An outbreak of West Nile Virus infection in the region of Monastir, Tunisia, 2003

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Background: A West Nile (WN) fever epidemic occurred in the region of Monastir, Tunisia, between August and October 2003.

Aim of the study: We attempt to describe the epidemiology, clinical presentation, and outcome of patients with confirmed West Nile virus (WNV) infection.

Methods: Three groups of specimens were prepared. One was made up of serum only ($n = 43$), the other of cerebrospinal fluid (CSF) only ($n = 30$), and the third group was made up of both ($n = 40$). These specimens were obtained from 113 patients. A serological diagnosis and evidence of WNV genome by nested reverse-transcriptase polymerase chain reaction (nRT-PCR) and TaqMan reverse transcription-polymerase chain reaction (RT-PCR) were carried out.

Results: Thirty-eight cases (33.6%) were serologically positive. Results of nRT-PCR showed a total of 10 positive cases of WNV (8.8%) detected in group 1 ($n = 1/43$), group 2 ($n = 5/30$), and group 3 ($n = 4/40$) whereas the PCR TaqMan showed 18 positive samples (15.9%) found in group 1 ($n = 3/43$), group 2 ($n = 9/30$), and group 3 ($n = 6/40$). All TaqMan PCR positive cases were nRT-PCR positive. In addition, four serologically probable cases were confirmed by TaqMan PCR. The attempts to isolate WNV by cell culture were unsuccessful. Considering the results of TaqMan assay and the serological diagnosis, WNV infection was confirmed in a total of 42 patients. The main clinical presentations were meningoencephalitis (40%), febrile disease (95%), and meningitis (36%). Eight patients (19%) died. The highest case-fatality rates occurred among patients aged ≥ 55 years. The phylogenetic analysis revealed that isolates of WNV were closely related to the Tunisian strain 1997 (PAH001) and the Israeli one (Is-98).

Conclusions: West Nile virus is a reemerging global pathogen that remains an important public health challenge in the next decade.

Keywords: West Nile virus, Outbreak, TaqMan RT-PCR, Epidemiology, Meningoencephalitis

Introduction

West Nile virus (WNV) is an arthropod borne virus that is classified taxonomically within the *Flaviviridae* family, genus *Flavivirus*, and is a member of the Japanese encephalitis (JE) virus serocomplex, which includes JE virus, St. Louis encephalitis (SLE) virus, and Murray Valley encephalitis virus.¹ It was first isolated from a woman with a febrile illness in 1937 in the West Nile (WN) district of Uganda.² Other isolates were later obtained from the blood of apparently healthy children in Egypt.³ The ecological studies undertaken in 1952–1954 in the Sindbis district in Egypt established the cycle of the virus, which involves

mosquitoes as vectors, birds as amplifying hosts, and humans and horses as sensitive dead-end hosts.^{4,5}

Initially, WN fever was considered as a minor arbovirus infection inducing in humans essentially a non-symptomatic disease or a mild influenza-like illness that goes undetected and is generally characterized by acute fever, headache, myalgia, malaise, nausea, and vomiting. More recently, WN fever has become a major public health concern. Several human outbreaks have been reported with fatal cases and severe neurological diseases including meningitis, encephalitis, and meningoencephalitis occurring principally among elderly people.^{6–10} In very few cases, fulminant hepatitis, pancreatitis, and myocarditis have also been reported.^{11,12} West Nile virus encephalitis or meningitis cannot be clinically distinguished from other viral central nervous system (CNS) infections.^{13,14} The treatment is currently non-specific and supportive.

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The WNV has a widespread geographical distribution throughout the tropical and temperate regions of the world and is a vector-borne pathogen of global importance. It is now endemic in Africa, the Americas, Europe, Asia, the Middle East, and Australia, where it infects birds, humans, horses, and other mammals. The WNV has been responsible for several significant human epidemics notably in Israel (1950s, 2000, 2005–2010), France (1962, 2003), South Africa (1974, 2008–2009), Russia (1999, 2007), Romania (1996–1997, 2003–2009), Italy (1998, 2008, 2009), Hungary (2000–2009), Spain (2004, 2009), and Portugal (2004).^{8,15–23} Between 2010 and 2012, large outbreaks occurred in southeastern Europe in the Central Macedonia Region in northern Greece, as well as in Romania, Serbia, Hungary, Italy, Spain, Volgograd, Russia, Turkey, Israel, and Croatia.^{10,24–34}

