

Detection of West Nile virus genome and specific antibodies in Iranian encephalitis patients

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SUMMARY

West Nile virus (WNV) is a mosquito-borne flavivirus which circulates in birds, horses and humans. An estimated 80% of WNV infections are asymptomatic. Fewer than 1% of infected persons develop neuroinvasive disease, which typically presents as encephalitis, meningitis, or acute flaccid paralysis. This study was conducted from January 2008 to June 2009 in Isfahan, Iran. Patients attending the emergency department with fever and loss of consciousness were consecutively included. Cerebrospinal fluids (CSF) were initially analysed through bacteriology and biochemistry examinations, resulting in those with evidence of meningitis being excluded. Patients' CSF and serum were diagnosed by serological and molecular assays. A total of 632 patients with fever and loss of consciousness were tested by CSF analyses. Samples of the remaining patients (39.4%) were referred for WNV investigation. Three (1.2%) of the patients were positive for both serum and CSF by RT-PCR, and six (2.4%) were positive only for IgG antibodies. History of insect bite, and blood transfusion and transplantation were risk factors for being positive by RT-PCR ($P=0.048$) and being IgG positive ($P=0.024$), respectively. The results of this study showed that the prevalence of West Nile fever is low in patients with encephalitis.

Key words: West Nile virus, encephalitis, Isfahan, Iran.

INTRODUCTION

West Nile virus (WNV) (family Flaviviridae, genus *Flavivirus*) is a small, enveloped, single-stranded, positive RNA genome virus. It was first isolated in 1937 [1]. Wild birds develop prolonged high levels of

viraemia and serve as amplifying hosts but generally remain asymptomatic. Nearly all human infections of WNV are due to mosquito bites; mosquitoes that transmit WNV are usually of the *Culex* species, which vary by geographical area. Other less common routes include occupational exposure to infected blood, blood transfusion and organ transplantation. The risk of neuroinvasive disease increases in the elderly and immunosuppressed individuals [2–4].

The virus was endemic throughout Africa, the Middle East, West and Central Asia, and the

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Mediterranean; however, most early epidemics occurred mainly in rural populations with few cases of severe neurological disease [5]. Naturally occurring WNV infection first appeared in North America in 1999 when an outbreak in New York City resulted in encephalitis in 62 patients and seven deaths [6]. This epidemic and similar outbreaks in Romania in 1996 and Russia in 1999 were the first reported epidemics in large urban populations and involved hundreds of patients with severe neurological disease [5]. Sequence studies of the earliest isolates from North America suggested that WNV was imported from the Middle East, possibly via infected humans arriving from Israel, from infected migratory birds or illicitly imported exotic birds, or via infected mosquitoes inadvertently transported in an aeroplane or other carrier [7, 8]. WNV has emerged as the most common cause of epidemic meningoencephalitis in North America and the leading cause of arboviral encephalitis in the USA [5].

In a study in 1974 in Iran, 100 sera from children aged 1–6 years were tested for the prevalence of antibodies against 15 different viruses. A positive reaction was detected in 10% of the sera with WNV [9]. In 1976, Saidi *et al.* reported human infection by WNV in Iran. The results of their study showed that out of a total of 698 blood and serum samples examined by the plaque reduction neutralization test for antibodies to WNV, 186 (26.6%) had antibodies. The highest prevalence of antibodies was found in the Dezful-Deigi region [10].

WNV is associated with febrile illness that occasionally causes neuroinvasive disease, particularly in the elderly or immunosuppressed host. West Nile fever (WNF) is characterized by fever, headache, malaise, back pain, myalgia, and anorexia. A maculopapular rash appears in about 50% of patients [11]. WNV infection can present as encephalitis, meningitis, or acute asymmetric flaccid paralysis. Encephalitis that is associated with muscle weakness and flaccid paralysis is particularly suggestive of WNV infection [3].

The diagnosis can be confirmed by demonstrating either specific IgM antibodies or viral nucleic acid in serum obtained in the acute phase of the infection and/or IgG antibodies in the late stage of the disease. Convalescent-phase serum should be tested for IgG antibodies if acute serum IgM antibody or plaque reduction neutralization tests are negative. In patients presenting with suspected meningitis, encephalitis, or acute flaccid paralysis, a lumbar puncture and testing

of cerebrospinal fluid (CSF) for detection of IgM antibodies as well as serological testing is recommended [12]. In cases with signs of CNS involvement, the CSF usually demonstrates a pleocytosis often with a predominance of lymphocytes as well as elevated protein concentration [12].

Objective

According to the national surveillance system of Arboviruses and Viral Haemorrhagic Fevers (Iran), there is little information about the epidemiology of WNF in Iran although a serological study which was recently performed on the sera of blood donors showed some seropositive sera; however, there has not yet been any molecular approach to the disease in Iran. With regard to recently reported disease outbreaks in neighbouring countries [13, 14] and the possible risk factors, knowledge of the epidemiology of the virus in Iran is important. As it was not possible to discover the cause of some of the reported encephalitis cases in Iran, we aimed to determine the prevalence of WNV encephalitis in Isfahan, central Iran.

