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Special edition: West Nile virus, malaria, dengue fever

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This special edition of *Eurosurveillance* presents a presents articles related to the emergence and re-emergence of West Nile virus infections, malaria and dengue fever in some Mediterranean and other southern European countries. A mix of surveillance reports, case reports and results from laboratory studies provide insight into recent outbreaks in 2011 and overviews on the epidemiological situation since 2005.

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Editorial board

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This is a photograph of the Asian tiger mosquito, *Aedes albopictus*.

An epidemiologically important vector, *Aedes albopictus*, from the Culicidae family, may transmit several viral pathogens, including West Nile virus and Dengue virus.

Prevalence of IgM and IgG antibodies to West Nile virus among blood donors in an affected area of north-eastern Italy, summer 2009

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Following reports of West Nile neuroinvasive disease in the north-eastern area of Italy in 2009, all blood donations dating from the period between 1 August and 31 October 2009 in the Rovigo province of the Veneto region were routinely checked to exclude those with a positive nucleic acid test for West Nile virus (WNV). Only one of 5,726 blood donations was positive (17.5 per 100,000 donations; 95% confidence interval (CI): 0.4–97.3). In addition, a selection of 2,507 blood donations collected during the period from 20 July to 15 November 2009 were screened by ELISA for IgG and IgM antibodies against WNV. A positive result was received for 94 of them. The positive sera were further evaluated using immunofluorescence and plaque reduction neutralisation test (PRNT), in which only 17 sera were confirmed positive. This corresponds to a prevalence of 6.8 per 1,000 sera (95% CI: 4.0–10.9). In a case-control study that matched each of the 17 PRNT-positive sera with four negative sera with the same date of donation and same donation centre, we did not find a significant association with age and sex of the donor; donors who worked mainly outdoors were significantly more at risk to have a positive PRNT for WNV.

Introduction

West Nile virus (WNV) was originally identified in 1937 in northern Uganda [1]. Birds, especially those in the *Corvidae* family are the natural reservoir of the virus, which is mainly transmitted to humans through the bite of infected *Culex* mosquitoes [2,3]. Transmission through receipt of blood products and transplantation has also been documented [4,5]. Rare cases of vertical transmission (i.e. transplacentally or through breast milk) and laboratory-acquired infection have also been reported [6].

During the last 20 years, several WNV outbreaks have occurred throughout the world. In addition to the United States [7], where the virus was first identified in 1999 [2,3,6], large WNV outbreaks have also been reported in Europe and in the Mediterranean basin [8,9]. The largest European outbreak occurred in 1996 in Bucharest, Romania [10]. In the Mediterranean area, the first outbreak of human encephalitis was identified in the Camargue, France, in 1962 [11], followed by other epidemics in the 1990s involving a relatively high number of human cases in Algeria [8], Tunisia [12] and Israel [13,14]. In the past few years, cases of WNV encephalitis have also been reported in the Volgograd region in Russia [15], in Hungary [16] and Romania [17], and very recently in Greece [18].

In Italy, the first outbreak of WNV infection was reported in 1998 in horses in Tuscany [19]. The virus re-emerged in north-eastern Italy in summer 2008, when equine cases of WNV neuroinvasive infection were notified in the regions Veneto and Emilia Romagna [20]. An extraordinary WNV surveillance programme was subsequently activated, which led to the notification of nine human cases of West Nile neuroinvasive disease (WNND) in the summer of 2008 [21,22] and a further 16 cases in late summer 2009; all cases occurred in the regions Emilia Romagna, Veneto and Lombardia, in wet areas surrounding the Po river [23-25].

Since WNV infection is generally asymptomatic with encephalitis occurring in less than 1% of cases, we conducted a seroepidemiology investigation in the Rovigo province in northern Italy, where a high incidence of WNND was observed, in order to estimate the extent of the epidemic and to better plan intervention strategies.

Methods

Setting

The study area was the province of Rovigo, Veneto region, north-east Italy. This province, which has around 250,000 inhabitants, borders with the Po river and the Emilia-Romagna region. All territory is a level land characterised by extraordinary biodiversity, mainly because of the presence of freshwater and brackish water wetlands, including flooded deciduous woodlands, open lagoons of shallow water and river mouths. The high humidity level makes this area particularly attractive for mosquitoes, in particular during summer. It is located at the crossroads of bird migration routes connecting Europe, the Mediterranean basin and Africa, hosting a high number and wide range of migrating birds throughout the year. Thus, the high concentration of mosquitoes and migrating birds creates opportunities for vector-borne viruses such as WNV [26].

Study design

The study involved the three blood donation centres of Rovigo province which collected about 18,500 blood donations in 2009. During the period from 1 August to 31 October 2009, all blood donations were routinely evaluated by nucleic acid amplification test (NAAT) for WNV to identify potentially viraemic donations. In order to study the prevalence of WNV in the area, we tested, during the period from 20 July to 15 November 2009, serum samples from 25 blood donations per day for IgG and IgM antibodies to WNV. Any IgM- or IgG-positive sample was further evaluated by immunofluorescence and by plaque-reduction neutralisation test (PRNT) for confirmation. The number of serum samples collected at each centre was proportional to the volume of donations performed in the year 2008. Thus, each day, five donations were sampled from Adria (Centre 1), five from Trecenta (Centre 2), and 15 from Rovigo (Centre 3), choosing serum samples from the first consecutive daily donors who gave their consent to the study. All samples were handled anonymously by technicians and researchers involved in this study.

Table 1

Serological results of serum samples positive in the West Nile virus ELISA screening, Rovigo province, Italy, 20 July–15 November 2009 (n=94)

IgG ELISA	IgM ELISA	IgG IFA	IgM IFA	Number of samples
Confirmed positive by PRNT				17
+	+	+	+	7
+	-	+	-	9
+	+	+	-	1
Not confirmed positive by PRNT				77
+	-	+	-	46
+	-	+	+	3
+	-	-	-	19
+	+	+	-	1
-	+	+	+	2
-	+	-	+	1
-	+	-	-	5

ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescence assay; PRNT: plaque reduction neutralisation test.

To evaluate potential risk factors associated with WNV infection, a case–control study matched by day and donation centre was performed. More specifically, for all donors that had IgG and/or IgM to WNV confirmed by PRNT, specific information about age, sex, address and type of job was retrospectively collected. For each positive donor, we collected the same information from four negative cases who were randomly chosen among the donors seen on the same day in the same centre. Although details on the type of job were collected, we decided after preliminary analysis to create a dummy variable classifying the job as done predominantly in the open air or not, e.g. a builder was classified as an open-air worker while a bank clerk's work was classified as indoors.

Laboratory testing

WNV NAAT screening was performed using Cobas Taq Screen West Nile Virus test on a Cobas s201 system (Roche Molecular Systems) on pools of aliquots from six individual plasma specimens. Specimens included in WNV RNA-positive pools were re-tested individually with the same WNV NAAT kit.

WNV IgM and IgG testing was done using the WNV IgM capture DxSelect ELISA and IgG DxSelect ELISA kits (Focus Diagnostics), respectively, as reported [18]. All serum samples which tested positive in the ELISA were further analysed by anti-West Nile virus IIFT IgG and IgM immunofluorescence assays (IFA) (Euroimmun AG), and, to rule out cross-reactivity with other flaviviruses and confirm the result, also with the PRNT, according to the previously described protocol [18].

Statistical analysis

Prevalence of antibodies to WNV was calculated as the ratio between sera confirmed positive by PRNT and all tested sera. The 95% confidence intervals (CI) of the prevalence were calculated using the binomial distribution. Prevalence estimates were also stratified by blood donation centre and by month of donation. Chi-square test was used to evaluate if the prevalence by blood donation centre and by month of donation was statistically significant.