In 2013, 226 human cases of WN fever have been reported in Croatia, Greece, Hungary, Italy, and Romania. In neighboring countries, 557 cases have been declared in Bosnia and Herzegovina, the Former Yugoslav Republic of Macedonia, Montenegro, Serbia, and the Russian Federation. Sporadic cases have been reported in Tunisia and Ukraine.³⁵ However, the most striking factor is its introduction in 1999 into the north-eastern United States, a country previously free of WNV, and its progressive spread to the North-South and East-West gradients to finally cover the North American territory in 2002 and 2004.³⁶ West Nile virus became endemic since its introduction in North America. So far, in the United States, a total of 16 196 patients with WNV neuroinvasive disease with onsets from 1999 through 2012 and 1549 deaths have been recorded.³⁷

The WNV strains form two major genetic lineages. Lineage 1 (L1), has a wide geographical distribution ranging from Europe, Africa, the Middle East, and America and was responsible for previous outbreaks in the Mediterranean and North America. Lineage 2 (L2) had only been found in sub-Saharan Africa and Madagascar until 2004, when it was isolated in a Hungarian goshawk with neurological disease.^{38,39} Recently, additional lineages in central and eastern Europe (lineages 3 and 4) and India (lineage 5) have been reported. The lineages 6 and 7 have also been proposed.⁴⁰

In the summer of 1997, two coastal districts of Tunisia: Sfax and Mahdia experienced an unprecedented epidemic of WN encephalitis/meningitis, with at least 173 hospitalized cases and 8 deaths.⁴¹ This was the first evidence of WNV in Tunisia, and the first known natural occurrence of a flavivirus. In August–October 2003, a new cluster of cases of suspected WNV infection presenting mainly with neurological symptoms appeared in Monastir region, which is a neighboring region to Mahdia.

The present study is the first report to describe the epidemiology, clinical presentation, and outcome of patients with confirmed WNV infection in the region of Monastir during the 2003 outbreak. The serological diagnosis, nested reverse-transcriptase polymerase chain reaction (nRT-PCR), and real time reverse transcription-polymerase chain reaction (TaqMan RT-PCR) using clinical samples for WNV detection were carried out. Cell culture was also used for virus isolation.

Material and Methods

Clinical specimens

Three groups of specimens were prepared. One was made up of serum only ($n = 43$), the other of cerebrospinal fluid (CSF) only ($n = 30$), and the third group was made up of both ($n = 40$). These specimens were obtained from 113 patients hospitalized at Fattouma Bourguiba University Hospital of Monastir presenting with fever, viral encephalitis, and meningitis during the epidemic infection that occurred in the region of Monastir between August and October 2003. Complete epidemiological and clinical data were available in all cases.

Laboratory methods

The samples were analyzed by enzyme-linked immunosorbent assay (ELISA). In addition, the specimens were tested by nRT-PCR and real time RT-PCR. We also attempted to isolate the virus from clinical specimens.

Case definition

The finding of any of the following was considered to represent laboratory evidence of WNV infection: (i) isolation by culture of WNV from serum or CSF; (ii) demonstration of genomic sequences in CSF or serum; (iii) demonstration of IgM antibody to WNV in CSF by IgM capture ELISA; and (iv) WNV IgM high titer and detection of WNV IgG and confirmation by neutralization. In the current study, all patients who were diagnosed as confirmed cases had either WNV-specific IgM antibody response in CSF or the RT-PCR was positive. Laboratory criteria for a probable case include the presence of WNV-specific IgM and IgG antibody response in serum in the absence of WNV-specific IgM antibodies in CSF.

Serological tests

Acute phase CSF and acute and convalescence phase serum samples (on the first day of hospitalization and within 10–14 days after symptom onset) were collected to be tested for the presence of IgM and IgG WNV-specific antibodies. The samples were transported at 4°C and stored at –25°C until testing. They were analyzed by ELISA (Anti-West Nile Virus ELISA (IgM) and Anti-West Nile Virus ELISA (IgG), EUROIMMUN, Medizinische Labordiagnostika AG),

which was performed by the Laboratory of Microbiology at Fattouma Bourguiba Teaching Hospital, Monastir.

