West Nile virus outbreak in humans, Greece, 2012: third consecutive year of local transmission

D Pervanidou (pervanidou@gmail.com)[1,2], M Detsis[1], K Danis[1,2], K Mellou[1], E Papanikolaou[1], I Terzaki[1], A Baka[1], L Veneti[1], A Vakali[1], G Dougas[1], C Politis[1], K Stamoulis[3], S Tsiodras[1,4], T Georgakopoulou[1], A Papa[5], A Tsakris[4], J Kremastinou[1], C Hadjichristodoulou[1,6]

1. Hellenic Center for Disease Control & Prevention, Athens, Greece
2. European Programme in Intervention Epidemiology Training (EPIET), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
3. Hellenic National Blood Centre, Athens, Greece
4. Medical Faculty, University of Athens, Athens, Greece
5. Medical Faculty, University of Thessaloniki, Thessaloniki, Greece
6. Medical Faculty, University of Thessaly, Larisa, Greece

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In 2010, the first outbreak of West Nile virus (WNV) infection in Greece was recorded, the largest in Europe since 1996. After 2010, outbreaks continued to occur in different areas of the country. Enhanced surveillance was implemented during transmission periods (June to October). We investigated the 2012 outbreak to determine its extent and identify risk factors for severe disease using regression models. Of 161 cases recorded in 2012, 109 had neuroinvasive disease (WNND). Two outbreak epicentres were identified: the southern suburbs of Athens in July and a rural area in East Macedonia &Thrace in August–September. The case fatality rate of the WNND cases was 17% (18/109). A lower case fatality rate was recorded in the two epicentres (7% (2/28) and 9% (4/46)): the higher case fatality outside the two epicentres might reflect a diagnostic bias. Age above 74 years (adjusted risk ratio (RR): 7.0; 95% CI: 2.2–22) and chronic renal failure (adjusted RR: 4.5; 95% CI: 2.7–7.5) were independently associated with WNND-related death. In three PCR-positive samples, sequencing revealed WNV lineage 2 identical to the 2010 strain. The occurrence of human cases in three consecutive years suggests that WNV lineage 2 has become established in Greece. Raising awareness among physicians and susceptible populations (elderly people and persons with comorbidities) throughout Greece is critical to reduce the disease impact.

Introduction

West Nile virus (WNV) is one of the most widely distributed arboviruses in the world, with endemic foci in Africa, the Middle East, west Asia, North and Central America, parts of Europe and Australia [1]. Human cases have been reported from several countries since the 1960s; however, the frequency of reported outbreaks has increased over the last 15–20 years [2,3]. About 20% of persons infected with WNV develop a mild disease, usually referred to as West Nile fever (WNF). In less than 1% of the cases, the virus causes a neuroinvasive disease (WNND) with serious neurological manifestations, i.e. encephalitis, meningitis, meningoencephalitis or acute flaccid paralysis [4]. Among patients with severe illness, the case fatality rate varies (e.g. approximately 10% in the United States [5] and 12–18% during transmission periods during 2010 to 2011 in Greece [6]).

Two main WNV genetic lineages are known: lineage 1, identified in the majority of the outbreaks in humans and horses in Europe and the United States and lineage 2, which until 2004 had not been detected outside Africa, but since then has repeatedly appeared – initially in Hungary in 2004 [7] and 2005–09 [8], in Russia in 2007 [9] and in Austria in 2008–09 [8,10].

Before 2010, symptomatic human cases of WNV infection had not been documented in Greece. However, serosurveys in the early 60s, 80s and in 2007 suggest that WNV or a related flavivirus had been circulating at low levels in Greece at least since the 60s [11-13]. The first recorded outbreak of WNV infection in Greece was in 2010: this was the largest reported outbreak in Europe since 1996 [14], with 262 recorded cases. Of these, 197 developed WNND, of whom 33 (17%) died [15]. A seroprevelence study conducted after the 2010 outbreak (between 25 November to 22 December 2010) indicated that 1 in 140 people infected with WNV developed WNND [16].

The outbreak in 2010 was first detected in the Central Macedonia region, in northern Greece [15,17]. Surveillance in the blood donor population and post-transfusion information during the 2010 outbreak

showed that the estimated risk of infected blood donations in the affected areas, associated with collecting blood from asymptomatic donors (based on the method proposed by Biggerstaff and Petersen [18], which is recommended in the 2012 European Union (EU) preparedness plan for WNV and blood safety [19]) was 2.95 per 10,000 population. Transfusion-transmitted WNV infection was recorded in two of 369 thalassaemic patients in 2010 (incidence 1:2,397 transfused units of red cell concentrates), before the implementation of blood safety measures in this year [20,21].

In 2011, cases of WNV infection occurred in the same districts as in 2010. In addition, the virus dispersed southward to the region of Thessaly, and further south to Eastern Attica, in proximity to the metropolitan area of Athens [6]. Overall, 100 human cases were identified, 75 of whom had WNND and nine (12%) of the WNND cases died.

