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

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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