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Molecular Detection of West Nile Virus in Pediatric Patients with Meningitis and Encephalitis

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ABSTRACT

Introduction: West Nile virus (WNV) is a mosquito-borne pathogen of the *Flaviviridae* family with a global distribution. It is a significant cause of neurological diseases, including meningitis and encephalitis, in children. This study employed a SYBR Green-based real-time reverse transcription polymerase chain reaction (RT-qPCR) assay to detect and quantify WNV RNA in cerebrospinal fluid (CSF) samples from children presenting with meningitis and encephalitis. **Methods**: CSF samples (n=160) were collected from children diagnosed with aseptic meningitis or encephalitis at a single medical center in Karaj, Iran, from September 2019 to October 2020. Samples were analyzed by RT-qPCR, with viral loads quantified against a standard curve generated from serial dilutions of a plasmid containing the WNV capsid (C) gene. **Results**: Two of 160 CSF samples (1.25%; 95% CI: 0.15–4.46%) were positive for WNV genomic RNA. The viral loads were 3.4×10^3 and 1.2×10^5 copies per reaction in a 3-year-old female and a 4-year-old male patient, respectively. **Conclusion**: The low prevalence of WNV observed, coupled with the limited sample size and reports of higher prevalence in other regions, highlights the need for larger, multicenter studies to better define WNV epidemiology in children.

INTRODUCTION

West Nile virus (WNV) is a zoonotic arbovirus of the genus *Flavivirus* within the family *Flaviviridae*. It has a global distribution and infects humans and other vertebrates [1, 2]. WNV is transmitted primarily through *Culex* mosquitoes, with birds serving as the primary reservoir hosts, while humans and horses are considered dead-end hosts [3]. In 1970, WNV was first detected in Iran through serological surveys [4, 5]. It was not until later that active infections were confirmed by reverse transcription polymerase chain reaction (RT-PCR) in patients presenting with fever and neurological symptoms [6].

WNV belongs to the Japanese encephalitis serocomplex within the *Flavivirus* genus [5, 7-9]. Among WNV lineages, lineages 1 and 2 are most frequently associated with neuroinvasive disease, a pathogenicity linked to mutations in the envelope (E) protein and variations in glycosylation patterns that enhance neurotropism [10–12].

Approximately 80% of WNV infections in humans are asymptomatic, while the remaining 20% of infected individuals develop symptomatic illness. Severe

neuroinvasive conditions—such as meningitis, encephalitis, or acute flaccid paralysis—occur in less than 1% of all infections [13–16]. In children, WNV infections are typically asymptomatic or manifest as a mild, self-limiting, flu-like illness [12, 17, 18]. In Iran, most WNV studies have relied on serological evidence, and few have utilized RT-PCR to detect WNV genomic RNA in CSF samples. Therefore, this study aimed to detect and quantify WNV RNA in CSF samples from pediatric patients with meningitis or encephalitis using a real-time RT-qPCR assay targeting the capsid (C) gene.

MATERIAL AND METHODS

Study population and sample collection. This prospective study was conducted from September 2019 to October 2020. A total of 160 CSF samples were collected from children under 5 years of age who were diagnosed with aseptic meningitis or encephalitis and admitted to Imam Ali Hospital in Karaj, Iran. The study adhered to ethical guidelines approved by the Research Ethics Committee of Islamic Azad University (Ethics code: IR.IAU.PS.REC.1399.059). Written informed consent was obtained from the legal guardians of all participants. A standardized questionnaire was used to collect demographic and clinical data, including age, sex, presenting symptoms (*e.g*., fever, diarrhea, headache), and the duration of illness before admission. All samples were pre-screened and confirmed negative by routine bacteriological and molecular testing for common causative agents, including Herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and enteroviruses. The remaining samples were then stored at −80°C until RNA extraction.

Viral RNA extraction and reverse transcription. Total RNA was extracted from 200 µL of each CSF sample using the BIOFACT HiGene™ Total RNA Prep Kit (BIOFACT, Daejeon, South Korea). The eluted RNA was then used as a template for complementary DNA (cDNA) synthesis. The reverse transcription was performed using the LyoFACT™ RT Pre-Mix (BIOFACT, Daejeon, South Korea) with 10 µL of template RNA. Briefly, the reaction was incubated at 42°C for 60 min, followed by enzyme inactivation at 85°C for 5 min.