WNV lineage 2 sequences (strain Nea Santa-Greece-2010) were obtained from blood donors, mosquitoes and birds in the transmission period (June to October) of both years [22-27].

In 2012, cases of WNV infection were first reported to the Hellenic Center for Disease Control & Prevention in June. Here we present the analysed epidemiological data gathered during the 2012 transmission period in order to describe the outbreak in terms of time, place and person and identify possible factors associated with disease severity.

Methods

Surveillance

Following the 2010 outbreak, physicians (public and private sector) in Greece were asked to include WNV infection in their differential diagnosis during the transmission period and notify daily suspected and laboratory-diagnosed cases to the Hellenic Center for Disease Control & Prevention. In parallel, during this period, daily information exchange with the laboratories involved in the diagnosis of WNV infection was established for timely case identification and investigation.

Outbreak case definition

The 2008 European Union case definition for WNV infection [28] was used with a slight modification, i.e. the definition of probable cases included clinical and laboratory – but not epidemiological – criteria).

Data collection

We collected information regarding the demographic characteristics, clinical manifestations, underlying chronic diseases and laboratory results of all the cases reported in 2012 by using standardised reporting forms. We telephoned treating physicians of all reported cases for data validation and follow up of the patients' clinical status. Moreover, in-depth telephone interviews with all cases or their close relatives (as proxy respondents, when cases had severe disease and/or cognitive problems) using a semi-structured questionnaire were conducted to obtain a detailed travel history during the incubation period (2–14 days before symptom onset) and identify the suspected place of exposure.

Cases reported as having encephalitis (including meningoencephalitis), meningitis or acute flaccid paralysis were classified as having WNND. WNND classification was based on the treating physicians' clinical assessment and laboratory data (detection of WNV nucleic acid and/or WNV-specific antibody response in cerebrospinal fluid (CSF) and/or imaging findings), when available. Deaths in persons with WNV infection were recorded during hospitalisation.

Municipalities (the lowest administrative unit) with at least one human laboratory- diagnosed case of WNV infection during the 2012 transmission period were classed as affected areas.

We assigned week numbers using the International Organization for Standardization (ISO) 8601 standard [29].

Blood safety measures

Measures for the protection of blood donations against WNV infection were implemented in the affected areas. These included blood donor deferral, blood screening for WNV RNA and haemovigilance (a set of organised surveillance procedures related to serious adverse or unexpected events or reactions in donors or recipients and the epidemiological follow-up of donors, according to the eligibility criteria of donors of whole blood and blood components as referred to in article 4 and annex III of the Commission Directive 2004/33/EC [30]).

Laboratory methods

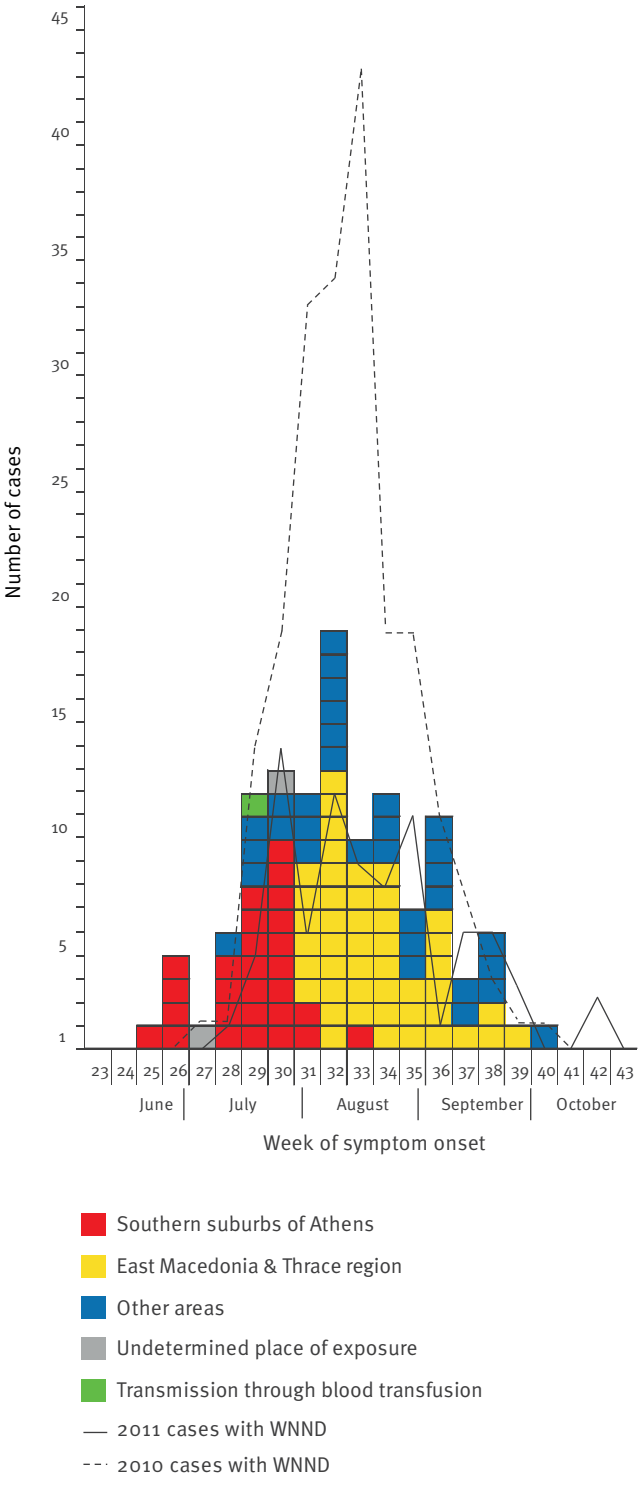
We obtained laboratory data from the four laboratories in which all the suspected WNV infection cases from all over Greece were tested: (i) National Reference Laboratory for Arboviruses, School of Medicine, Aristotle University of Thessaloniki; (ii) Department of Microbiology, School of Medicine, University of Athens; (iii) Department of Microbiology, Infectious Disease Hospital of Thessaloniki; and (iv) Department of Diagnostic Services, Hellenic Pasteur Institute.