RNA extraction

Virus RNA was isolated from CSF and serum by using the Magna Pure LC DNA isolation kit® (Roche Diagnostics, Penzberg, Germany). RNA was extracted from 200 µl of serum or CSF samples, eluted in a final volume of 100 µl of elution buffer, and was stored at -70°C until used. A negative control of RNase-free water and a positive control of WN-NY99 culture supernatant of 10² PFU were included in each assay.

Nested reverse-transcriptase polymerase chain reaction

The primers used for nRT-PCR were designed to amplify a conserved region of 176 bp at the 3'-untranslated region (UTR) (unpublished data). The annealing temperature was 55°C. Nucleotide positions of the primers according to Eg 101 strain and sequences are presented in Table 1.

The reverse transcription step was undertaken with the one step RT-PCR kit® (Promega, Corporation, Madison, WI, USA) using 10 µl of RNA and 50 pmol of each primer (F2/R2) in a 50 µl total reaction volume by following the manufacturer's protocol with the following cycling times and temperatures: 1 cycle at 48°C for 45 minutes and 94°C for 2 minutes and 40 cycles at 94°C for 30 seconds, 55°C for 50 seconds, and 68°C for 50 seconds, plus a final extension step at 68°C for 7 minutes.

For the nested reaction, 2 µl of the first stage amplification product was added to 50 pmol of second primers (F3/R3) in a 50 µl total reaction volume. This reaction mixture was amplified by using the following conditions: 94°C for 5 minutes and 40 cycles at 94°C for 1 minute, 72°C for 90 seconds, and a final elongation at 72°C for 7 minutes.

The nRT-PCR products were run on 2% agarose gel containing ethidium bromide (UltraPure™, Invitrogen, Cergy Pontoise, France) and were visualized under ultraviolet light. The PGEM marker (Promega Corporation, Madison, WI, USA) was used

to estimate the length of products. A positive nRT-PCR reaction was expected to produce a 176 bp band.

TaqMan assay

The WNV TaqMan primers and probe specific for the 5'-UTR were designed with the Primer-Express Software package (PE Applied Biosystems, Foster City, CA, USA). The TaqMan probe was labeled at the 5'-end with the FAM reporter dye and at the 3'-end with the quencher dye TAMRA as described previously.⁴²

The TaqMan assay was undertaken with 5 µl of RNA combined with 50 pmol of each primer and 10 pmol of the FAM- and TAMRA-labeled probe in a 50 µl of a total reaction mixture using the TaqMan RT-PCR Ready-Mix Kit® (PE Applied Biosystems). The samples were subjected to 45 cycles of amplification in an ABI Prism 7700 sequence detection system instrument (PE Applied Biosystems) according to the manufacturer's protocol for TaqMan assay RT-PCR cycling conditions.⁴²

Virus isolation by in vitro cell culture

The virus isolation was performed on SW13 cell line (ATCC CCL 105). Cells were grown in 24 well microplates in RPMI 1640 medium (Invitrogen), washed twice with phosphate-buffered saline and infected with 200 µl of patient samples. The samples were allowed to adsorb for 1 hour at 37°C with gentle swirling every 15 minutes. Eagle's minimal essential medium with 5% fetal calf serum, 100 U/ml penicillin, 200 µg/ml streptomycin, and 1 mg/mL l-glutamine (GIBCO BRL, Invitrogen, Carlsbad, CA, USA) was then added to the infected cells. Four passages were performed onto SW13 cell line. These cells were monitored daily for 7 days for cytopathic effect (CPE).

Sequencing and phylogenetic analysis

The amplified DNA products were purified using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA, USA). The primers F2, R2, F3, and R3 were used for sequence analysis using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE applied Biosystems). The PCR conditions used

Table 1 Sequences and position of primers used in nested reverse-transcriptase polymerase chain reaction (nRT-PCR) and PCR TaqMan

PCR	Primers	Position	Sequences (5'-3')
Nested PCR	F2	10535-10556	GTTGAGTAGA CGGTGCTGCCTG
	R2	10750-10770	CTAGGGCCGCGTGGGGCGTTG
	F3	10571-10591	GAGG ACTGGGTGAACAAAGCT
	R3	10727-10746	TGCCTTTGTTAACCCAGTCC
PCR TaqMan	WN NCS	10668-10684	5-CAGACCACGCTACGGCG-3'
	WN NCR	10770-10756	5-'CTAGGGCCGCGTGGG-3'
	Probe	10691-10714	5'TCTGCGGAGAGTGCAGTCTGCGAT-3'

WN: West Nile.