Real-time quantitative PCR (RT-qPCR) assay. A SYBR Green-based RT-qPCR assay was developed to amplify a 158 bp fragment of the WNV capsid (C) gene. The primers used were WNV-C-F (Forward): 5′-TGTCTAAGAAACCAGGAGGGC-3′ and WNV-C-R (Reverse): 5′-AAACGCCAAAAGAGCCAACA-3′. For using as a positive control and quantification standard, a synthetic plasmid (5,200 bp) containing conserved regions of the WNV C gene representative of lineages 1 and 2 was constructed. Following synthesis, the plasmid concentration was determined by spectrophotometry. This quantified plasmid was used to generate a standard curve via serial tenfold dilutions prepared in nuclease-free water. The initial plasmid copy number concentration was calculated using the standard formula: Copy number per µL = (Plasmid concentration [g/µL] × 6.022×10^{23} [molecules/mol]) / (Plasmid length [base pairs] × 660 [g/(mol·bp)]).

RT-qPCR reactions were performed on a Rotor-Gene system (Qiagen, Hilden, Germany). The thermal cycling protocol consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and extension at 72°C for 20 s. Standard curves were generated by plotting mean quantification cycle (Cq) values against the $\log_{10}$ of plasmid copy numbers per reaction. A no-template control (NTC) was included in each run. The *in silico* analytical specificity of the primers was evaluated using the Basic Local Alignment Search Tool (BLAST), which showed no significant sequence homology with other clinically relevant *Flaviviridae* members, predicting high specificity for WNV.

RESULTS

RT-qPCR assay performance and validation. The assay successfully amplified plasmid DNA dilutions ranging from 1.0×10^1 to 1.0×10^6 copies per reaction. The Cq values were linearly correlated with the $\log_{10}$ of viral genome copies, generating a standard curve with a coefficient of determination (R^2) of 0.946 and a slope of –3.454. From this slope, the PCR efficiency was calculated to be 95% (Figure 1). The lower limit of quantification (LLOQ) was 1.0×10^1 copies per reaction. Amplicon specificity was verified through melting curve analysis, which showed a single, distinct peak for all positive samples, indicating the absence of non-specific products (Figure 2).

Detection and prevalence of WNV in CSF samples. WNV genomic RNA was detected in two of the 160 CSF samples analyzed, corresponding to a prevalence of 1.25% (95% confidence interval [CI]: 0.15–4.46%). The positive samples were from a 4-year-old male and a 3-year-old female. Notably, both positive cases were identified from samples collected during July 2020, suggesting a potential seasonal pattern of infection. Detailed clinical characteristics of these patients are summarized in Table 1.

Viral load and clinical correlation. The viral loads in the two positive CSF samples were quantified at 1.2×10^5 and 3.4×10^3 copies per reaction for the 4-year-old male and 3-year-old female patients, respectively. A comparison of clinical records suggested a potential correlation between viral load and disease severity (Table 1). Both patients presented with fever and myoclonic jerks. The male patient, who had the higher viral load, also exhibited headache, neck stiffness, and seizures, leading to a diagnosis of Meningitis and Encephalitis. In contrast, the female patient, with the lower viral load, initially presented without these additional neurological signs but later developed seizures during hospitalization and was diagnosed with aseptic meningitis.