Sensitivity, specificity, positive predictive (PPV) and negative predictive value (NPV) of IgG and IgM with respect to PRNT were also calculated, assuming that all sera not evaluated by PRNT were negative. This assumption favours highest estimates for specificity and NPV.

Odds ratios (OR) were calculated to evaluate the association between age, sex and open-air/indoors job of the donor with confirmed positivity by PRNT compared

Table 2

Sensitivity, specificity, negative and positive predictive value of serological tests for West Nile virus, compared with PRNT, Rovigo province, Italy, 20 July to 15 November 2009 (n=2,507)

	PRNT-positive	PRNT-negative	PPV	NPV	Sensitivity	Specificity
WNV ELISA						
IgM-positive/IgG-positive	8	1	88.9	99.6	47.1	100.0
other	9	2,489				
IgM-positive/IgG-negative	0	8	0.0	99.3	0.0	99.7
other	17	2,482				
IgM-negative/IgG-positive	9	68	11.7	99.7	52.9	97.3
other	8	2,422				
ELISA-positive	17	77	18.1	100.0	100.0	96.9
ELISA-negative	0	2,413				
WNV IFA [a]						
IgM-positive/IgG-positive	7	5	58.3	99.6	41.2	99.8
Other	10	2,485				
IgM-positive/IgG-negative	0	1	0.0	99.3	0.0	100.0
other	17	2,489				
IgM-negative/IgG-positive	10	47	17.5	99.7	58.8	98.1
other	7	2,443				
IFA-positive	17	53	24.3	100.0	100.0	97.9
IFA-negative	0	2,437				

ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescence assay; NPV: negative predictive value; PPV: positive predictive value; PRNT: plaque reduction neutralisation test.

[a] WNV IFA is evaluated as a second line test for WNV ELISA-positive samples.

Note: It was assumed that all sera tested negative by ELISA would also have been negative in IFA and PRNT even when these tests were not performed.

Table 3

Adjusted odds ratios of being PRNT-positive for West Nile virus, associated with blood donor characteristics, conditional logistic model, Rovigo province, Italy, 20 July–15 November 2009 (n=17)

		PRNT-positive		PRNT-negative[a]		AOR	95% CI	p
		N	%	N	%			
Age (years)	‹40	5	23,8	16	76,2	1.00		
	40-46	4	18,2	18	81,8	0.42	0.08-2.14	0.30
	47-53	4	19,0	17	81,0	0.48	0.09-2.45	0.37
	›53	4	19,0	17		0.69	0.15-3.23	0.64
Sex	Male	16	20,5	62	79,5	1.00		
	Female	1	14,3	6	85,7	0.88	0.09-8.75	0.92
Indoor/outdoor	Indoors	5	16,2	57	83,8	1.00		
working activity	Outdoors	11	38,5	8	61,5	5.07	1.01-25.37	0.05
	Unknown	1	25.0	3	75.0	2.74	0.20-37.38	0.45

AOR: adjusted odds ratios; CI: confidence interval; PRNT: plaque reduction neutralisation test.

[a] PRNT-negative blood donors were matched to positive ones by day and centre of donation.

to matched sera not tested/not confirmed by PRNT. The OR was calculated taking into account matching by day and blood donation centre. A conditional logistic model was used to simultaneously adjust the OR for the effect of each evaluated factor with respect to the others.

Results

During the period from 1 August to 31 October 2009, 5,726 blood donations were collected in Rovigo province and tested by WNV NAAT. One blood donation, not included in the seroprevalence study, was WNV RNA-positive (17.5 per 100,000 donations; 95% CI: 0.4–97.3). Details on this positive case have been reported previously [25].

Among the 2,507 serum samples evaluated for IgG and IgM, 94 (3.7%) were positive in the WNV ELISA IgG and/or IgM screening and further tested by IFA and PRNT (Table 1). Of the 94 ELISA-positive samples, 70 (75%) were also WNV IFA-positive and 17 (18%) were confirmed by PRNT. The PRNT-confirmed cases included eight (47%) cases with both WNV IgM and IgG and nine (53%) with only WNV IgG detected by ELISA and/or IFA.

The estimated overall prevalence of WNV antibodies confirmed by PRNT was 6.8 per 1,000 tested sera (95% CI: 4.0–10.9). Stratifying by month of donation no particular fluctuations were observed, with prevalences that varied from 6.2 per 1,000 in September to 7.6 in October (p=0.99, Chi-square test, data not shown). The prevalence differed significantly by blood donation centre, with the highest prevalence in Trecenta (17.9 per 1,000 donations) and the lowest in Adria and Rovigo (4.0 per 1,000 donations) ($p<0.01$).

Table 2 shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of WNV ELISA alone and WNV ELISA followed by WNV IFA as a second-line test compared with PRNT results as the gold standard, and assuming that all ELISA-negative samples that were not also evaluated by PRNT, would have been negative in the PRNT. The sensitivity of WNV IgM detection for predicting WNV infection was low, whether by ELISA alone or by a combination of ELISA and IFA; however, the possibility of false negative results of PRNT at early phases of infection was not excluded in this study. The sensitivity of WNV IgG detection, either alone or in combination with WNV IgM detection by ELISA was about 50%. The addition of WNV IFA as a second-line test for ELISA-positive samples did not improve test sensitivity and PPV.

Table 3 shows the association of the blood donors' characteristics with being WNV positive in PRNT in a matched case-control analysis. While age and sex were not found to be significantly associated with being WNV-positive in PRNT, working outdoors was associated with a statistically significant higher risk (>5 times) of WNV infection than working indoors (p=0.05).

Finally, a map of the province was produced, plotting the geographical coordinates of the PRNT-positive donors and the cases of WNND notified in 2009 and resident in the Rovigo province. Most subjects with a PRNT-confirmed WNV infection lived close to rivers in western areas of the Rovigo province, without any apparent clustering or association with the place of residence of subjects diagnosed with WNND (data not shown).

Discussion

In accordance with national guidelines [27] that recommend screening of blood donations in affected areas where at least one human case with WNND has been detected, all blood donations performed in Rovigo province during summer 2009 were individually tested by NAAT. Only one of 5,726 blood donations resulted positive by NAAT, which corresponds to an estimated risk of WNV transmission of about 17.5 per 100,000 blood donations. With the limitation of the small samples size in our study, this risk appears to be lower than that reported in the United States during the peak of the WNV epidemic in 2002 [28] and in Canada in the period from 2006 to 2007 [29].

In order to estimate the extent of WNV infection among humans, we also conducted a serosurvey in a selection of blood donations in the study area; the estimated overall prevalence of anti-WNV antibodies was 6.8 per 1,000 sera (95% CI: 4.0–10.9). This prevalence estimate was lower than that found in a previous survey (15.6 per 1,000), that was conducted in the same province but was restricted to a population considered at risk of environmental exposure represented by farm employees who worked in areas where WNV-seropositive horses had been identified [22].

A retrospective screening of solid organ donors in several Italian regions found a higher prevalence of anti-WNV antibodies than the present study on blood donors [30]. This discrepancy could be accounted for by differences in target populations and sampling strategies as well as laboratory methods used for testing and for confirmation of positive results. We considered as positive only those samples which were confirmed by neutralisation assays. In fact, less than 20% of WNV ELISA-positive samples were confirmed by PRNT. After combining ELISA and IFA, the specificity did not increase significantly for those samples which were positive in both tests. Since the PPV of a test tends to decrease in low prevalence areas, our results suggest that neutralisation assays should be used to confirm positive results, especially in areas with a low risk of infection.

The prevalence in our study varied widely among geographical areas, ranging from about 18 per 1,000 blood donations in Trecenta to 4.0 per 1,000 in Adria and Rovigo. Moreover, the geographical distribution of infected individuals suggested that most cases were resident in areas near rivers and other water sources.