Serum and CSF specimens were tested for IgM and IgG against WNV by ELISA (WNV IgM capture DxSelect and WNV IgG DxSelect, respectively, Focus Diagnostics Inc, Cypress, CA, United States). A real-time reverse-transcription (RT)-PCR [31] and an RT-nested PCR [32] were used.

After the diagnosis of the first human case, screening of donated blood for WNV RNA with targeted individual donation (ID) nucleic acid amplification testing (NAT) using the Procleix WNV Assay [33] or minipool

Figure 1

Laboratory-diagnosed (confirmed and probable) cases of West Nile neuroinvasive disease by week[a] of symptom onset, Greece, 2010 (n=197), 2011 (n=75), 2012 (n=109)[b]



WNND: West Nile neuroinvasive disease.

[a] Week number is according to the International Organization for Standardization (ISO) 8601 standard.

[b] Each box represents one laboratory-diagnosed case of WNND reported in 2012.

(MP) NAT of equal aliquots of six individual donations using the Gobas TaqScreen West Nile Virus Test [34] was implemented in the affected areas, from 11 July to 10 November. The screening was carried out in five Blood Centres (three in Athens, one in Thessaloniki and another in Alexandroupoli).

Data analysis

Descriptive analysis of the surveillance data was conducted, including the geographical and temporal distribution of WNND cases, age, sex, clinical manifestations, underlying diseases and clinical outcome.

We calculated risk ratios (RRs) to compare the incidence of WNND in different populations. Urban and rural areas were defined according to the Hellenic Statistical Authority data [35]: townships with more than 2,000 residents were classified as urban.

To identify predictive factors of developing WNND versus WNF, we calculated odds ratios (ORs), as WNF reported cases represented a small fraction of all WNF infections in the population [16]. An association was considered statistically significant when the p value was ≤0.05.

We constructed multiple logistic regression models to identify factors independently associated with disease severity. Initials models included all variables for which the p value was ‹0.1 or the OR was ›1.10 or ‹0.90. We removed variables one at a time, depending on the significance testing (p‹0.05) by the likelihood ratio test. We estimated adjusted RRs from binomial regression analysis including all variables that remained significant in the final logistic regression model. Imported cases and asymptomatic infections detected through haemovigilance were not included in the analysis.

The analysis was carried out using STATA version 12 software (Stata Corporation LP, Texas, United States).