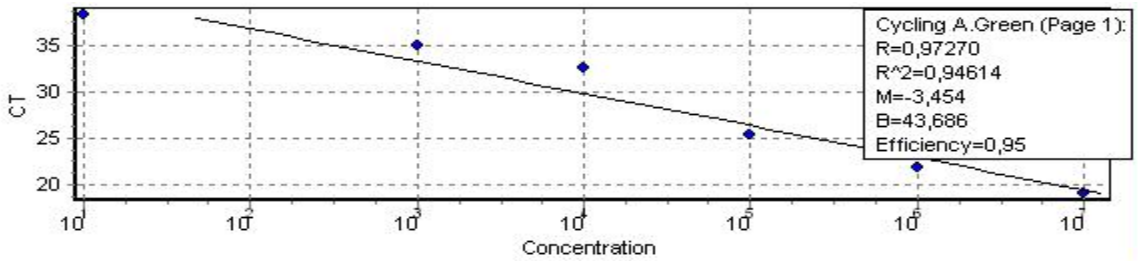

Fig. 1. Standard curve and performance of the WNV RT-qPCR assay. The standard curve was generated by plotting the quantification cycle (Cq) values (Y-axis) against the $\log_{10}$-transformed copy number of a serially diluted plasmid standard (X-axis). The assay demonstrated linearity across a dynamic range of 10^1 to 10^6 copies per reaction. The key performance parameters, as determined by the instrument software, include a coefficient of determination (R^2) of 0.946, a slope (M) of –3.454, and a corresponding amplification efficiency of 95%.

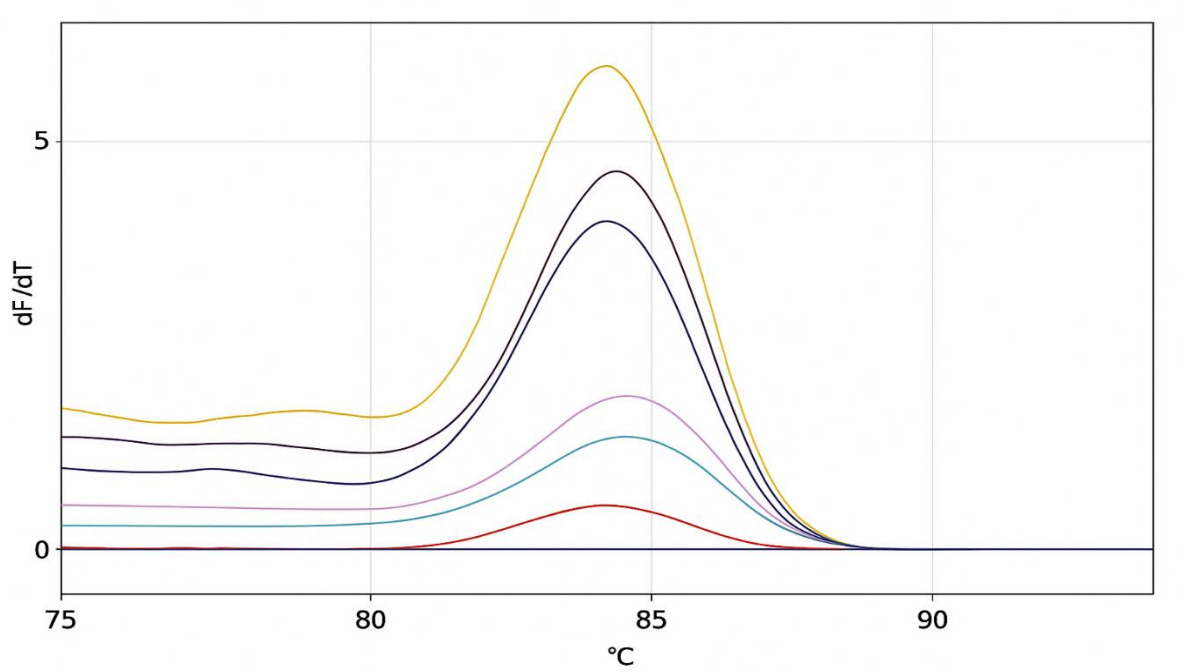

Fig. 2. Melting curve analysis confirming the specificity of the WNV RT-qPCR amplicon. The analysis plots the negative first derivative of fluorescence to temperature (dF/dT) *vs.* Temperature. A single, sharp peak was observed for all serial dilutions of the plasmid standard, with a consistent melting temperature (Tm) of approximately 84°C. This single peak confirms the amplification of a specific, homogeneous DNA product and demonstrates the absence of primer-dimers or other non-specific artifacts in the reaction.

Table 1. Demographic, clinical, and virological characteristics of the two WNV-positive pediatric patients

Sex	Age (years)	Fever	Viral load (copies/rxn)	Myoclonic jerks	Headache	Neck rigidity	Seizure	Meningitis	Encephalitis
Male	4	+	1.2×10^5	+	+	+	+	+	+
Female	3	+	3.4×10^3	+	–	–	+	+	-

Note: +, present; –, absent; y, years; rxn, reaction.