This is consistent with other studies on WNV and other mosquito-borne infections, such as La Crosse encephalitis [3,31]. However, we could not evaluate the association between WNV seroprevalence and proximity to water sources in our samples because of the lack of available geo-reference data on WNV-negative blood donations. For the same reason, any factor explaining the geographical variation could not be evaluated due to limited environmental data.

With regard to risk factors for infection, no statistically significant difference was found for age or sex, while a statistically significant association was found with working outdoors compared with other jobs. These findings, which suggest a higher risk of exposure to mosquito bites, contradict studies conducted in Romania, where having spent more than six hours outdoors during the day was not found associated with West Nile virus infection [32].

In conclusion, this study estimated the seroprevalence of WNV among blood donors of an affected area of Italy as 6.8 per 1,000 sera and indicates that WNV seroprevalence can vary widely even between different geographical areas within the same province. Although WNV NAT-positive samples were rare in our population, blood screening is needed in order to reduce the risk of WNV transmission to vulnerable recipients.

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Surveillance of West Nile virus Disease, Tel Aviv District, Israel, 2005 to 2010

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We present the findings of a six-year surveillance period (2005–2010) of human West Nile virus (WNV) infection in Tel Aviv district, Israel. Initial notifications of positively identified patients received from the Central Virology Laboratory were followed by epidemiological investigations of the local district health office. During 2005–2010, 104 patients, 79 with WNV neuroinvasive and 25 with WNV non-neuroinvasive disease were reported. The median age of the patients with a neuroinvasive disease was 74 years (range: 15 to 95 years) and 53 of such patients had encephalitis, 14 had acute flaccid paralysis, and 12 had meningitis. The case-fatality rate in these patients was 8%. The average annual incidence of neuroinvasive disease during 2005–2010 was 1.08 per 100,000 population. The incidence declined by 86% steadily between 2005 and 2009 (p for trend=0.005), but increased by more than six-fold in 2010. Elderly (≥65 years) men, comprising 25 patients of whom 24 were chronically-ill, had the highest incidence of WNV encephalitis (p<0.001). These findings are concordant with previous data, at the national level, published in Israel and the United States. Notably, the percentage of previously healthy patients, who developed a neuroinvasive disease was the highest (37%, p=0.001) in the surveillance period in 2010.

Introduction

West Nile virus (WNV) is a mosquito-borne arbovirus of the family *Flaviviridae*. Numerous avian species serve as the amplifying hosts. These include, migratory birds, such as the white storks which travel across Israel each autumn, as well as urban species, such as the common house sparrows. Transmission to humans mainly occurs by mosquito vectors, principally by mosquitoes of the genus *Culex*, during their active period, usually between mid-summer and early autumn. WNV has rapidly expanded in both the eastern and western hemispheres in the past two decades [1,2].

Human West Nile fever, caused by laboratory-confirmed WNV infection, was reported in Israel for the first time in the early 1950s, with several outbreaks in that decade, and an additional outbreak in 1980 [3]. The largest outbreak (439 serologically-confirmed cases with 29 deaths) of human WNV infection in Israel occurred in 2000, with a 73% rate of neuroinvasive disease in hospitalised patients [3,4]. Following this outbreak, a national surveillance system was established for humans and mosquitoes [5] and since 2001 WNV infection is notifiable in Israel. The surveillance system detects new cases of human WNV infections and characterises viral genotypes. The system is based on initial notifications of probable or confirmed cases of WNV infection by the Central Virology Laboratory (CVL) and on the subsequent epidemiological investigations of these patients by the local district health offices.

This report summarises a six-year period (2005–2010) of human WNV surveillance in the Tel Aviv district. This most densely populated urban district of Israel included 7,425 residents per square kilometre at the end of 2009, according to the Israel Central Bureau of Statistics [6].

Methods

Epidemiological investigation

The Tel Aviv District Health Office routinely receives notifications from the CVL about probable or confirmed cases of WNV infection in patients who are residents of the district. The local health office initiates an epidemiological investigation by collecting available information on the patients and by using a standard national-based questionnaire. For hospitalised patients, information is gathered from the patients' hospital records, and by conducting an interview with the patient or with a close family member, in order to obtain information regarding risk factors for WNV infection such as occupation, residence or travel near water bodies, recent exposure to mosquitoes, migratory birds and domestic fowl. For non-hospitalised patients, another source of information, in addition to the patient's interview, is usually the family physician. The gathered demographic, clinical, laboratory and

epidemiological data are reported to the Division of Epidemiology, Ministry of Health, Jerusalem.

Laboratory analysis

During the study period, the CVL used a combination of serology assays comprising in-house synchronised IgM-capture (until August 2007), indirect IgG, and indirect IgG avidity ELISAs [4], and from September 2007, the IgM InBios commercial kit (West Nile Detect IgM Capture ELISA, Seattle, Washington, USA) [7]. Results were interpreted according to the manufacturer's instructions. IgM and IgG tests were conducted simultaneously. The avidity IgG test was performed on single samples with IgM and IgG positive results. Unresolved cases were further tested by virus neutralisation assay [8].

Case definition

The following official national laboratory criteria of WNV infection are used by the CVL to define probable and confirmed cases.

• A confirmed case is defined as having at least one of the following criteria: (i) Serological conversion or at least two-fold increase in ELISA test results of IgM antibodies and/or IgG antibodies levels [4,7] in blood or in cerebrospinal fluid (CSF), in paired samples taken at least seven days apart; (ii) IgM antibodies level higher than IgG antibodies level and also IgG avidity lower than 30% in one blood sample; (iii) At least ten-fold higher optical density (OD) of IgM antibodies in CSF in one sample, compared to a reference cut-off level; (iv) Positive result of West Nile viral RNA in CSF, body fluids or body tissues, by real-time RT-PCR assays detecting WNV lineages 1 and 2 [8-10].

• A probable case is defined as detection of WNV IgM antibodies, without detection of IgG antibodies, in one blood or CSF sample or the lack of increase in IgM antibodies, without the presence of IgG antibodies, between paired samples of blood collected one to seven days apart.

The data in this report were summarised and analysed from the district health office's files of individual patients who had an onset of laboratory-confirmed or probable WNV infection, between 1 January 2005 and 31 December 2010. No clinical criteria were used, in addition to the laboratory criteria, for the case definition.

Clinical criteria

The term 'neuroinvasive disease' as used in our analysis refers to WNV-associated diagnoses of encephalitis, meningoencephalitis, meningitis and acute flaccid paralysis (AFP). The clinical definitions of encephalitis, meningitis and AFP, were adopted from previously described definitions [11]. Patients who have had a combined clinical picture of encephalitis and meningitis, hence, meningoencephalitis, were classified as having encephalitis, as in previous reports [12,13]. Also, any presentation of AFP combined with other illness (i.e. encephalitis) was classified as AFP only [12].

We assumed that in non-neuroinvasive patients, there would be a high level of cohort incompleteness due to lack of serological testing and therefore, lack of WNV diagnosis. These patients largely present with a mild nonspecific illness, which is often not followed by seeking medical care [14]. Thus, this report mainly analyses the cohort of WNV neuroinvasive disease patients who usually require diagnosis and treatment within a hospital setting, and therefore, this cohort is more likely to be complete.

Patients were also classified as to whether they had a pre-existing chronic medical condition, which was further classified as to whether it was an immunocompromised state. Any chronic medical condition, excluding psychiatric conditions, was included.

We abstracted these data, post hoc, at the information summary stage of the study from written medical diagnoses in patients' hospital records, where available.

Statistical analysis

For the purpose of p value calculations, we used the exact two-tailed Mann-Whitney U test in case of mean age differences, and not the Student's t-test, because of the small sample size. For the same reason, we used two-tailed Fisher's exact test in case of categorical data and Spearman's rank correlation coefficient for the trend of incidence. Also, the chi-square test with Yates' correction was used for testing differences in the average annual incidence between certain subgroups. A two-tailed p value inferior to 0.05 was considered significant. Statistical analyses were performed using SPSS version 15.0 software (Chicago, Illinois, USA).

