 and the absorbance was recorded at 490 nm in an ELISA reader.

2.5. RT-PCR

All the DENV- and CHIKV-negative samples were tested for the presence of WNV-specific RNA by RT-PCR, real-time RT-PCR, and RT-LAMP assays. RNA was extracted from the patient serum, plasma, and infected culture supernatant using the QIAamp Viral RNA Mini Kit in accordance with the manufacturer's protocol (Qiagen, Germany). The RNA was then eluted in 50 μl of nuclease-free water and used as template in the RT-PCR. A one-step RT-PCR was performed using the Access Quick RT-PCR Kit (Promega, Madison, WI, USA), in accordance with the manufacturer's protocol, employing primer pairs targeting the *env* gene designed from the nucleotide sequence of the reference Eg101strain (GenBank accession number **AF260968**; a set of forward primer WNV F: 5′ TGG ATT TGG TTC TCG AAG G-3′ (genome position 1028–1046) and the reverse primer WNV R: 5′ GGT CAG CAC GTT TGT CAT T-3′ (genome position 1228–1210)).

The amplification was performed in a 50-μl total reaction volume with the Promega Access Quick One-Step RT-PCR Kit, with 50 pmol of each forward and reverse primer and 2 μl of extracted viral RNA, in accordance with the manufacturer's instructions. Positive and negative controls were included in each assay run, and all precautions to prevent cross-contamination were observed.

2.6. Real-time RT-PCR

The WNV *env* gene-specific real-time RT-PCR was performed employing the US Centers for Disease Control and Prevention (CDC) recommended oligonucleotide primers as well as RT-LAMP outer primers.[10,14] Briefly, the amplification was carried out in 25-μl reaction mixtures using Brilliant SYBR Green Single-Step QRTPCR Master Mix (Stratagene, USA) containing 12.5 μl of 2× reaction mix, 0.4 μl of reference dye (ROX), 1 μl (10 pmol) of each forward and reverse primer, 1 μl of RNA, 0.1 μl of reverse transcriptase, and 9.0 μl of nuclease-free water. 'No-template', 'no-primer', and buffer controls were also included in the tests.

2.7. RT-LAMP

RT-LAMP was performed at a total reaction volume of 25-μl using the Loopamp RNA Amplification Kit (Eiken Chemical, Tokyo, Japan) in accordance with the reported protocol.[12] Real-time monitoring was accomplished by incubating at 63 °C for 60 min in a Loopamp Realtime Turbidimeter (LA-200; Teramecs, Japan). Real-time monitoring of the RT-LAMP amplification of virus template was observed through spectrophotometric analysis by

recording the optical density at 400 nm every 6 s with the aid of the Loopamp Realtime Turbidimeter (LA-200).

2.8. Genotyping

The real-time RT-PCR-positive amplicons were gel purified and subjected to double-stranded sequencing with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI 310 sequencer (Applied Biosystems, California, USA). The genotype of the WNV, based on the partial *env* gene sequence, was determined by nucleotide sequencing and compared with 25 other globally diverse WNV isolates (Table 1). A dendrogram was constructed by pair-wise comparison of 200 nucleotide sequences of the partial *env* gene (positions 1028–1228, with respect to the Eg101 genome), which classified all isolates into five different lineages. The phylogenetic tree was constructed with the neighbor-joining method, with a bootstrap analysis of 1000 replicates, using MEGA software, version 2.1.[15]

3. Results

Of the 170 patients seen at the Aravind Eye Hospital, 105 were seronegative for DENV and CHIKV. Seventy-eight of these patients were clinically suspected to have WNV but had negative test results, and 27 had evidence of WNV by real-time RT-PCR and RT-LAMP (see below). These 27 patients had developed an acute posterior uveitis following a febrile illness; 17 were men (63%). Their age at presentation ranged from 9 to 59 years, with a mean age of 30 years and median age of 26.5 years. Of these 27 patients, eight were from the town of Madurai (Tamil Nadu), 15 were from other parts of Tamil Nadu, and four were from the neighboring states of Kerala and Andhra Pradesh (Figure 1). The clinical characteristics of these patients, who showed various presentations, are outlined in Table 2.