Results

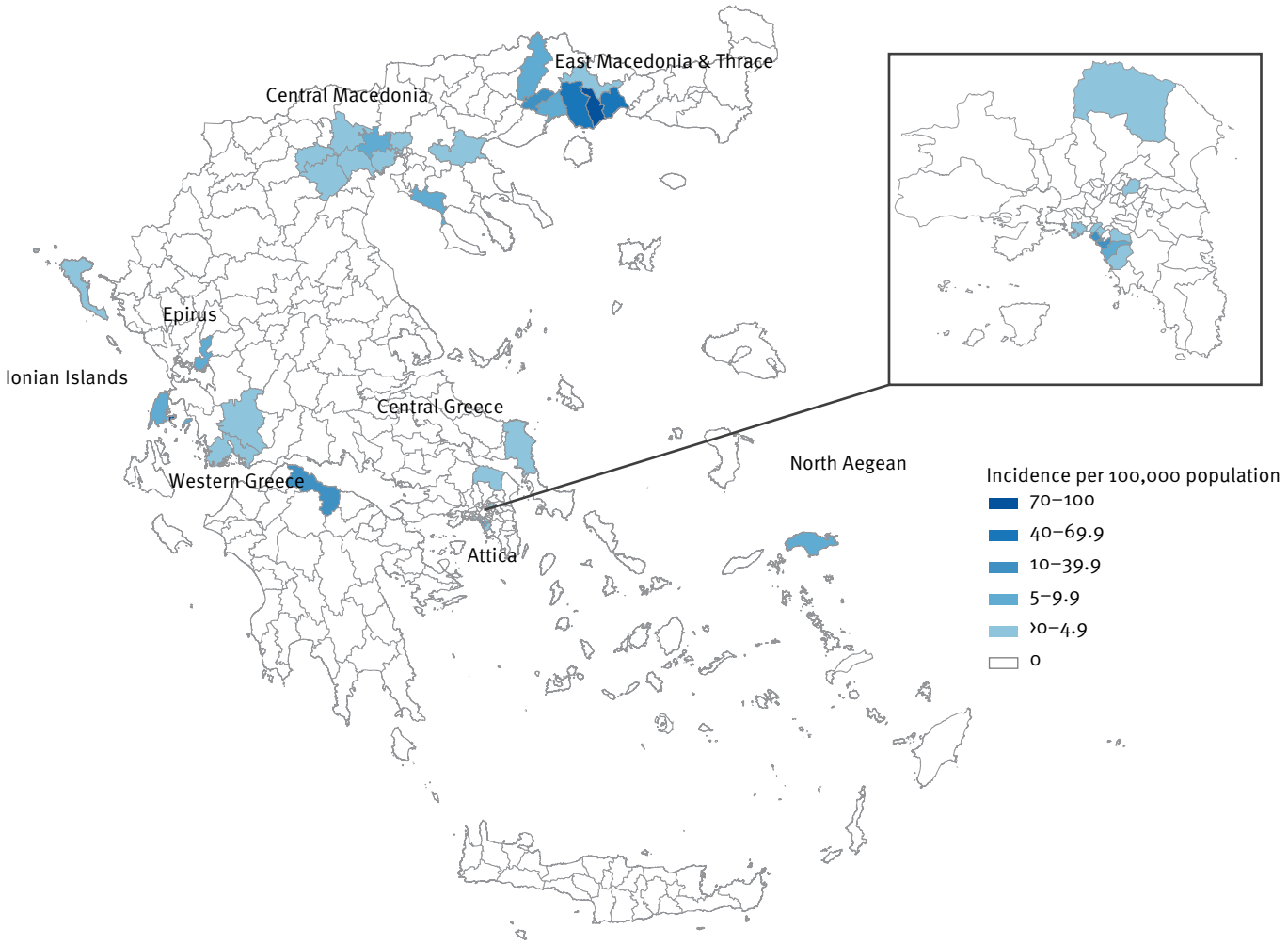
Descriptive analysis

In 2012, 163 cases of WNV infection were recorded. Two of the cases were classified as imported from the United States. Of the 161 locally acquired cases, 109 (47 confirmed and 62 probable) were classified as WNND and 52 (one confirmed and 51 probable) as WNF. The overall WNND incidence was one case per 100,000 population. One of the WNND reported cases was a Greek traveller whose infection was diagnosed in Germany [36]. Close relatives of 83% (n=91) of the 109 WNND cases, with severe disease and/or cognitive problems, were interviewed.

All cases occurred within a 16-week interval from 20 June (week 25) to 7 October 7 (week 41) 2012 and the outbreak peaked in the second week of August (Figure 1). The median period from symptom onset to diagnosis

Figure 2

Incidence (per 100,000 population) of West Nile neuroinvasive disease by suspected municipality of exposure, Greece, June–October 2012 (n=106)[a]



[a] The place of exposure could not be determined for two cases. One patient acquired the infection through blood transfusion and is also not included in the map.

for the WNND cases was 7 days (range: 2–53) and the mean was 9.29 (SD: 6.98) days.

Haemovigilance procedures demonstrated that one WNND case acquired the infection through blood transfusion: this patient was admitted to the intensive care unit and recovered. Another person transfused with a blood component derived from the same unit of blood from the implicated donor was also infected but did not develop symptoms. It is important to emphasise that blood collection from the implicated donor and both transfusions took place before diagnosis of the first case of WNV infection in Greece in 2012, which triggered the implementation of blood safety measures against WNV infection. Details of these haemovigilance findings and data on surveillance in the blood donor population will be presented elsewhere.

For two WNND cases, the probable place of exposure could not be determined, due to their complicated travel history during the incubation period. The remaining 106 WNND cases were infected in 19 of the 74 regional units of the country, in eight regions (Figure 2); 55% (58/106) of the cases occurred in eight regional units that had not been previously affected.