The annual average population estimates for the years 2005–2009, used as denominators for incidence calculations, were taken from the Israel Central Bureau of Statistics. The annual average population estimate for 2010 was based on an estimated annual population growth rate of 1.725%, which was derived from the average population growth rate during 2005–2009.

Results

Between 1 January 2005 and 31 December 2010, 104 confirmed or probable cases of WNV infection were reported in the Tel Aviv district. Of these 104 cases, 94 (90%) had an onset of illness in the period from July to the end of October (Figure 1). Eighteen of the 23 (78%) neuroinvasive and non-neuroinvasive cases in 2010, and 18 of the 25 (72%) cases in 2005, occurred in July and August, compared to the occurrence of only 16 of the 56 (29%) cases in the years 2006–2009 combined during the same period.

Seventy-nine patients have had a neuroinvasive disease (Table), all of whom were hospitalised: 53 (67%) had encephalitis, 12 (15%) had meningitis and 14 (18%)

had AFP, of whom two patients had Guillain–Barré syndrome. The median age of all patients with a neuroinvasive disease was 74 years (range: 15 to 95 years) and 51 (65%) of these patients were aged 65 years or older. Fourty (51%) of these patients were males.

Of 79 patients with neuroinvasive disease, 25 (32%) were elderly men (≥65 years), of whom 24 were chronically-ill. Similarly, 26 (33%) elderly women including 25 chronically-ill were part of the 79 patients presenting with neuroinvasive disease.

Patients with encephalitis had a significantly higher mean age than patients with meningitis and AFP (74 years; 95% confidence interval (CI): 70–78 years vs 59 years; 95% CI: 51–67 years, p=0.02). The mean age of patients with meningitis was significantly lower than the mean age of patients with encephalitis and AFP (47 years; 95% CI: 35–60 years vs 73 years; 95% CI: 69–76 years, p<0.001).

Of all 79 patients with a neuroinvasive disease, 69 (87%) had a pre-existing chronic medical condition.

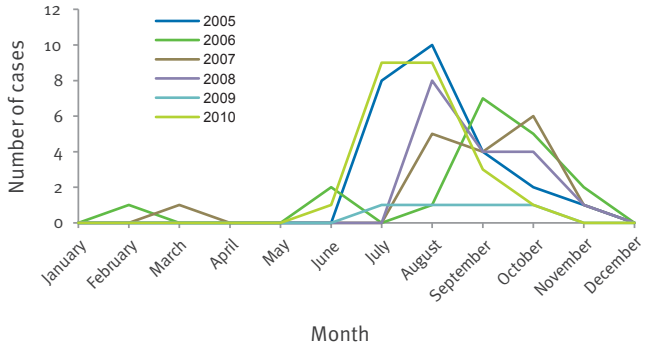
The group of patients with meningitis had a significantly lower percentage of chronic illness when compared to the group of patients with encephalitis (8/12, 67%; 95% CI: 35%-90% vs 49/53, 92%; 95% CI: 82%-98%, respectively, p=0.03). The percentage of patients with a pre-existing chronic medical condition was significantly lower in 2010, when compared to the period between 2005 and 2009 (12/19, 63%; 95% CI: 38%–84% vs 57/60, 95%; 95% CI: 86%–99%, p=0.001).

The case-fatality rate (CFR) of patients with a neuroinvasive disease was six of 79 (8%). The patients who died were older than 74 years of age, five of six were females, five of six had a pre-existing chronic medical condition, and four of six had a diagnosis of WNV encephalitis.

During the surveillance period, the annual percentage of patients with encephalitis, meningitis or AFP ranged between 55% and 90%, 0% and 25%, and, 10% and 33%, respectively (Figure 2).

Figure 1

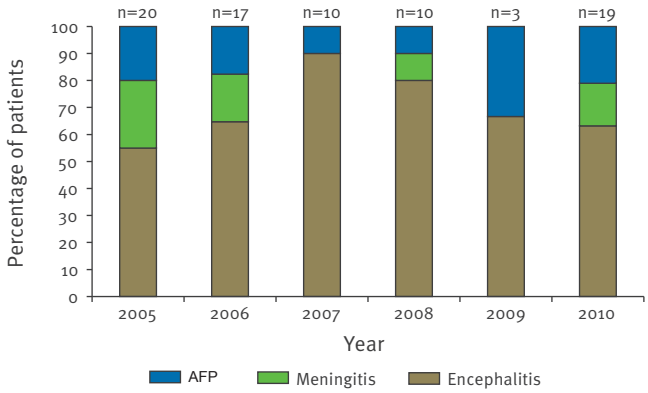
Human West Nile virus infection confirmed and probable cases, by month of illness onset, 2005–2010, Tel Aviv district, Israel (n=104)



Human West Nile virus infection confirmed and probable cases include 79 cases of neuroinvasive disease and 25 cases of non-neuroinvasive disease.

Figure 2

Percentage of patients with West Nile virus neuroinvasive disease, by year and clinical diagnosis, Tel Aviv district, Israel, 2005–2010 (n=79)



AFP: acute flaccid paralysis.

Table

Characteristics of cases of human West Nile virus neuroinvasive disease by clinical diagnosis, Tel Aviv district, Israel, 2005–2010 (n=79)

Characteristic	Encephalitis	Meningitis	AFP	**Total**
	(n=53)	(n=12)	(n=14)	**(n=79)**
Median age, years (range)	75 (38-95)	45 (15-79)	77 (41-95)	**74 (15-95)**
Male sex (%)	26 (49)	6 (50)	8 (57)	**40 (51)**
Deaths (%)	4 (8)	1 (8)	1 (7)	**6 (8)**
Immunocompromised (%)	5 (9)	0 (0)	1 (7)	**6 (8)**
Any chronic medical condition[a] (%)	49 (92)	8 (67)	12 (86)	**69 (87)**

AFP: acute flaccid paralysis.

[a] Most commonly included was a history of one or more illnesses such as essential hypertension, hyperlipidemia, and/or diabetes mellitus.

The average annual incidence of neuroinvasive disease between 2005 and 2010 was 1.08 per 100,000 population (Figure 3). The incidence of neuroinvasive disease declined significantly, between 2005 and 2009, from 1.69 to 0.24 per 100,000 population (an 86% decrease, rs=-0.97, p for trend=0.005), but increased more than six-fold in 2010, to 1.47 per 100,000 population, compared to the previous year. The change in trend, during 2005–2010, of the WNV-associated central nervous system diagnoses (encephalitis and meningitis) was similar to the trend of the WNV-associated peripheral nervous system illness (AFP).

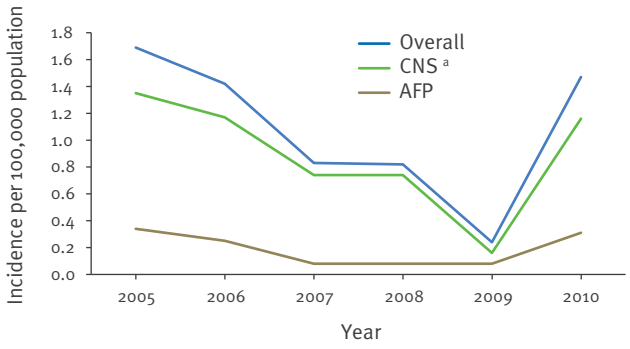
The average annual incidence of neuroinvasive disease, by age, between 2005 and 2010 (Figure 4) demonstrated a peak in incidence among patients aged 75 years or older (7.03 per 100,000 population), while no patients aged less than 15 years were reported. The peak of the average annual incidence of encephalitis and of AFP was in patients aged 75 years or older (5.18 and 1.48 per 100,000 population, respectively) (Figure 5).