Twenty-five patients complained of sudden deterioration of vision in one or both eyes and two had ocular pain in addition to defective vision. Blurring of vision occurred bilaterally in nine patients and unilaterally in 18 patients accounting for 36 eyes. Of 36 eyes, 15 had normal vision (Figure 2). Slit lamp examination showed quiet anterior chamber and no vitreous inflammation in 25 patients, and only two patients had 2+ cells and mild flare in the anterior segment. Fundus examination showed the presence of neuroretinitis in 11 patients, retinitis alone in six, and vasculitis in nine patients, as predominant posterior segment signs. During the active phase, other features seen in the posterior segment examination included discrete deep, white retinitis, retinal edema along the retinal vessels near the disc, and macular star.

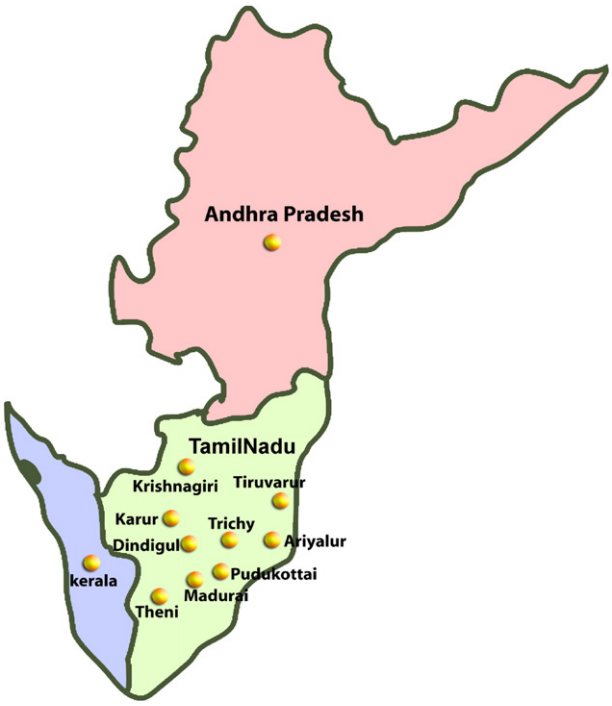

Figure 1. Map showing the affected areas of the three different states of South India during the epidemic (December 2009–May 2010).

A comparative analysis of blood specimens obtained from the 170 patients with suspected WNV infection was conducted using serology, RT-PCR, real-time RT-PCR, and RT-LAMP. Out of the 170 samples, 25 were positive for chikungunya IgM antibody (15%), 10 for chikungunya antigen (6%), and 30 were positive for dengue IgM (18%). The remaining 105 seronegative samples were further

Table 1
Details of West Nile viruses used in this study to compare sequence phylogeny

S. No.	Virus strain	Year of sample collection	Country	GenBank accession No.
1	Mex03	2004	Mexico	AY660002
2	NY99	2005	USA	DQ211652
3	TVP-9376	2005	USA	AY848697
4	IS-98STD	2002	France	AF481864
5	TX2002 2	2005	USA	DQ164205
6	Goose-Hungry/03	2005	Hungary	DQ118127
7	Ast02-2-692	2006	Russia	DQ411035
8	PaH001	2003	France	AY268133
9	WN Italy 1998-equine	2001	USA	AF404757
10	96-111	2004	France	AY701412
11	KN3829	2003	France	AY262283
12	RO97-50	2000	USA	AF260969
13	LEIV-Vlg00-27924	2003	Russia	AY278442
14	Eg101	2000	USA	AY260968
15	Kunjin virus	1988	Australia	D00246
16	Rabensburg isolate 97-103	2004	Austria	AY765264
17	H442	2007	South Africa	EF429200
18	SA381/00	2007	South Africa	EF429199
19	Goshawk-Hungary/04	2005	Hungary	DQ116961
20	B956	2004	USA	AY532665
21	SPU116/89	2007	South Africa	EF429197
22	SA93/01	2007	South Africa	EF429198
23	804994	2005	India	DQ256376
24	LEIV-Krnd88-190	2003	Russia	AY277251