METHODS AND MEASURES

Study design

This study was conducted in Isfahan, a city located about 340 km south of the capital city, Tehran; Isfahan is the capital of Isfahan Province and Iran's third largest city. The city is located in the lush plain of the Zayandeh River, at the foothills of the Zagros mountain range. The city enjoys a temperate climate and regular seasons. No geological obstacles exist within 90 km north of Isfahan, allowing cool northern winds to blow from this direction. Situated 1590 m (5217 ft) above sea level, Isfahan is very hot during the summer with maximum temperatures typically around 36 °C.

Patients attending the emergency department of Alzahra Hospital with fever and loss of consciousness were consecutively included. This study was conducted from January 2008 to June 2009. Inclusion criteria were having documented fever; oral temperature ≥ 38.7 °C and Glasgow Coma Scale (GCS) score of ≤ 14 . Exclusion criteria were contraindication for CSF sampling and other causes for loss of consciousness such as trauma. The sampling method was non-probability sampling.

History of insect bite, organ transplantation, blood transfusion and travelling to an endemic area in the previous month were assessed using a questionnaire. CSF analyses including Gram stain, cell count and biochemistry were performed for all patients. Patients with evidence of meningitis were excluded: positive Gram stain or culture, CSF/serum glucose <50%, CSF protein >50 mg/dl, presence of >6 white blood cells in the CSF. CSF and serum samples of the patients were centrifuged and stored in -70°C . All samples were then sent to the Pasteur Institute, Iran for diagnosis of WNV using specific IgM and IgG antibodies and the real-time polymerase chain reaction (RT-PCR) method.

Serological assay

For IgG ELISA, wells were coated overnight at 4°C with mouse hyperimmune ascitic fluid diluted 1:1000 in 0.05% Tween-20 PBS containing 5% skim milk as a saturating reagent. This solution was used to dilute antigen and sera. Native antigen at 1:500 dilution was incubated for 1 h at 37°C and after washing with 0.05% Tween-20 PBS, the clinical or animal sample diluted 1:100 was added for 1 h at 37°C . Peroxidase-labelled anti-human or anti-animal immunoglobulin was added at 1:1000 for 1 h at 37°C . After 10 min incubation with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL, USA), the OD was read at 450 and 620 nm. For IgM ELISA, the ELISA plates were coated with goat IgG fraction to human IgM (anti- μ chain) diluted in PBS $\times 1$ and incubated overnight at 4°C . After addition of diluted native antigen, diluted immunoascite was then added. After a definite incubation, peroxidase-labelled anti-mouse immunoglobulin was added and incubated. The plates were then washed three times with PBST containing 0.5% Tween. Finally, hydrogen peroxide and TMB were added and after a short incubation, the enzymatic reaction was stopped by the addition of 4 N sulphuric acid. The plates were then read by an ELISA reader at 450 nm.

Molecular assay

RT-PCR reaction was performed in 50 μl total volume as follows: 23 μl RNase free water, 10 μl buffer ($\times 5$), 2 μl dNTP mix, 1 μl primers A and B (10 μM) (primer A: 5'-TTGTGTTGGCTCTCTTGCGTTCCTT-3'; primer B: 5'-CAGCCGACAGCACTGGACATTCATA-3'), 2 μl enzyme mix, 1 μl RNase

inhibitor, 10 μl extracted RNA. All processes were performed on ice, in a clean room. For all materials Qiagen kit (Qiagen, USA) was used except for RNase inhibitor. Negative and positive controls were prepared using the same method. The PCR program was as follows: amplification of each sample was performed in a SmartCycler run with SmartCycler software (USA). Samples were subjected to one cycle at 50°C for 30 min, 95°C for 15 min, and then 95°C for 30 s, 54°C for 30 s and 72°C for 60 s, with a final extension of 72°C for 5 min.

Data analyses

The statistical analysis were performed using SPSS software version 16 (SPSS Inc., USA), and the significance level was set at $P < 0.05$. Quantitative variables were expressed as means and standard deviations (s.d.). The χ^2 test was used to check the differences between percentages.

RESULTS

During January 2008 to June 2009, a total of 632 patients attended our emergency department with fever and loss of consciousness for whom CSF analyses were performed. Bacterial meningitis was diagnosed in 383 patients; with either positive Gram stain, culture or biochemical evidence. Samples of 249 patients were sent for WNV investigation.

The remaining patients ($n = 249$) were 126 (50.6%) male and 123 (49.4%) female with a mean age of 35.76 years (s.d. = 15.37, range 10–81 years). CSF and serum analyses results and risk factors among patients with positive results are presented in Table 1.