The average annual incidence of neuroinvasive disease had a similar pattern among men and women (Figure 6) with the exception that the incidence in male patients aged 65–74 years, was almost three-fold higher than in female patients of the same age group (4.11 per 100,000 population vs 1.41 per 100,000 population, respectively). The latter difference could not be confirmed with statistical significance, but this could be due to the small number of cases in each of the male and female subgroups.

During the surveillance period, elderly men (≥65 years) had the highest average annual incidence of WNV encephalitis in the cohort, and it was significantly higher than the rest of the patients with encephalitis, aged between 35 and 64 years (4.54 per 100,000 population vs 1.19 per 100,000 population, p<0.001).

Figure 3

Annual incidence of human West Nile virus neuroinvasive disease by year and clinical diagnosis, Tel Aviv district, Israel, 2005–2010

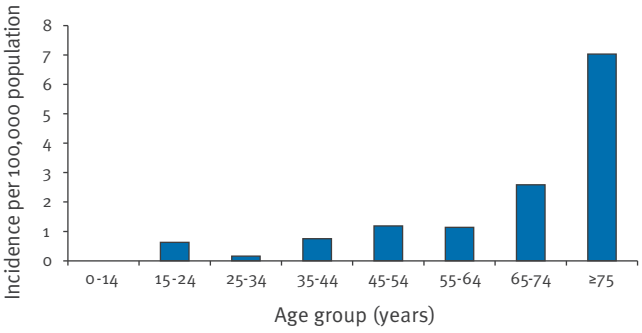


AFP: acute flaccid paralysis; CNS: central nervous system group of diagnoses.

[a] The CNS group of diagnoses includes encephalitis and meningitis.

Figure 4

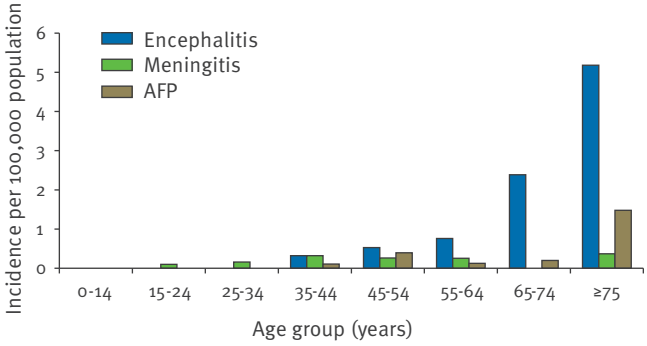
Average annual incidence of human West Nile virus neuroinvasive disease by age group, Tel Aviv district, Israel, 2005–2010



Average annual incidence is calculated using Israel Central Bureau of Statistics average population estimates of Tel Aviv district for 2008.

Figure 5

Average annual incidence of human West Nile virus neuroinvasive disease by age group and clinical diagnosis, Tel Aviv district, Israel, 2005–2010

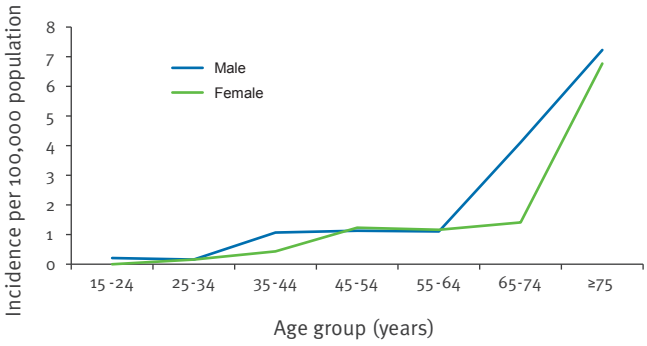


AFP: acute flaccid paralysis.

Average annual incidence is calculated using Israel Central Bureau of Statistics average population estimates of Tel Aviv district for 2008.

Figure 6

Average annual incidence of human West Nile virus neuroinvasive disease by age group and sex, Tel Aviv district, Israel, 2005–2010



Average annual incidence is calculated using Israel Central Bureau of Statistics average population estimates of Tel Aviv district for 2008.

Similarly, male patients aged 75 years or older had the highest average annual incidence of encephalitis or AFP in the cohort during the surveillance period, which was significantly higher than the rest of the patients, aged between 35 and 74 years, with encephalitis (5.42 per 100,000 population vs 1.46 per 100,000 population, p<0.001) or AFP (1.81 per 100,000 population and 0.33 per 100,000 population, p=0.004). In contrast, female patients aged 75 years or older had the highest average annual incidence of meningitis, but it was not significantly higher than the rest of the patients with meningitis, aged between 15 and 74 years (0.62 per 100,000 population vs 0.17 per 100,000 population, p=0.3).

Discussion

The data that were summarised and analysed in this study reflect a six-year period of WNV surveillance in Tel Aviv district. After several years of successive declines in WNV neuroinvasive disease, its incidence increased sharply in 2010.

We observed, as previously reported by Lindsey et al. [12], a higher incidence of encephalitis in elderly male patients during our surveillance period. These patients were also chronically ill, which may serve as an independent risk factor for WNV neuroinvasive disease [15]. However, five of the six fatalities in the observation period were female patients.

Two-thirds of the patients with a neuroinvasive disease had encephalitis (53/79, 67%), followed by patients with AFP (14/79, 18%). Of those latter 14 patients with AFP, four were diagnosed in 2010 alone. WNV-associated AFP was hardly observed during the large outbreak of 2000 in Israel: 98% of 233 hospitalised patients were diagnosed as having West Nile fever, encephalitis or meningitis but not AFP, which was reported as myelitis by Chowers et al. [13].

In our series, patients with meningitis were significantly younger than patients with other forms of neuroinvasive disease. This age discrepancy was described previously by Lindsey et al. [12].

Our reported CFR for the patients with neuroinvasive disease (6/79, 8%), who were all hospitalised, was similar to the overall CFR in Israel during the outbreak in 2000 (29/439, 7%) [4]. The CFR in our study was however half the CFR of hospitalised patients (33/233, 14%), for whom data were obtained in 2000 [13]. Our reported CFR was also similar to that in the United States (US), which was 9% of the patients with a neuroinvasive disease during 1999–2008, most of whom were hospitalised [12].

Remarkably, the highest percentage of WNV neuroinvasive disease occurring in previously healthy patients was observed in 2010, when compared to the period between 2005 and 2009 (7/19, 37% vs 3/60, 5%, p=0.001).

In our series, meningitis affected patients who were significantly younger on average than patients with other forms of neuroinvasive disease. The year 2010 did not appear to present an exception, although the total number of cases was small (39 years on average (n=3) for patients with meningitis vs 47 years in all years of the surveillance period (n=12)). Because of their younger age, patients with WNV meningitis would be less likely to have underlying medical conditions. Therefore, we examined whether the overall increase of previously healthy patients presenting with WNV neuroinvasive disease in 2010 could have been due to an increase in the number of patients with WNV meningitis in that year; however, of the patients with a neuroinvasive disease in 2010, only three of 19 had meningitis, which was well within the range of annually reported cases of WNV meningitis in the district, between 2005 and 2009 (0/10 in 2007 and 0/3 in 2009 up to 5/20 in 2005).

We also verified if the number of previously healthy patients presenting with a neuroinvasive disease had increased in other years than 2010, such as in 2005, in which a peak in the proportion of meningitis was observed (5/20). There was, however, no lower proportion of chronically-ill patients (17/20) in that year.

Additionally, to our knowledge, there was no change in the surveillance practice over the study time period that could account for the highest percentage of previously healthy patients presenting with WNV neuroinvasive disease observed in 2010.