Table 2
Clinical characteristics of patients with febrile illness, December 2009 to May 2010, in South India

Characteristic	Number	%
Chronicity		
Acute	27	100%
Chronic	Nil	
Laterality		
Unilateral	18	66.7%
Bilateral	9	33.3%
Pattern of anterior inflammation		
Granulomatous	Nil	
Non-granulomatous	2	7.4%
Location		
Posterior	25	92.6%
Diffuse	2	7.4%
Neuroretinitis	11	40.7%
Retinal vasculitis	9	33.3%
Retinitis	6	22.2%
Vascular occlusion	2	7.4%
Bilateral combined vascular occlusion	2	7.4%
Foveolitis	1	3.7%
Recurrences	Nil	Nil

processed for WNV detection by IgM capture ELISA and molecular methods. Out of the 105 samples, 35 (33%) were positive for WNV IgM antibody, 15 (14%) were positive for WNV by RT-PCR, and 27 (26%) were found to be positive for WNV by real-time RT-PCR and by RT-LAMP assays (Table 3). All real-time RT-PCR and RT-LAMP positive samples also showed positivity by CDC reported real-time RT-PCR assay. Comparative evaluation with acute-phase patient serum samples revealed 100% concordance between the real-time PCR and RT-LAMP assays. These assays had an overall higher sensitivity than the conventional RT-PCR as they picked up 12 additional samples with a low copy number of template. There was no cross-reactivity of real-time RT-PCR and RT-LAMP assays with other closely related flaviviruses or with healthy individual panel samples.

Real-time RT-PCR amplicons of the 27 WNV-positive samples were purified and sequenced by Sanger's dideoxy chain termination method (Figure 3). The nucleotide sequence of the partial *env* gene junction (201 bp) of the WNV conserved region was deciphered. The nucleotide sequences were subjected to BLAST search to find the closest sequence identity. The WNV sequences revealed closest sequence identity with isolates of genogroup I lineage circulating in North Africa and South America. These sequences were compared with WNV prototype strain (Eg101). All these sequences were aligned with the homologous regions (nucleotides 1–201) of prototype Eg101 strain and submitted to GenBank. The GenBank accession numbers are given in Table 4. The alignment did not reveal any base insertion or deletion in this region. On comparison to the prototype strain, the majority of mutations were found to be silent. The deduced amino acids were also aligned following the nucleotide alignment pattern. The majority of amino acid changes were found to be of conservative type. On comparison of the gene region, it was found that all WNV isolates were very closely related (90.0% at the nucleotide level and 91.7% at the deduced amino acid level) to the prototype strain. The phylogenetic analysis was carried out based on the nucleotide sequence of the partial gene sequence region (nucleotide positions 1028–1228). A dendrogram based on the *env* gene junction region clearly revealed that all the WNV isolates were grouped in lineage I (Figure 4).

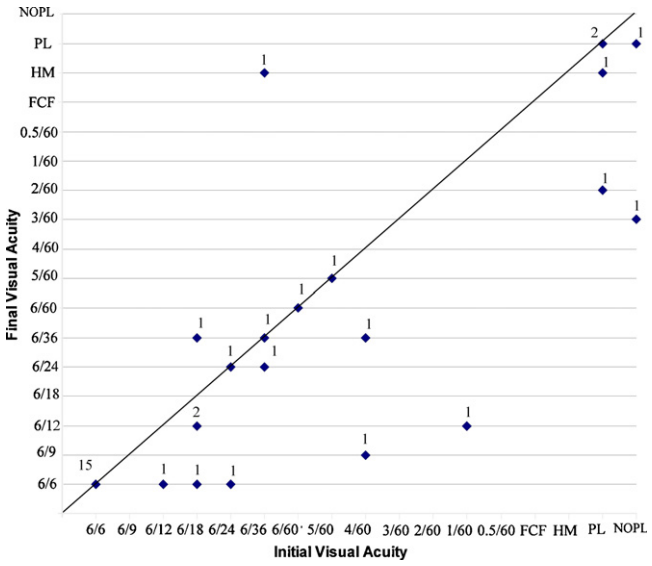

Figure 2. Scatter plot showing initial and final visual acuity. The diagonal line shows eyes with no change in vision. Points above the line show eyes that worsened and points below the line show eyes that improved.