NCS: WN 3' non-coding forward (WN3'NC-forward)

NCR: WN 3' non-coding reverse (WN3'NC-reverse)

Table 2 Comparison of serologic, nested reverse-transcriptase polymerase chain reaction (nRT-PCR), and TaqMan assay results

		Positive confirmed cases					
Clinical cases		Serology	nRT-PCR	TaqMan	nRT-PCR and TaqMan	Serology and nRT-PCR	Serology and TaqMan
Cohort 1 (<i>n</i> = 43)	Sera	0	1	3	1	0	0
Cohort 2 (<i>n</i> = 30)	CSF	23	5	9	5	5	9
Cohort 3 (<i>n</i> = 40)	Sera	0	1	1	1	0	0
	CSF	15	3	5	0	3	5
Total: 153		38 (33.6%)	10 (8.8%)	18 (15.9%)	7 (6.2%)	8 (7%)	14 (12.3%)

were 96°C for 5 minutes, then 25 cycles at 96°C for 30 seconds, 55°C for 20 seconds, and 60°C for 2 minutes. The sequencing was conducted on Applied Biosystems 3730 1 DNA Analyser (PE applied Biosystems). A search for homologies to known sequences was conducted using the nucleotide database of the Basic Local Alignment Search Tool (BLAST) at <http://www.ncbi.nlm.nih.gov/BLAST/> and consensus sequences were aligned with the corresponding sequences deposited in the Genbank database with Clustal X.⁴³ The phylogenetic analyses were performed using Phylogeny Interference Program Package (PHYLP)⁴⁴ and the phylogenetic tree was generated by Tree View software.⁴⁵

Statistical analysis

To determine risk groups and mortality, a statistical analysis (chi-2 test) with a level of significance of 95% was performed using Epi Info version 6.04 (CDC, Atlanta, GA, USA).

Results

West Nile virus detection

Of a total of 113 clinical cases between sera and/or CSF collected in the region of Monastir from patients admitted to Fattouma Bourguiba hospital, 38 cases (33.6%) were serologically positive according to the case definition. Results of nRT-PCR showed a total of 10 positive WNV cases (8.8%) detected in group 1 (*n* = 1/43), group 2 (*n* = 5/30), and group 3 (*n* = 4/40) (Table 2), whereas the quantitative RT-PCR (TaqMan) showed 18 positive samples (15.9%) found in group 1 (*n* = 3/43), group 2 (*n* = 9/30), and group 3 (*n* = 6/40). All TaqMan PCR positive cases were nRT-PCR positive. In addition, four serologically probable cases were confirmed by TaqMan PCR, of whom two were detected by nRT-PCR (Table 2). The results of serology, nRT-PCR, and TaqMan assay are compared in Table 2. Considering the results of TaqMan assay and serological diagnosis, WNV infection was confirmed in a total of 42 patients. The attempts to isolate the WNV using SW13 cell culture were unsuccessful. No cases were positive by isolation of the virus from serum or CSF.

Epidemiological and clinical characteristics

The demographic and clinical data are shown in Table 3.

Monastir region is a costal district geographically neighboring to Mahdia (Fig. 1), which experienced the first evidence of WNV in Tunisia in the summer of 1997.

Among the 42 positive cases, 27 (64%) were males, 15 (36%) were females with a male : female infection ratio of 1.8 (*P* = 0.1). The median age of the patients was 53.5 years (range, 10–80 years) (Fig. 2). Twenty-two patients (52%) were at least 55 years old (*P* < 0.1), of whom 8 (36.3%) had diabetes mellitus at presentation (Table 3). The onset of illness ranged from August to October 2003 with the peak of the outbreak occurring in mid-September (week 37) and waning with the onset of cooler temperatures (Fig. 3).

Of all the patients, 17 (40%) had meningoencephalitis, 15 (36%) had meningitis, and 10 (24%) had an illness characterized by fever and headache. Patients with meningoencephalitis are older than those with meningitis (median age, 63.6 vs 51.2 respectively; *P* = 0.001).