In the years following the major national outbreak of WNV infection in 2000, various strains of WNV lineage 1 predominated in Israel either in isolated foci or in the entire country [16]. Representative Israeli WNV genomic sequences of viruses isolated from mosquitoes and humans between 2000 and 2009 were deposited in the GenBank/EMBL/DDBJ database under accession numbers GU246634–GU246714 and HM152773–HM152780, respectively.

The emergence of new viral genotypes simultaneously with higher number of human cases was observed in Israel in 2000, 2005, and 2007 [16]. Particularly, in 2005, an increased nationwide WNV activity, with 102 laboratory-confirmed human patients [16] of whom 20% were from the Tel Aviv district, which was a part of the epicentre, was reported [17]. Molecular analysis of human and mosquito isolates revealed a genotype most similar to the one that was isolated in the equine WNV outbreak in Morocco in 2003 [18], and which had not been isolated previously from humans or mosquitoes in Israel [17].

Notably, 2005 and 2010 shared some epidemiological similarities, as we observed in the district: both years had a similar incidence of WNV neuroinvasive disease, higher than the other years of surveillance, including a higher incidence of AFP, which is a long-term

complication of considerable morbidity and mortality [19,20]. In addition, the majority of WNV infection cases in both years had occurred early, already in July and August, compared to later months of occurrence, in the other years.

One possible explanation to the increased incidence of morbidity observed in 2010, which was also accompanied by a higher fraction than before of previously healthy patients could be the recent emergence of another WNV strain or variant, as in 2005. A genotype characterisation of the 2010 WNV strain is however not available to date.

Another explanation for the 2010 epidemiological characteristics could be the early arrival of an extremely hot summer, already in May. This might have contributed to the early peak of WNV season in 2010, in the district. The period of May–July 2010 was warmer than the perennial average of the years 1981–2000 [21-23]. In addition, August 2010 was the warmest measured month in Israel [24,25].

The summer of 2010 was extremely hot also in areas, which usually have a temperate climate, such as northern Greece and Romania. A human WNV outbreak emerged in northern Greece for the first time, early in July 2010 [26]. An additional noticeable outbreak, which also began in early July 2010, was reported in Romania, where a neuroinvasive lineage 2 WNV strain was detected for the first time [27]. Whether there is a possible association between recent climatic extremes in the region, WNV activity, and its mosquito vectors should be extensively studied.

Conclusions

We report on the epidemiological and clinical characteristics of human WNV infection in the Tel Aviv district between 2005 and 2010. As such, it may be limited in time, place and person. Nevertheless, our main body of findings, such as the patients' characteristics, was concordant with previous data, at the national level, published in Israel and the US.

Any successful WNV surveillance system should integrate and maintain both epidemiological and laboratory capabilities for prolonged periods of time, particularly in endemic and densely populated areas, such as the Tel Aviv district.

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Human cases of West Nile virus Infection in north-eastern Italy, 15 June to 15 November 2010

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In 2010, for the third consecutive year, human cases of West Nile virus (WNV) infection, including three confirmed cases of neuroinvasive disease and three confirmed cases of West Nile fever, were identified in north-eastern Italy. While in 2008 and 2009 all human cases of WNV disease were recorded in the south of the Veneto region, cases of WNV disease in 2010 additionally occurred in two relatively small northern areas of Veneto, located outside those with WNV circulation in the previous years. WNV IgG antibody prevalence in blood donors resident in Veneto was estimated as ranging from 3.2 per 1,000 in areas not affected by cases of WNV disease to 33.3 per 1,000 in a highly affected area of the Rovigo province. No further autochthonous human cases of WNV disease were notified in Italy in 2010. The recurrence of human cases of WNV infection for the third consecutive year strongly suggests WNV has become endemic in north-eastern Italy.

Introduction

In Italy, the first outbreak of West Nile virus (WNV) infection was reported in 1998 and occurred among horses in the Tuscany region [1]. The virus re-emerged in 2008, when equine and human cases of West Nile neuroinvasive disease (WNND) were notified in the Veneto and Emilia Romagna regions in north-eastern Italy [2]. In Veneto, six clinical cases of WNV infection were identified with disease onset from August to September 2008 and all were from the Rovigo province [3,4]; three further human cases of WNND were notified in Emilia Romagna in September and October 2008 [4,5]. Veterinary and entomological surveillance documented that WNV infection was widespread in the same areas in north-eastern Italy, with notification of 794 equine WNV infections in 251 stables and viral isolation in resident bird species and mosquitoes [2]. Compared to 2008, a trend towards an increasing number of human WNV infections and a spread to a wider geographical area was noticed in 2009 [2], when 17 cases of WNND were notified in northern Italy, including six from Veneto, nine from Emilia-Romagna, and two from the Lombardia region [6-8]. In 2009, we isolated the virus from a blood donor and sequenced its whole genome (Ita09, GenBank accession number GU011992) [7]. Phylogenetic analysis classified the Ita09 isolate as Lineage 1, clade 1a, within the Mediterranean subtype [7], which includes the majority of strains responsible for outbreaks in Europe and in the Mediterranean basin. The full length genome of WNV Ita09 was almost identical to that of WNV isolated from two magpies the year before in the same area [7] and to WNV sequences obtained from mosquito pools collected in Emilia-Romagna in 2009 [9], thus suggesting that WNV might have become endemic in some areas in northern Italy. In these areas, the presence of *Culex pipiens* vector at high density and resident bird species susceptible to WNV infection, like magpies, carrion crows, and rock pigeons, could play an important role in WNV persistence and maintenance during epizootic periods [2].

In this context, in 2010, enhanced National and Regional Surveillance Plans for WNV surveillance were implemented in Italy. This study reports further human cases of WNV infection, who were identified in Veneto in 2010, also in areas north of those affected in 2008 and 2009.

Methods

National Surveillance Plan

During spring 2010, the Ministry of Health published a National Plan for WNND Human Surveillance in Italy, which detailed the activities to be carried out between 15 June and 15 November, the annual period, when the risk for WNV infection is high. In 2010, the surveillance area was enlarged to include municipalities where autochthonous human and veterinary cases of WNV infection had been notified in previous years, as well

as surrounding areas within 20 km from the municipalities. Activities included (i) surveillance of human cases of WNND, (ii) active surveillance of WNV disease and serosurveillance of WNV infection in workers employed in farms where equine cases of WNV infection had been identified, (iii) WNV nucleic acid amplification test (NAAT) screening of blood and haematopoietic stem cell donations in areas under surveillance and, of tissue and solid organ donations, on the whole national territory, (iv) measures for mosquito vector control.

Regional Surveillance Plan

In 2010, the Veneto region implemented the activities of the National Surveillance Plan and intensified the surveillance of human cases of WNV infection by activating an enhanced regional surveillance plan for West Nile fever, as well as seroprevalence studies on blood donors resident in at-risk areas of Veneto.

Case definition of West Nile neuroinvasive disease and West Nile fever

According to the Regional Surveillance Plan implemented in 2010, cases of WNND were defined as being older than 15 years, having fever ≥ 38.5 °C, and neurological symptoms such as encephalitis, meningitis, Guillain-Barré syndrome or acute flaccid paralysis. Cases of West Nile fever were defined as being over 15 years-old, having fever ≥ 38.5°C (or history of fever in the last 24 hours) for a period no longer than seven days occurring from 15 July to 15 November, no history of recent travel to tropical countries, and absence of other concomitant diseases which could account for the febrile illness.