4. Discussion

WNV is an emerging virus infection across the globe. Several outbreaks of varying degrees of severity have been reported from different countries.[16–18] However human ocular infection with WNV has not been reported in India so far. Based on clinical presentation, it is difficult to distinguish between the different *Flavivirus* group infections, as their systemic clinical signs overlap and the clinical picture is non-specific. Laboratory support is crucial for a definitive diagnosis of dengue, chikungunya, or WNV.[19]

In the present study, of 27 WNV patients, six did not have any systemic illness and two had a severe systemic illness with viral meningitis. During the previous outbreak of chikungunya viral infection in South India in 2005/2006, constitutional symptoms and arthralgia were very common clinical signs. Several patients in the 2009/2010 outbreak presenting with these symptoms developed post-febrile visual morbidity and were thus screened for chikungunya and dengue antibody by serology. These patients tested negative for all these serological tests. In addition to reported systemic symptoms such as headache, myalgia, arthralgia, malaise, nausea, vomiting, and pharyngitis, 11 patients had facial pigmentation and generalized hyper-pigmentation, and six patients also reported generalized anasarca during the febrile stage.

Although non-granulomatous uveitis and vitritis have been reported previously in WNV ocular infection,[20,21] in 25 out of 27 patients in our series, inflammatory cells were absent in the anterior segment and vitreous, despite the presence of retinitis. However, clear vitreous has also been observed by previous investigators in patients with WNV ocular disease.[22] Among

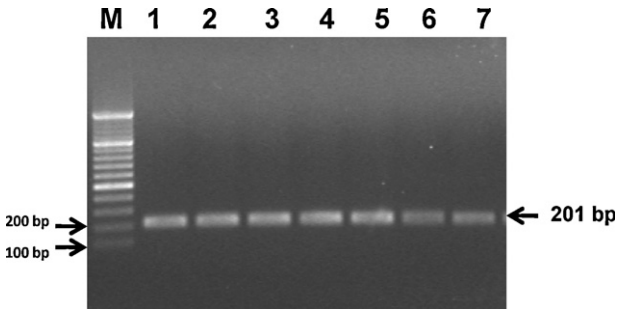

Figure 3. West Nile virus envelope gene-specific RT-PCR amplicons (201 bp) as observed on 2% agarose gel analysis under UV illumination.

Table 3
Comparative analysis of the results of tests for the detection of West Nile virus in suspected cases of West Nile infection

Results	RT-PCR ($n = 105$)[a]	Real-time RT-PCR ($n = 105$)[a]	RT-LAMP ($n = 105$)[a]	IgM detection ($n = 105$)[a]
Positive	15 (14%)	27 (26%)	27 (26%)	35 (33%)
Negative	90 (86%)	78 (74%)	78 (74%)	70 (67%)

RT-PCR, reverse transcription polymerase chain reaction; RT-LAMP, reverse transcription loop-mediated isothermal gene amplification.
[a] n = the number of suspected cases screened for West Nile infection.

various other ocular findings, multifocal chorioretinitis and retinal vascular involvement have been recognized as common, self-limiting ocular manifestations.[23,24] Kaiser et al. described a diabetic patient who had moderate visual impairment and multiple branch artery occlusions.[25] Khairallah et al. related the linear distribution of chorioretinitis with retinal nerve fiber organization,[26] suggesting a contiguous spread of WNV from the central nervous system via the optic nerve fibers to the outer retina, retinal pigment epithelium, and choroids.[27] Multiple small, round, scattered chorioretinal lesions in the posterior and peripheral fundus and optic neuritis have also been reported previously.[28,29] Linear chorioretinitis was not seen in our patients; instead, a uniformly similar fundus picture of clear vitreous, retinitis near the disc, vasculitis, and macular star with hyperemic disc were predominantly noted in the majority of patients. Common causes of retinitis and vasculitis in our population include toxoplasmosis, syphilis, herpetic viral retinitis, and occasionally tuberculosis. All these causes are known for their intense vitreous reaction, which was not present in these patients. Systemic evaluation also ruled out the possibility of tuberculosis or syphilis. Toxoplasmosis and syphilis serology were negative. Interestingly two patients who had high-grade fever presented to us with combined retinal arterial and venous occlusion, and they had very poor visual recovery.