Table 3 Demographic and clinical presentation of the positive cases of West Nile virus (WNV)

	Number of positive cases	Percentage (%)	<i>P</i> value
Gender			
Male	27	64	0.1
Female	15	36	
Age (years)			
≥55	22	52	<0.1
<55	20	48	
Symptoms			
Meningitis	15	36	0.001
Meningoencephalitis	17	40	
Fever	40	95	
Headache	27	65	
Neck stiffness	19	45	
Nausea and vomiting	19	45	
Asthenia	6	15	
Arthralgia and myalgia	15	35	
Altered mental states	15	35	
Photophobia	2	5	
Eruption	2	5	
Diplopia	2	5	
Rash	2	5	
Medical history			
Diabetes	8	19	<0.1

Figure 1 Distribution of human cases of West Nile virus (WNV) infection, Monastir region, Tunisia August–October, 2003.

Nearly all the patients presented with fever (95%) and temperatures ranged from 37.5 to 40°C. Other symptoms included vomiting (45%), headache (65%), altered mental states (35%), arthralgia and myalgia lasting 3–6 days (35%), and stiff neck (45%). A rash was reported in two patients (5%) and was described as an erythematous macular, papular, or morbilliform eruption.

Pleocytosis (mean CSF white cell count, range 5–490) with lymphocytic predominance, an elevated

protein concentration (≥ 0.4 g/l), mild leukocytosis ($\geq 145/\text{mm}^3$), low level of serum sodium, and normal CSF glucose level (≥ 0.5) were found in all patients.

Only the age of 55 years or older was associated with a significantly higher likelihood of encephalitis and meningoencephalitis. Eight patients died with an overall case-fatality rate of 19%. The age of 55 years or older was the factor most strongly associated

Figure 2 Age distribution of patients with confirmed West Nile virus (WNV).

Figure 3 Reported cases of West Nile (WN) infection by week of symptom onset, Monastir region, Tunisia, August–October 2003.

with death. The mean age of patients who died was 69 years (range, 55–80 years) ($P < 0.005$).

The average length of acute-care hospital stay was 9 days, with a median of 7 days (range, 2–37 days). Patients with meningoencephalitis had a longer median length of stay than those having meningitis (37 vs 18 days, respectively). For the remaining cases, recovery was generally rapid and complete.

Phylogenetic analysis

The sequencing of amplified fragments was performed to characterize the isolated viruses. The sequences were aligned using the Clustal X program. A phylogenetic tree including the WNV isolates and their relationship with other WNV strains of different lineages is represented in Fig. 4. As shown in the figure, the WNV isolates fall into lineage 1. The phylogenetic analysis revealed that our isolates (designed respectively by Tun1-03, Tun2-03, Tun3-03, and Tun4-03) were closely related to the Tunisian strain 1997 (PAH001) and the Israeli one (Is-98). These results suggest that the virus amplified in this study derived from the single introduction that occurred and circulated in Tunisia, 1997.

Discussion

West Nile virus is an emerging pathogen that is causing diseases in humans in Europe, the Mediterranean basin and worldwide.

The main aim of this study was to carry out an epidemiological and clinical study of a suspected WNV infection that occurred in August–October, 2003, in the region of Monastir on the central coast of Tunisia. To our knowledge, this is the first report to describe the 2003 outbreak in this region.

The patients were admitted to hospital mainly because of neurological symptoms. Cerebrospinal fluid and serum samples were tested by serology (IgM and IgG ELISA) and nested and TaqMan RT-PCR. The attempts were also made to isolate the virus on SW13 cells.

The serological testing of CSF showed 38 positive cases (33.6%) of WNV infection, which confirms that IgM ELISA remains a technique of reference.⁴⁶ However, in some cases, particularly when patients die during the first week of illness, serology may have limited use due to low levels of host antibodies against WNV.⁴⁷

Using molecular approaches, the WNV was detected in 10 cases (8.8%) by nRT-PCR, from whom there were two probable cases. In comparison, 18 cases (15.9%) were detected by the real time RT-PCR. The results have shown firstly the ease of the virus detection from either CSF or serum by the two molecular used approaches, nested and TaqMan RT-PCR.^{48,49} Compared to the nRT-PCR, the TaqMan was a highly sensitive, specific, and rapid technique for viral RNA

detection. It is a quantitative test over a wide dynamic range.⁵⁰ In addition, the assays are done in a closed system and the reaction mixture is discarded, which decreases the risk of contamination.^{40,42,50,51}