As shown in Table 1, from 249 serum samples, three (1.2%) were positive for both serum and CSF by RT-PCR, and six (2.4%) were positive only for IgG antibodies. Of the patients with a positive RT-PCR result, one (33.3%) had insect bite in his history (compared to 1.25% of other patients, $P = 0.048$) and for patients with positive IgG antibodies one (16.6%) had a history of transplantation and another (16.6%) a history of transfusion, compared to 0% and 3.4% of other patients ($P = 0.024$ and $P = 0.180$), respectively. These data could indicate a correlation between detection of the virus genome and acute infection caused by insect bite compared to the IgG-positive patients with a history of transplantation.

Table 1. Summary of patients with positive WNV laboratory test among those with viral encephalitis referred to Alzahra Hospital, January 2008 to June 2009, Isfahan, Iran

Sex	Age	RT-PCR				Insect bite	Transfusion	Transplantation	Travelling
		Serum	CSF	IgG	IgM				
M	28	+	+	-	-	-	-	-	-
M	17	+	+	-	-	+	-	-	-
M	56	+	+	-	-	-	-	-	-
M	49	-	-	+	-	-	+	-	-
M	38	-	-	+	-	-	-	-	-
M	62	-	-	+	-	-	-	-	-
M	42	-	-	+	-	-	-	+	-
M	27	-	-	+	-	-	-	-	-
F	38	-	-	+	-	-	-	-	-

Relationships of gender to positive PCR result ($P=0.128$) or IgG results ($P=0.112$) were not statistically significant.

DISCUSSION

In our study, 1.2% of the patients were WNV-positive for both serum and CSF by RT-PCR, and 2.4% were positive only for IgG antibodies; mosquito bites and transplantation were risk factors for WNV infection. Although the results showed that most patients admitted to hospital were more often IgG positive rather than IgM and RT-PCR positive, both diagnostic assays for different stages of the disease are helpful during admission of patients.

Our study showed insect bite as a risk factor for WNV infection. This result is predictable as mosquito bites are one of the most important routes of disease transmission [15]. In addition, it was demonstrated that transplantation is a risk factor for WNV infection. There are different reports of WNV transmission from organ donors to transplant recipients [15, 16] and our study authenticated this mode of transmission.

This is the first report of WNV detection by RT-PCR from Iran. Other studies in Iran have shown antibodies against WNV in blood samples [9, 10] as well as the current study on serological findings of the patients. Regarding the results, about 2.4% of probable WNF patients with encephalitis signs had positive IgG which showed a previous contact with the virus and about 1% were positive by RT-PCR, which shows a recent infection. Although nationwide sampling may yield different results and would be more representative of the presence of WNV in Iran, our

results demonstrated a 2.4% seropositive prevalence in the representative samples which is comparable with 2.6% of individuals in the USA who were seropositive for WN encephalitis [17].

Since 2000 the national surveillance system of Arboviruses and Viral Haemorrhagic Fevers in Iran has monitored the country for endemic and probable arboviruses including Crimean-Congo haemorrhagic fever (CCHF), dengue fever (DF), Rift Valley fever (RVF) and WNF, while the ArboNET national surveillance system has tracked WNV in the USA. Therefore, with equipment allowing rapid serological and molecular diagnosis of WNV in the serum and CSF of probable cases, patients receive effective treatment that should lead to a decrease in the mortality rate. In 2002, a multistate outbreak was reported throughout the midwestern United States involving 2946 neuroinvasive disease cases [18]. In 2003, 1481 cases were reported, of whom 217 had neuroinvasive disease and 10 died [19]. During 2004–2006, fewer than 50 cases of neuroinvasive disease were reported each year, only to be followed by a large outbreaks in Alberta, Saskatchewan, and Manitoba in 2007 with 2338 cases reported, including 130 cases with neuroinvasive disease [20].

Since the first discovery of WNV, infrequent human outbreaks have been reported mostly in groups of soldiers, children, and healthy adults in Israel and Africa [21]. These outbreaks were associated with only minor illness in most patients [22]. However, since the mid-1990s, outbreaks of WNV infection associated with severe neurological disease have occurred in Algeria (1994, 1997), Tunisia (1997), Romania (1996), Russia (1999), Israel (2000), the USA (1999, 2002–2007), Sudan (2002), and Canada

(2002, 2003, 2007). In each of these outbreaks, mortality among patients with meningitis and encephalitis was about 10% and occurred more often in elderly patients [8, 13, 23, 24].

Our results demonstrate that about 3.6% of patients with encephalitis symptoms had been infected by WNV. Therefore WN encephalitis should be given consideration along with other agents of encephalitis. The national surveillance system should monitor and control the distribution of WNV throughout the country.

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DECLARATION OF INTEREST

None.

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