This study identified the presence of WNV infection in southern India by real-time RT-PCR and RT- LAMP assays and found neuroretinitis and retinitis to be the most common ocular findings. We also found that the majority of patients had a good prognosis. Real-time RT-PCR and RT-LAMP assays can be used as diagnostic tools, and the RT-LAMP assay is more cost-effective with the same effectiveness as RT-PCR and real-time RT-PCR. Real-time RT-PCR assays using either probes with Taqman technology or SYBR Green have been developed for the detection of WNV infection.[30–32] Recently, other PCR-based methods such as the RT-LAMP assay have been developed by targeting the *env* gene of WNV. The RT-LAMP assay is a novel method of gene amplification that amplifies

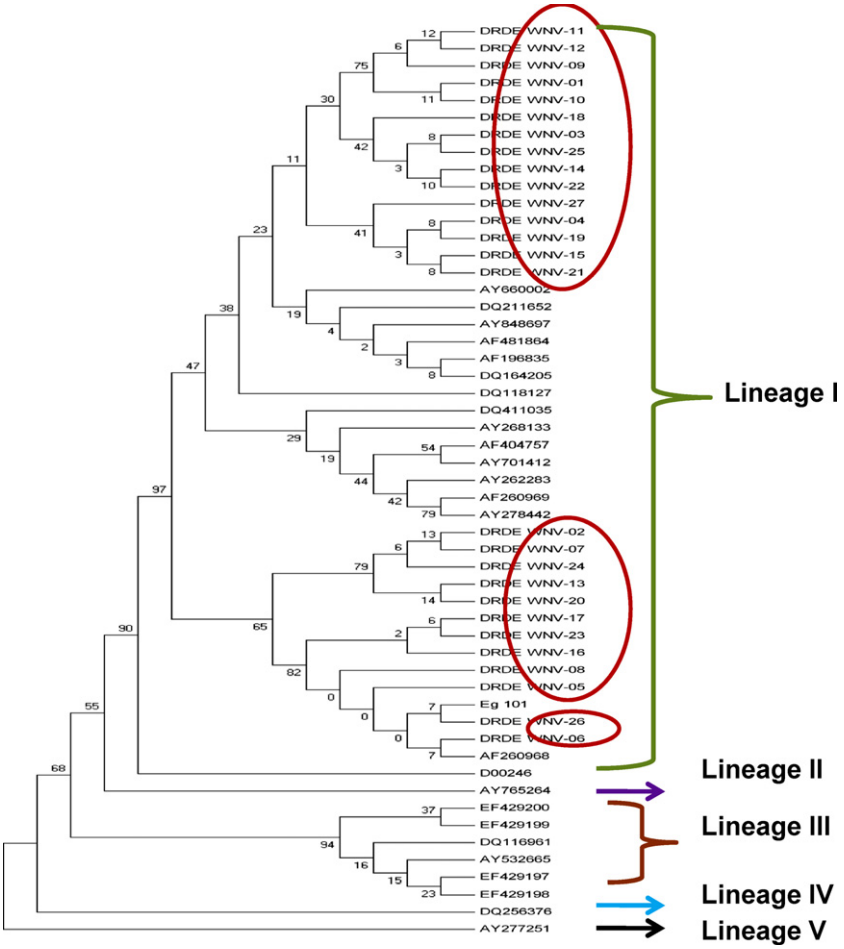

Figure 4. Phylogenetic analysis based on the 201-bp (positions 1028–1228) partial envelope gene of West Nile virus associated with ocular manifestations in Madurai, Tamil Nadu, India with respect to other global isolates. The encircled clusters are the samples sequenced in this study; their GenBank accession numbers are given in Table 4.