The recent development of real time RT-PCR has combined the amplification and detection steps and has thus increased throughput dramatically, as well as improved quantitation, specificity, and accuracy,^{42,50,51} but in human disease, these assays detect infection in only 57% in CSF and 14% in sera. This reduced ability to detect WNV in CSF or serum is likely due to the short-lived viremia in humans with WNV infection that occurs before clinical symptoms develop, as has been reported previously.^{42,52} Some patients, particularly those who are immunocompromised may never produce antibody, but such patients are more likely to have virus detected by PCR.⁵³ However, our study had some limitations. The techniques used for the genomic amplification could not detect lineage two strains. Future investigations implicating diagnostic sensitivity, specificity, and reproducibility are needed.

The attempts to isolate the WNV by cell culture from human samples are frequently unsuccessful because of the low viral titer in clinical specimens.^{48,49,53} Virus isolation in cell culture is not only time-consuming but is also generally low-yield because of low concentrations of viable particles in tissues and fluids.⁵⁴ It would require a blood sample collected early after the onset of fever or an abnormally lasting replication of the virus due to immunodepression.

In the recent years, the frequency of WNV outbreaks in humans has increased. These outbreaks have been associated with a high incidence of severe disease. Since 1999, WNV has flourished in the North American ecosystem where it caused, in 2002, the largest arboviral meningoencephalitis outbreak ever recorded in this area before reaching central and southern America.³⁶

The data obtained in the current study suggest that the 2003 epidemic in Tunisia started in mid-August and abated by the beginning of October like the epidemic in the Sousse area.⁵⁵ The 2000 epidemic in Israel started at the end of July–early August, peaked in the second week of September, and abated by the end of October.⁵⁶ However, the 1997 epidemic in the region of Mahdia and Sfax started in September and ended by mid-December like the 1996 epidemic in southeast Romania.^{41,57} In comparison, the summer of 2010 was extremely hot in areas, which usually have a temperate climate, such as northern Greece and Romania.^{10,58} A human WNV outbreak emerged in northern Greece for the first time, early in July 2010 and peaked in mid-August.⁵⁸ The time of the year in which WN fever outbreaks occur has remained constant for the last decade. They generally coincided with the increased population of the *Culex*

Figure 4 Phylogenetic tree of West Nile virus (WNV) strains isolated during the outbreak in the region of Monastir, Tunisia, 2003.

mosquitoes during summer or early fall in temperate regions and during the rainy seasons in tropics but little published information on the epidemiology and ecology of WNV in the tropics is available.^{23,27,55}

The relation between WNV infection and gender is not clear. In the previous outbreaks in Israel, most patients were without predominance of either sex, but many recent outbreaks showed that more than 50% were males.^{7,12,27} The present study demonstrated a male to female infection ratio of 1.8 ($P = 0.1$). In the Sousse area, the sex ratio was of 3.2. In addition, our previous investigations conducted on two populations of Tunisian blood donors living in areas where human outbreaks of WNV have occurred (Monastir and Mahdia areas) showed that the prevalence of anti-WNV IgG was significantly lower in females than in males.⁵⁹ Male sex may be an important risk factor for developing WNV disease.^{10,60–62} The hypothesis of a prolonged male activity outdoors, especially in the evenings when the *Culex*'s activity is optimal was raised but more evidence is needed to support such hypothesis.

West Nile virus, initially regarded as benign, typically causes a self-limited illness characterized by fever, asthenia headache, vomiting, myalgia, and arthralgia.^{14,63} This self-limited syndrome is so non-specific that it often escapes medical attention. However, recently, a link between WNV infection and severe CNS disease has been observed. During the last decade, only few encephalitis cases were reported in Israel, India, and the Mediterranean region.⁷ A major change in neurovirulence was evident with the occurrence of three epidemics of WNV, gaining more attention: the first in Romania in 1996, where high rates of neurological disorders and death were recorded (up to 10%), the second in New York in 1999, in which several cases of flaccid paralysis were described, and in Israel in 2000 with unprecedented high rates of encephalitis.^{7,57,64} Central nervous system involvement has been reported likewise in France, Russia, Greece, and Serbia.^{12,13,27,65,66} Similarly, our results indicate that the WN fever was a severe disease with significant CNS involvement (78%) and high mortality (19% death rate in the study group). Our results were similar to that reported in 1996 Romanian outbreak when we compare the percentage of meningitis (36 vs 40%, respectively) and meningoencephalitis (40 vs 44%, respectively). In the Sousse area, the clinical presentation was meningitis (52.3%), meningoencephalitis (33.3%), and encephalitis (14.3%).⁵⁵