During the 2012 transmission period, two main outbreak epicentres were identified. From 20 June (week 25) to 16 August (week 33), 26% (n=28) of the 106 WNND cases occurred in the southern suburbs of Athens in Attica (incidence: 3.6 per 100,000 population): this had not been previously considered an established area for WNV. Six weeks after the beginning of the outbreak, and as the number of new cases was substantially decreasing in Attica, a second epicentre was detected in a newly affected rural wetland area in the

Table 1

Characteristics of cases with West Nile neuroinvasive disease, Greece, June–October 2012 (n=109)

Characteristic	Number of cases (%)[a]	Incidence (per 100,000 population)[b]	Risk ratio (95% CI)
Age group in years			
<20	2 (2)	0.09	Reference
20–29	3 (3)	0.23	2.5 (0.42–15)
30–39	3 (3)	0.17	1.9 (0.32–11)
40–49	4 (4)	0.23	2.6 (0.47–14)
50–59	12 (11)	0.80	8.7 (1.9–39)
60–69	29 (27)	2.34	26 (6.1–107)
70–79	31 (28)	2.94	32 (7.7–134)
≥80	25 (23)	4.22	46 (11–194)
Sex			
Female	38 (35)	0.67	Reference
Male	71 (65)	1.27	1.9 (1.3–2.8)
Place of exposure[c]			
Urban	55 (52)	0.67	Reference
Rural	51 (48)	1.87	2.8 (1.9–4.1)

[a] Percentages do not sum to 100% as a result of rounding.

[b] Population data from Hellenic Statistical Authority (EL. STAT.) [56].

[c] Three cases are not included (two cases with undetermined place of exposure and one case infected through blood transfusion).

Source: Hellenic Center for Disease Control & Prevention.

region of East Macedonia & Thrace, with 43% (46/106) of the WNND cases (incidence: 17.6 per 100,000 population), from 30 July (week 31) to 27 September (week 39) (Figure 1). Among these cases, 41 were recorded in the regional units of Xanthi and Kavala. The remaining cases mainly occurred in the regions of Central Macedonia (n=15) and Western Greece (n=7).

Among the WNND cases, 52% (55/106) were residents of urban areas, while the incidence of WNND in rural settings was almost three times higher than that in urban areas (Table 1). All 28 WNND cases in the southern suburbs of Athens and 11/46 of the cases in the region of East Macedonia & Thrace were from urban areas. In rural areas, the incidence of WNND was significantly higher among people who were male (RR: 2.3; 95% CI: 1.2–4.2, p=0.005), whereas in urban areas, the difference between the sexes was not statistically significant (RR: 1.6; 95% CI: 0.91–2.7, p=0.10).

The median age of WNND cases was 70 years (range: 11–95) and 65% (71/109) were male. The incidence of WNND cases increased from 0.17 per 100,000 in the 30–39 year-olds to 4.22 per 100,000 in those who were ≥80 years-old (Table 1). The median age and sex distribution of patients with WNND in the two epicentres did not significantly differ (p=0.93 and p=0.87, respectively).

Encephalitis/meningoencephalitis (83%; 90/109) was the most prominent clinical syndrome, followed by meningitis (16%; 17/109) and acute flaccid paralysis (5%; 5/109). In two patients, acute flaccid paralysis was the only symptom.

Fever was the most commonly reported symptom among WNND cases (99%; 106/107), followed by fatigue (84%; 83/99), confusion (79%; 82/104), weakness (74%; 73/98), headache (69%; 69/100), myalgia (62%; 56/91), arthralgia (56%; 48/85), chills (55%; 52/94), gastrointestinal symptoms (48%; 52/109), extrapyramidal signs/tremor (29%; 29/100), rash (16%; 16/101) and limb paresis (15%; 15/100).

Of the 109 WNND cases, 83 (76%) had at least one underlying chronic disease, with 54/109 (50%) cases having two or more coexisting conditions. The most commonly reported underlying conditions among the 109 WNND cases were hypertension (45%; n=49), diabetes mellitus (35%; n=38), heart disease (21%; n=23), cancer (11%; n=12), chronic neuropsychiatric disease (10%; n=11), stroke (9%; n=10), chronic renal failure (6%; n=7) and chronic respiratory disease (6%; n=6). Chronic neuropsychiatric disease included dementia, epilepsy, Parkinson disease and psychosis.

Of 80 WNND cases with the relevant information, 56 reported having agricultural/gardening activities and/or other outdoor activities in the countryside, while 21 of 94 cases reported having outdoor activities at night.

All 109 WNND identified cases were hospitalised and 18 (17%) were admitted to an intensive care unit (ICU).

Predictive factors of disease severity

Predictive factors of WNND versus WNF

The median age of WNND cases (70 years; range: 11–95) was significantly higher (p=0.001) than that of the diagnosed WNF cases (63 years; range: 14–92).

In the univariable analysis, the odds of WNND among all reported cases of WNV infection increased significantly (OR: 1.03; 95% CI: 1.01–1.05) with increasing age and was significantly higher among cases who were male (OR: 2.8; 95% CI: 1.3–5.8) (Table 2).