DISCUSSION

WNV is a mosquito-borne arbovirus with a global distribution across Africa, Europe, the Middle East, and West Asia, where it poses a significant public health concern by causing neurological disorders [5, 6, 19].

Although WNV is a less frequent cause of neuroinvasive disease in children compared to other viral pathogens, its diagnosis remains critical. During outbreaks, WNV diagnosis typically relies on serological assays or molecular testing of CSF; however, the limited availability of molecular diagnostics can hinder a timely diagnosis [12]. While serological methods are valuable, the diagnostic delay associated with seroconversion may impede prompt treatment decisions [6]. In contrast, RT-qPCR enhances the rapid diagnosis of WNV-associated meningitis and encephalitis by detecting WNV genomic RNA directly in CSF, enabling more timely clinical evaluation [12].

In this study, we detected WNV genomic RNA in 1.25% (95% CI: 0.15–4.46%) of CSF samples from pediatric patients in Karaj, Iran, with aseptic meningitis or encephalitis. This low prevalence is consistent with the established understanding that WNV neuroinvasive disease (WNND) is a relatively rare diagnosis in children compared to other etiologies of central nervous system infection [20]. However, the prevalence in our cohort is markedly lower than the 30% WNV positivity reported in a Croatian study of patients with suspected neuroinvasive disease [18]. This discrepancy may be attributable to several factors, including differences in local WNV endemicity, patient selection criteria, or the analytical sensitivity of the diagnostic assays used.

The epidemiology of WNV is complex, influenced by the interplay of *Culex* mosquito vectors, avian amplifying hosts, and local environmental conditions. Our finding showed that both WNV-positive cases occurred in July 2020 aligns with this ecological model, as this period corresponds to peak WNV transmission in the Northern Hemisphere, driven by elevated mosquito activity during warmer months. Furthermore, the variable clinical presentations of the two positive patients—ranging from aseptic meningitis to more severe meningoencephalitis—underscore the challenge of clinical diagnosis. Given that, initial symptoms like fever and seizures can be non-specific, these cases highlight the importance of molecular testing for an accurate and timely etiological diagnosis in pediatric patients presenting with neuroinvasive disease. The SYBR Green-based RT-qPCR assay used in this study demonstrated high analytical sensitivity and amplicon specificity for detecting WNV RNA in CSF, a performance consistent with that of previous reports [20, 21]. This approach offers an advantage over probe-based assays (*e.g.*, TaqMan), as SYBR Green method is less susceptible to false-negative results caused by point mutations within the probe-binding site [21].

In conclusion, WNV genomic RNA was detected in 1.25% of CSF samples from Iranian pediatric patients with aseptic meningitis or encephalitis. Nevertheless, several limitations of this study must be acknowledged. The low prevalence observed, the single-center design, and the limited sample size mean that our findings may not fully capture the true burden of WNV in the broader Iranian pediatric population. This limitation is underscored by reports of higher prevalence in other regions [22, 23]. Therefore, larger, prospective, multicenter studies are warranted to provide a more comprehensive assessment of WNV epidemiology and its clinical impact on children across different regions of Iran.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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AI DISCLOSURE

No artificial intelligence (AI) tools or algorithms were used in the design, execution, analysis, or writing of this study. All data collection, interpretation, and manuscript preparation were conducted manually by the authors.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are not publicly available due to privacy and ethical restrictions concerning patient data but are available from the corresponding author on reasonable request.

AUTHORS' CONTRIBUTIONS

SDMN: Investigation; Project administration; Writing – original draft; Writing – review & editing. ZI, FRS, BK, MHF and MF: Investigation; Project administration; Formal analysis; Writing – review & editing. ZF: Investigation; Project administration; Writing – review & editing. MG: Supervision; Methodology; Writing – review & editing.

ETHICS STATEMENT

The study protocol was approved by the Research Ethics Committee of Islamic Azad University (Ethics Code: IR.IAU.PS.REC.1399.059). The study was performed in accordance with the ethical principles of the Declaration of Helsinki of 1964 and its later amendments. Written informed consent was obtained from the legal guardians of all participants prior to their inclusion in the study.

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