Cases of WNND and West Nile fever were further classified as possible, probable or confirmed. Possible cases of WNND or West Nile fever fulfilled the clinical case definition. Probable cases fulfilled the clinical case definition and at least one of the following laboratory criteria: presence of IgM antibodies against WNV by ELISA; seroconversion by ELISA; fourfold increase of IgG antibodies in acute- and convalescent-phase serum samples (preferably with 15 to 20 days between the two samples) by ELISA. Confirmed cases fulfilled the clinical case definition and at least one of the following laboratory criteria: isolation of WNV from blood and/or, for WNND, from cerebrospinal fluid (CSF); presence of IgM antibodies in CSF by ELISA (for WNND); detection of WNV RNA by RT-PCR in blood and/or CSF (for WNND); detection of increasing levels of IgM and IgG antibodies against WNV by ELISA, confirmed by plaque-reduction neutralisation test (PRNT).

Case laboratory investigations

Possible cases of WNND and West Nile fever occurring in Veneto were referred to the Regional Reference Laboratory. WNV RNA in plasma and CSF samples was detected by using two different real-time RT-PCR methods, targeting WNV lineage 1 [10] and both WNV lineage 1 and lineage 2 [11]; detection of IgM and IgG antibodies against WNV in serum and CSF samples was done by ELISA (WNV IgM capture DxSelect ELISA and IgG DxSelect ELISA kits, Focus Diagnostics, Cypress, California). To confirm the specificity of antibody response, ELISA-positive samples were further tested by PRNT90, with cutoff 1:10 for positive results. PRNT was conducted in a biosafety level 3 laboratory, according to the protocol described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 of the World Organisation for Animal Health (OIE) [12].

Active surveillance of stable workers and household contacts

Active surveillance of WNV infection was done for workers employed in farms and for subjects aged ≥15 years residing in farms where equine cases of WNV infection had been identified. Members of households with confirmed cases of WNND or West Nile fever and close contacts of identified human or equine cases of WNV disease were also surveyed. Laboratory tests included detection of IgM and IgG antibodies against WNV in serum and CSF samples by ELISA and confirmation by PRNT, as described above.

Screening of blood and organ donations

In 2010, during the period of surveillance according to the National Surveillance Plan, NAAT screening was performed for all blood and haematopoietic stem cell donations from the Rovigo and Venice provinces, where human cases of WNND had been identified in 2009. In September 2010, following the notification of the first case of WNND, NAAT screening was extended to the Vicenza province. Screening of tissue and organ donations was performed on the whole national territory.

WNV NAAT screening of blood donors was performed by using Cobas TaqScreen West Nile Virus test on Cobas S201 system (Roche Molecular Diagnostics) or the PROCLEIX WNV Assay on PROCLEIX TIGRIS System (Novartis Diagnostics). WNV NAAT-positive cases were confirmed by detection of seroconversion or increasing levels of IgM and IgG antibodies against WNV by ELISA, confirmed by PRNT, as described above.

Screening of tissue and organ donations was done by WNV NAAT using Cobas TaqScreen West Nile Virus test on a Cobas S201 system (Roche Molecular Diagnostics) and by IgM and IgG ELISA, as described above. Laboratory results had to be provided within 72 hours from donation.

West Nile virus IgG seroprevalence study

The prevalence of WNV IgG antibodies was investigated in serum samples collected from 4,450 blood donors (about 6% of blood donation collected during the study period), who were referred to four blood donation centres in different areas in Veneto, in the period from 1 August to 1 December, 2010 (Table). Sample size was determined on the basis of an expected prevalence of 6.8 per 1,000, as determined by a WNV seroprevalence study performed in 2009 [13]. The design and

the results of the seroprevalence study performed in 2009 are available in the paper by Pezzotti et al. [13]. In 2010, serum samples from 48 blood donations per day were collected for WNV IgG antibody testing. In particular, each day, five donations were sampled from Rovigo, centre 3, 12 from Padova, South-East, 16 from Padova, North, and 15 from Verona, choosing serum samples from the first consecutive daily donors who gave their consent for the study. The number of serum samples collected at each centre was proportional to the volume of donations performed in the year 2009. All samples were handled anonymously by technicians and researchers involved in this study.

WNV IgG testing was done and any IgG-positive sample was further evaluated by PRNT for confirmation, as described above. In addition, in IgG-positive samples, the presence of WNV IgM antibodies was also determined as described above.

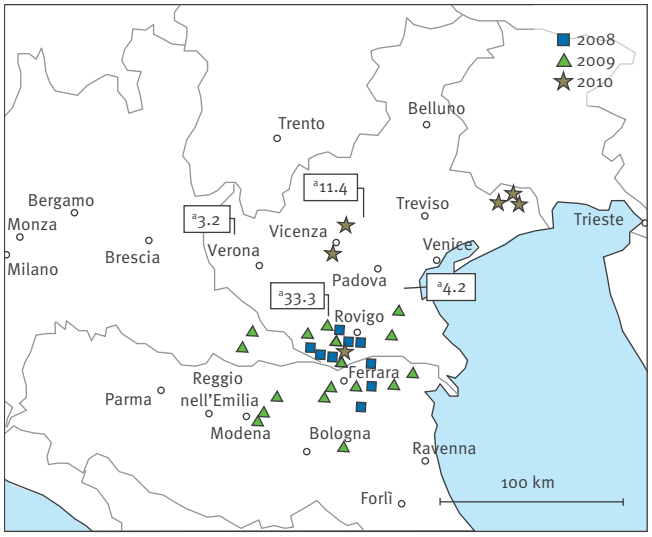
Results

Human cases of West Nile neuroinvasive disease

During the surveillance period in 2010, three males, aged 41–68 years, of 57 possible cases of WNND were confirmed by laboratory tests (all WNND cases were IgM and IgG-positive, confirmed by PRNT, while WNV RNA was undetectable in serum and CSF). Disease onset was at the end of August (two cases from the Venice and Vicenza provinces) and in the middle of October (one case from the Venice province) (Figure). Symptoms included fever, vomiting, headache, altered mental status, and, in one patient, urinary retention. All patients fully recovered.

Figure

Map of north-eastern Italy representing autochthonous human cases of West Nile neuroinvasive disease and West Nile fever notified in Italy, 2008–2010 (n=32)



[a] West Nile virus IgG antibody prevalence per 1,000 blood donors, determined in 2010 in four blood donor centres in Veneto.

A further case of WNND was diagnosed in the Rovigo province in the middle of November 2010. The case, aged in its late 40s, had been hospitalised one month before for viral encephalitis in Romania, in the Braila region, close to areas, where several human cases of WNV disease had been reported in 2010 [14]. This case was therefore considered as an imported case. The patient, who suffered from fever, headache, paraplegia, diarrhoea, myalgia, and pyramidal deficits in both limbs, showed progressive neurological improvement. Laboratory investigation confirmed the presence of IgM and IgG antibodies against WNV.

Human cases of West Nile fever

Of 38 possible cases of WNV infection, three (two males and one female, aged 40–67 years) were positive for WNV IgM and IgG and confirmed by PRNT test, but none of them was WNV RNA-positive. Symptoms included fever, arthralgia, and asthenia in two patients, and fever, headache, abdominal pain, vomit, and diarrhoea in one. One of these patients, after two weeks of fever and arthralgia, showed asthenia, disorientation and retarded movements. All patients fully recovered. Of these three patients, one was resident in the Rovigo province and was the first case of WNV infection identified in 2010, with symptom onset at the beginning of July, one was resident in the Venice province, and one in the Vicenza province (Figure). A further patient from Rovigo, with a retrospective diagnosis of fever, arthralgia, rash, asthenia, and PRNT-confirmed WNV IgG, but IgM-negative, was defined as a probable case.

Active surveillance of stable workers and household contacts

Twelve of 23 household contacts with WNV disease patients were investigated and, among them, an asymptomatic subject resident in Vicenza was found to be WNV IgM and IgG-positive. Active surveillance of WNV infection was also done in all seven workers employed in stables in the Venice province, where equine cases of WNND had been identified [15]. All of them were WNV seronegative.