Patients with WNND were twice as likely to have at least one underlying condition than patients with WNF and almost three times more likely to have more than one underlying condition (Table 2).

Age (≥75 years) and male sex were the only factors associated with the presence of WNND in the final logistic regression model (Table 2).

Table 2

Demographic characteristics and underlying conditions of reported cases with West Nile neuroinvasive disease (n=109) and West Nile fever (n=52), Greece, June–October 2012

Characteristic	WNND Number (%)	WNF Number (%)	Crude odds ratio (95% CI)	Adjusted odds ratio[a] (95% CI)
Age group in years				
<75	67 (61)	43 (83)	Reference	Reference
≥75	42 (39)	9 (17)	3.0 (1.3-7.7)	3.5 (1.5-8.1)
Sex				
Female	38 (35)	31 (60)	Reference	Reference
Male	71 (65)	21 (40)	2.8 (1.3–5.8)	3.1 (1.5–6.4)
Co-morbidity				
No underlying conditions	26 (24)	21 (40)	Reference	NA
One underlying condition	29 (27)	16 (31)	1.5 (0.63–3.4)	
≥2 underlying conditions	54 (50)	15 (29)	2.9 (1.3–6.5)	
≥1 underlying conditions	83 (76)	31 (60)	2.2 (1.0–4.6)	
Underlying conditions				
Hypertension	49 (45)	19 (37)	1.4 (0.68–3.0)	NA
Heart disease	23 (21)	9 (17)	1.3 (0.51–3.4)	
Diabetes	38 (35)	13 (25)	1.6 (0.73–3.7)	
Cancer	12 (11)	3 (6)	2.0 (0.51–12)	
Chronic neuropsychiatric disease	11 (10)	3 (6)	1.8 (0.45–11)	
Stroke	10 (9)	1 (2)	5.2 (0.69–228)	
Chronic renal failure	7 (6)	1 (2)	3.5 (0.43–161)	
Chronic respiratory disease	6 (6)	1 (2)	3.0 (0.34–139)	

NA: not applicable; WNF: West Nile fever; WNND: West Nile neuroinvasive disease.

[a] Adjusted for age and sex.

Source: Hellenic Center for Disease Control & Prevention.

Predictive factors of encephalitis/meningoencephalitis versus meningitis

The median age (72 years; range: 19–95) of cases with encephalitis/meningoencephalitis was significantly higher (p<0.001) than that of cases with meningitis (57 years; range: 11–80). The risk of developing encephalitis increased by 4% for each yearly increase in age (OR for trend: 1.04; 95% CI: 1.0–1.1).

Patients with at least one underlying condition (OR adjusted for age: 4.2; 95% CI: 1.0–17) were more likely to develop encephalitis/meningoencephalitis than meningitis.

Predictive factors of fatal outcome

A total of 18 WNND cases died, indicating an overall case fatality rate of 17%. The case fatality rate in the two main epicentres (southern suburbs of Athens, East Macedonia & Thrace) was lower, 7% (2/28) and 9% (4/46), respectively, than the rate recorded outside the main epicentres (34%; 12/35).

The median age of the fatal cases was 79 years (range: 72–95), with the age-specific case fatality rate in patients aged ≥75 years reaching 36% (15/42) (Table 3).

The case fatality rate did not differ significantly between sexes (p=0.69). Eight of the 18 WNND fatalities were hospitalised in an ICU. The median interval from WNV symptom onset to death was 16 days (range: 4–108).

Of the 18 patients with a fatal outcome, 16 had encephalitis/meningoencephalitis (one of whom also had acute flaccid paralysis) and two had meningitis. The risk of fatal outcome among WNND cases did not significantly differ (p=0.54) between cases with encephalitis/meningoencephalitis and cases with meningitis.

All patients who died had at least one underlying condition. Co-morbidity was a significant (p=0.009) risk factor for death, with the case fatality rate being almost six times higher among patients with chronic renal failure. Age (≥75 years) and chronic renal failure remained independent predictive factors of death in the multivariable analysis (Table 3).

The case fatality rate, adjusted for age and chronic renal failure, did not differ significantly in the two epicentres (p=0.667); however, it was significantly higher in Western Greece (RR: 4.9; 95% CI: 1.4–17) and Central

Table 3

Factors predicting death for cases with West Nile neuroinvasive disease, Greece, June–October 2012 (n=109)