Furthermore, a clear correlation between age and increased severity and death was noted. Patients aged ≥ 55 years were more likely to present with meningoencephalitis and had an increased mortality rate. During the Romanian outbreak in 1996, all the patients were over 50 years and so were the patients

during the outbreak in the Sousse area in 2003, the Russian and the Croatian outbreaks in 2012.^{12,13,34,55} The association between age and severe disease has been also reported in Italy, Greece, and Serbia.^{29,65} The contributing factor of age may relate to a decrease in the integrity of the blood–brain barrier and facilitate access of WNV to the CNS.

In our study, all the fatalities occurred in the group of patients aged ≥ 55 years. The reasons for the high death rates observed in the present epidemic are not clear. The fatality rate among patients (19%) was higher than that described in Israel in 2000 (14.1%), in New York and New Jersey (11.0%), and in Romania in 2010 (10%).^{57,63,67} The overall case-fatality rate among patients with West Nile neuroinvasive disease (WNND) was also high in Serbia (16%) and Greece (17%).^{24,27,65} The reasons for the observed differences are not clear, but they may be related to changes in WNV virulence, the immunological background in a population, the co-morbid conditions, and other factors.

The pathogenesis of severe infection with WNV is not well understood, but the pronounced risk of neurological infection and the grim outcome in the elderly raise the question of whether old age is a risk factor in itself or whether coexisting conditions associated with advanced age such as immune senescence and responsiveness, coexisting illness (diabetes mellitus, hyperglycemia, hypertension, and cerebrovascular disease),⁶⁵ and profound weakness account for the observed increased risk since they may facilitate the passage of such a neurotrophic virus across the blood–brain barrier and predispose infected individuals to neurological complications. Low background immunity may also account for higher morbidity and death since the outbreak in the region of Monastir was the first reported in that area.

The treatment of the WNV infection is supportive. Although no specific drugs achieved acceptance for the treatment of severe presentations, clinicians opted for ribavirin in the treatment of our hospitalized patients. It is a synthetic nucleoside affecting mainly RNA metabolism and has proven some *in vitro* activity against WNV infection.⁶⁸

The phylogenetic analysis revealed that our WNV isolates fall into lineage 1. They were closely related to the Tunisian strain 1997 (PAH001) and the Israeli one (Is-98). These results suggest that the WNV strain derived from the single introduction that occurred and circulated in Tunisia, 1997. The emergence of new pathogenic WNV strain(s) and the peculiarities of the human host response could explain the large increase in the severity of human disease in this outbreak. Studies in North America support this hypothesis.^{69,70}

However, only hospitalized patients were tested for WNV in Monastir area. Non-hospitalized patients

with WNV infection were not studied. Consequently, the full spectrum of symptoms of WNV illness, especially the mild disease, could not be discovered, and the total burden of the disease might have been underestimated. Regarding equidae, a study was achieved during a cross-sectional survey to characterize the nationwide serological status of WNV in 2009, in Tunisia. The estimated overall seroprevalence rate was 28%, and interestingly, a rate of 64% was observed in Monastir region.⁷¹ The reoccurrence of human cases in consecutive years and the spread of the virus in new areas⁷² suggest that WNV is established in Tunisia, and its transmission may continue to occur in the future. The potential for causing large-scale outbreaks, with substantial illness and death calls for the need of effective vaccines and treatment. The insufficient immunization coverage and population mobility may explain in part the permanence of these epidemics, which also reinforces the need to rebuild the public health infrastructure to deal with outbreaks of vector-borne diseases and to develop effective surveillance, prevention, and control strategies. The virus will remain an important public health challenge in the next decade.

Disclaimer Statements

Contributors Riabi Samira carried out the molecular methods and the analysis and interpretation of these Data, Imed Gaaloul and Mahjoub Aouni carried out the correction of the manuscript, Maha Mastouri and Mohsen Hassine carried out the collection of samples and the serologic methods. Mahjoub Aouni is the director of this work and responsible for the general supervision of the research group. All authors read and approved the final manuscript.

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