Table 4
Details of West Nile viruses sequenced in this study

S. No.	Virus strain	Year of sample collection	Country	GenBank accession No.
1	DRDE WNV-01	2010	India	JN591727
2	DRDE WNV-02	2009	India	JN591728
3	DRDE WNV-03	2009	India	JN591729
4	DRDE WNV-04	2009	India	JN591730
5	DRDE WNV-05	2010	India	JN591731
6	DRDE WNV-06	2010	India	JN591732
7	DRDE WNV-07	2010	India	JN591733
8	DRDE WNV-08	2010	India	JN591734
9	DRDE WNV-09	2010	India	JN591735
10	DRDE WNV-10	2010	India	JN591736
11	DRDE WNV-11	2010	India	JN591737
12	DRDE WNV-12	2010	India	JN591738
13	DRDE WNV-13	2009	India	JN591739
14	DRDE WNV-14	2009	India	JN591740
15	DRDE WNV-15	2010	India	JN591741
16	DRDE WNV-16	2010	India	JN591742
17	DRDE WNV-17	2010	India	JN591743
18	DRDE WNV-18	2010	India	JN591744
19	DRDE WNV-19	2010	India	JN591745
20	DRDE WNV-20	2010	India	JN591746
21	DRDE WNV-21	2010	India	JN591747
22	DRDE WNV-22	2009	India	JN591748
23	DRDE WNV-23	2009	India	JN591749
24	DRDE WNV-24	2009	India	JN591750
25	DRDE WNV-25	2009	India	JN591751
26	DRDE WNV-26	2010	India	JN591752
27	DRDE WNV-27	2010	India	JN591753

nucleic acid with high specificity, efficiency, and rapidity, under isothermal conditions, with a set of six specially designed primers that recognize eight distinct sequences of the target.

In the present study, the detection of WNV RNA in 14% of samples by RT-PCR and the 26% positivity by real-time RT-PCR and RT-LAMP, confirmed that the causative agent of this epidemic was WNV. All 78 patients who had clinically suspected WNV but whose real-time RT-PCR and RT-LAMP results were negative presented more than 7 days after the onset of fever; this may be the reason for the negative test results due to low transient viremia. The maximum number of PCR-positive results was observed on day 2 of illness.

In order to furnish the direct sequence information from the patient about the virus genotype, a short 201-bp fragment of the *env* gene from the real-time RT-PCR was selected for sequencing without passaging in a heterologous system. The *env* gene is the ideal target for genetic diversity studies owing to its immunodominant nature, which makes it subject to positive selection pressure. Previous phylogenetic analysis based on the 201-bp partial *env* gene sequence initially grouped WNV into two main phylogenetic lineages.[33] Strains from lineage I were restricted to North Africa, Asia, Europe, and North and South America, as well as Australia. Lineage II strains were in turn thought to circulate exclusively in southern Africa and Madagascar. These investigations subsequently subdivided lineage I strains into three subclades, with clade 1A representing strains from Africa, Asia, the Middle East, and America, clade 1B representing Australian strains (Kunjin), and clade 1C including isolates from India. It was then proposed that clade 1C represented a separate lineage (lineage V). Two new lineages have recently been described, namely Rabensburg virus (lineage III), isolated from a Culex mosquito at the border between Austria and the Czech Republic, and a proposed lineage IV strain identified in ticks in the Caucasus. Although it may be plausible that these new viruses represent individual flaviviruses that belong to the Japanese encephalitis serocomplex, phylogenetic analysis groups them with WNV.[34]

In conclusion, this is the first report on the implications of West Nile virus with ocular infection in India. The molecular techniques of real-time RT-PCR and RT-LAMP are useful for early, reliable, and rapid confirmation when there is a clinical suspicion.

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Conflict of interest: This is to certify that the authors do not have any commercial or other associations that might pose a conflict of interest.

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