Characteristic	Number of deaths (n=18)	Case fatality rate (%)[a]	Crude risk ratio (95% CI)	Adjusted risk ratio[b] (95% CI)
Age group in years				
<75	3[c]	4.48	Reference	Reference
≥75	15	35.71	8.0 (2.5–26)	7.0 (2.2–22)
Sex				
Female	7	18.42	Reference	NA
Male	11	15.49	0.84 (0.36–2.0)	
Clinical manifestation				
Meningitis	2	11.76	Reference	NA
Encephalitis/meningoencephalitis	16	17.78	1.5 (0.38–6.0)	
Co-morbidity				
1 underlying condition	5	17.24	Reference	NA
2 underlying conditions	7	20.00	1.16 (0.41–3.3)	
≥3 underlying conditions	6	31.58	1.83 (0.65–5.2)	
Underlying conditions				
Hypertension				
No	9	15.00	Reference	NA
Yes	9	18.37	1.22 (0.53–2.9)	
Heart disease				
No	12	13.95	Reference	NA
Yes	6	26.09	1.9 (0.79–4.4)	
Diabetes				
No	9	12.68	Reference	NA
Yes	9	23.68	1.9 (0.81–4.3)	
Chronic neuropsychiatric disease				
No	14	14.29	Reference	NA
Yes	4	36.36	2.5 (1.0–6.4)	
Cancer				
No	15	15.46	Reference	NA
Yes	3	25.00	1.6 (0.55–4.8)	
Stroke				
No	15	15.15	Reference	NA
Yes	3	30.00	2.0 (0.69–5.7)	
Chronic renal failure				
No	13	12.75	Reference	Reference
Yes	5	71.43	5.6 (2.8–11)	4.5 (2.7–7.5)
Chronic respiratory disease				
No	16	15.53	Reference	NA
Yes	2	33.33	2.1 (0.63–7.3)	

NA: not applicable.

[a] Case fatality rate refers to the percentage of fatalities among cases within a specific characteristic category.
[b] Includes variables that remained significant in the final binomial regression model.
[c] Belonged to the 70–74 year age group.

Source: Hellenic Center for Disease Control & Prevention.

Macedonia (RR: 4.1; 95% CI: 1.2–15), compared with that of cases in the southern suburbs of Athens.

Laboratory results

Of the 161 locally acquired cases, 45 had a WNV-specific IgM antibody response in CSF (confirmed cases), 113 had WNV-specific IgM antibody response in serum (probable cases, CSF sample was not available) and in three (confirmed) cases (one from the south suburbs of Athens and two from East Macedonia), WNV nucleic acid was detected in blood or urine. In these three cases, sequencing revealed WNV lineage 2, with sequences with 100% genetic similarity (in the amplified fragment of the NS3 gene) with the Nea Santa-Greece-2010 strain (GenBank Accession number HQ537483) [23]). The whole genome sequence was obtained from a urine sample from a patient with WNND in the regional unit of Kavala, East Macedonia (strain Greece/2012/Kavala, GenBank accession number KF179639; the strain presented 99.7% sequence identity with the Nea Santa-Greece-2010 strain) [37].

As in previous years, there was no cross-reactivity with tick-borne encephalitis virus, while cross-reactivity was seen with Dengue virus (DENV); however, when a positive result was obtained for DENV, the titres were lower than those against WNV [38]. None of the patients had been previously vaccinated against yellow fever or Japanese encephalitis.

Of the 36,911 blood units tested by NAT (76.4% (n=28,205) ID-NAT and 23.6% (n=8,706) MP-NAT), four (1:9,228) were positive, including the one found through haemovigilance procedures.

Discussion

Human WNV infections were notified in Greece in 2012 for the third consecutive year in the context of an enhanced surveillance system in place since 2010. A new geographical pattern of WNV circulation was observed, with a more dispersed distribution of cases and two outbreak epicentres (one in a rural and one in an urban area) with different temporal patterns. The reason for this distinct temporal distribution is not clear. It might reflect different microclimatic conditions (such as temperature), vector distribution or bird migration patterns between the two areas. In addition, more than half of the reported cases occurred in areas that had not been affected in previous years, outlining the difficulty in predicting WNV circulation and defining areas at risk for following years. In 2013, cases continued to occur in previously affected areas, but also in one new regional unit [39].

This outbreak was the largest recorded in the European Union (EU) in 2012 and the second largest among the EU countries reporting to the European Centre for Disease Prevention and Control (ECDC) and information compiled by ECDC (the largest being in Russia, with 447 cases, and then followed by Israel with 83 cases, Serbia with 69, and Italy with 50) [40]. The 2012 incidence of WNND cases in Greece was 50% higher than in 2011, but remained lower than that in 2010. The first human cases occurred earlier than in previous years (26 and 16 days earlier than the first case of 2011 and 2010, respectively), probably due to the higher temperatures recorded in June 2012, especially in central and southern Greece. In the southern suburbs of Athens, the mean temperature in June 2012 was 28.6 °C, whereas in June 2011 and 2010 it was 25.4 °C and 26.1 °C, respectively [41]*. However, the effects of climatic conditions e.g. temperature and humidity, on local vector populations need further study.

All three obtained sequences from the PCR-positive samples belonged to WNV lineage 2, with 100% genetic similarity with the strain detected in 2010, suggesting that this strain has become established in Greece. In addition, WNV lineage 2 sequences were also obtained from Culex spp. mosquitoes collected in the regional unit of Xanthi, in the municipality with the highest incidence in Greece in 2012; as in the strain circulating in the two previous transmission periods, the strain contained the $H_{249}P$ substitution in the NS3 protein (GenBank accession number JX860675) [42], which might be associated with increased virulence of the strain [23].

One WNND case acquired the infection through blood transfusion before the implementation of blood safety measures, including screening of donor blood for WNV RNA by NAT in the affected areas in 2012. It should be noted that the blood donor involved gave blood eight days before the first confirmed human case was diagnosed and reported to the Hellenic Center for Disease Control & Prevention.

The higher incidence of WNND among male patients is consistent with findings from other countries [43], probably reflecting behavioural factors leading to increased exposure to mosquitoes, especially in rural areas. The majority of the WNND cases reported outdoor activities, which has also been identified as a risk factor for developing the disease [44]. Older age (≥75 years) was also found to be significantly associated with a higher risk of severe neurological disease. The association between age and severe disease has been well established [14,43, 45-52].

The overall case fatality rate (17%) among patients with WNND was higher than that in other countries [14,43, 45-48,53] and similar to that recorded in the 2010 outbreak in Greece (17%)* and the 2008–11 outbreak in Italy (16%) [54]. The high case fatality rate in regions with fewer reported cases might reflect a diagnostic bias: in areas where physicians were not sensitised to test all suspected cases for WNV infection, only the most severe cases were likely to be diagnosed, including those with a fatal outcome. More research is required to investigate the reasons for the regional difference in case fatality rate.

In our study, advanced age was found to independently predict WNND-related death, a finding compatible with many previous studies [14,43,45,46,48-50,52]. The literature is inconsistent regarding whether pre-existing medical conditions are predictive of WNND or death [49-52,55]. In our study, co-morbidity was not found to predict WNND; however, chronic renal failure was significantly associated with a fatal outcome. Specific underlying conditions (such as diabetes, immunosuppression, history of stroke) have been identified as risk factors for fatal outcome [48,50-52,55], while some other studies showed an association between chronic renal disease and severe WNV infection [55] or death [51], as in our study.

Limitations should be taken into account when interpreting our findings. The cases detected by the enhanced surveillance system represent the severe cases of WNV infection. Moreover, diagnosed WNF cases are considered to represent a small fraction of all WNF cases (probably the more serious in the spectrum of mild illness), as mild WNF cases are less likely to be diagnosed and reported. Moreover, central nervous system involvement was not always validated by laboratory or imaging results. Finally, the ORs and case fatality rates should be interpreted with caution, as small numbers are involved for some risk factors and outcome categories.

In conclusion, the occurrence of human cases of WNV infection in three consecutive years and the spread of the virus in newly affected areas suggest that WNV, and specifically WNV lineage 2, is established in Greece and transmission is expected to continue in the future. WNV circulation continued in 2013, with 86 diagnosed cases (51 of whom had WNND) [39].The established enhanced surveillance of WNV infection among humans and animals, comprehensive vector control and the implementation of the recommended blood safety and haemovigilance measures during the transmission period constitute the cornerstones of successful management of this seasonal public health threat.

Ongoing health education campaigns, including seminars and dissemination of information material, targeting specifically susceptible populations (i.e. the elderly and those with co-morbidities, including chronic renal failure) and physicians throughout the country may lead to more effective disease prevention and decreased numbers of fatalities.

* Authors' correction

The following corrections were made at the request of the authors on 4 April 2014: the 2011 and 2010 mean temperatures in the southern suburbs of Athens were corrected. In addition, some details of references 20, 21, 35 and 56 were amended. On 5 April 2014, the case fatality rate in the 2010 outbreak in Greece was amended.

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Conflict of interest

None declared.

Authors' contributions

Danai Pervanidou, Kostas Danis, Marios Detsis, Theano Georgakopoulou, Sotirios Tsiodras, Agoritsa Baka, Jenny Kremastinou and Christos Hadjichristodoulou contributed to the surveillance and outbreak response.

Danai Pervanidou conducted the analysis and wrote the first draft of this manuscript. Kostas Danis and Kassiani Mellou contributed to the data analysis and the writing of the first draft. Christos Hadjichristodoulou supervised the surveillance activities.

Evaggelia Papanikolaou, Irene Terzaki, Lambrini Veneti, Anna Vakali and Georgios Dougas provided the surveillance data from the case investigations.

Anna Papa and Athanasios Tsakris provided the laboratory data of the human cases.

Constantina Politis and Kostas Stamoulis provided the haemovigilance findings and data on surveillance in the blood donor population.

All authors were members of the WNV outbreak response team. All authors read and critically revised the first as well as the subsequent drafts of this manuscript and approved the final version.

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