Surveillance of blood and organ donations

Of 46,045 screened blood donations, two were WNV RNA-positive and were collected in the middle of September from asymptomatic subjects, one resident in the Rovigo province and the other in the Venice province, in the same area where symptomatic cases of WNV infection had occurred. WNV infection was confirmed in both subjects by seroconversion and by real-time RT-PCR targeting WNV lineage 1, thus indicating that these were cases of WNV lineage 1 infection. Due to the low viral load, viral genome sequencing was unsuccessful. No cases of WNV infection were identified among tissue and organ donations.

West Nile virus IgG seroprevalence study in blood donors resident in the Veneto region

Compared to 2009, the seroprevalence investigation in 2010 was extended to more provinces than the Rovigo

province, by adding blood donor centres in the Padova and Verona provinces, as detailed in the Table. In 2010, WNV IgG seroprevalence ranged from 3.2 to 33.3 per 1,000 in the different centres. When compared with 2009, a two-fold increase of IgG WNV seroprevalence was observed in the blood donation centre Rovigo, centre 3, which was included in the study both in 2009 and 2010 (Table).

Distribution of cases of West Nile virus disease

All confirmed autochthonous human cases of WNV disease, including WNND and West Nile fever, identified in Italy in the period from 2008 to 2010 are shown on the map in the Figure. Estimated values of WNV seroprevalence in blood donors from the four centres evaluated in 2010 are also indicated on the map (Figure). While in 2008 and 2009 all human cases of WNV disease were identified in the south of Veneto and in neighbouring Emilia Romagna and Lombardia, in 2010, human cases of WNV disease also occurred in two relatively small areas north of the Venice province, as well as in the Vicenza province in Veneto.

Discussion and conclusions

This study reports six cases of symptomatic and three cases of asymptomatic WNV infection, detected in north-eastern Italy in 2010, an area where WNV seroprevalence was estimated to range from 3 to 33 per 1,000 in 2010. To our knowledge, no further autochthonous human cases of WNV disease were notified in Italy in 2010.

2010 is the third consecutive year that human cases of WNV infection are identified in north-eastern Italy. New areas in the Vicenza and Venice provinces of Veneto, located outside those with WNV circulation in the previous years, have been affected in 2010. A new geographic pattern of WNV spread has also been documented by equine, avian, and entomologic surveillance performed by the Regional and National Reference Centre for Exotic Diseases, which identified cases of WNV infection in horses, resident birds, and mosquitoes in several areas in the north-east (Modena, Treviso, Venice, Verona, Rovigo, and Bologna provinces), the centre (Campobasso province), and the south (Foggia and Trapani provinces) of Italy, some of which have not been previously affected by WNV [15]. Since WNV was circulating in animals in several areas of Italy in 2010, the identification of human cases of WNV disease only in Veneto could be related to the enhanced regional surveillance programme in this region, which was activated in 2010.

Besides autochthonous human cases of WNV infection, we diagnosed a case of WNND imported from Romania, where an epidemic outbreak was ongoing with 57 human cases notified in 2010 [14]. This case report, like the recently described cases of West Nile fever imported from Israel to the Netherlands [16], emphasise the importance of surveillance also for potential imported cases of WNV infection. These imported cases serve as sentinels of the increase in the incidence of WNV disease occurring in 2010 in European and Mediterranean countries, where cases of WNV infection are notified every year, such as Romania (57 human cases in 2010), Hungary (three cases in 2010), Israel (24 cases in 2010), and Russia (480 cases in 2010) [14,16-19]. Moreover, a large human epidemic outbreak with 261 confirmed cases of WNV disease occurred in Greece in 2010, where WNV infection had not been documented in humans before [20]; seven human cases of WNV infection were also confirmed in Turkey [21]. In addition, in 2010, equine outbreaks were reported in Morocco, Portugal, Spain, and Bulgaria [22,23].

In this regard, a recent study on the presence of neutralising antibodies against WNV, detected by neutralisation assay, in intravenous immunoglobulin preparations produced from human plasma samples

Table

Prevalence of serum West Nile virus IgG antibodies in blood donors from the Veneto region, Italy, 2009–2010 (n=6,957)

Province, blood donor centre	Number of samples		Number (%) of WNV IgG antibody-positive samples		Estimated WNV IgG prevalence per 1,000 blood donors [95% CI]	
	2010	2009	2010	2009	2010	2009
Rovigo, centre 1	NR	494	NR	2 (0.40%)	NR	4.0 [0–9.6]
Rovigo, centre 2	NR	1,509	NR	6 (0.40%)	NR	4.0 [0.8–7.2]
Rovigo, centre 3	511	504	17 (3.33%)	9 (1.79%)	33.3 [17.7–48.8]	17.9 [6.3–29.4]
Padova, South-East	719	NR	3 (0.42%)	NR	4.2 [0–8.9]	NR
Padova, North	1,662	NR	19 (1.14%)	NR	11.4 [6.3–16.5]	NR
Verona	1,558	NR	5 (0.32%)	NR	3.2 [0.4–6.0]	NR

NR: not recorded.
WNV: West Nile virus.

collected in Austria, Germany and the Czech Republic demonstrated increasing titres of neutralising antibodies from 2006 to 2010 [24]. Our study also could suggest an increase of WNV IgG seroprevalence in blood donors resident in areas of WNV circulation from 2008 to 2010. The prevalence of WNV IgG antibodies in Veneto ranged from 0.3% in areas not affected by WNV circulation to 3% in affected areas, in line with a previous study performed on solid organ donors in Italy in 2009 [25] and with recent WNV seroprevalence data from Greece [26].

In conclusion, for the third consecutive year, human cases of WNV infection have been identified in north-eastern Italy, suggesting WNV has become endemic in this area. In addition, in 2010, veterinary and entomologic surveillance identified WNV circulation in Italian areas that have not been previously affected by WNV infection. An increased incidence of WNV infection in humans and horses has been also reported in other European and Mediterranean countries. This epidemiological situation urges European countries to enhance surveillance of WNV disease, mosquito control activities, and implementation of measures to prevent transmission to humans through blood transfusion and organ donation.

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Outbreak of West Nile virus infection in humans, Romania, July to October 2010

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A total of 57 cases of West Nile virus infection (54 with neuroinvasive infection and three with fever) were identified in Romania between July and October 2010. The median age of the cases was 53.4 years, with the highest incidence in the age group 60–69 years. The case fatality rate was 8.8%. Cases were distributed in 19 districts in the southern, western, central and eastern parts of the country. Molecular investigation revealed lineage 2 West Nile virus, related to the Volgograd 2007 strain.

Introduction

On 28 August 2010, the National Reference Laboratory for Vector-borne Diseases in the Cantacuzino Institute in Romania reported to the National Centre for Surveillance and Control of Communicable Diseases (NCSCCD) 10 positive results for West Nile virus (WNV) in samples of patients distributed in nine different Romanian districts. Six of these 10 cases were male and four were female, with a median age of 56 years (range: 32–79 years).

Romania recorded the first large outbreak of West Nile neuroinvasive disease (WNND) in Europe in 1996, with 393 confirmed cases. This was also the first such outbreak in urban settings. Cases were confined to Bucharest, its rural surroundings and 14 districts in the Danube Plain [1,2]. In response to this outbreak, in 1997, the Ministry of Health set up a regional, hospital-based surveillance system and sporadic cases were recorded every year in the districts neighbouring the Danube River [3]. In 2009, the surveillance system was extended at national level, following the confirmation of two cases of West Nile fever (WNF) in humans in the central part of Romania and the detection of WNV-specific IgG antibodies among horses in many other areas of the country.

Within the routine WNV surveillance activities in Romania, the following case definition is used for a suspected case with WNV infection: a person over 15 years of age who presents with fever and meningitis, encephalitis or meningoencephalitis between May and October and who reports a history of mosquito bites.

Two sets of samples are collected for each suspected case: for patients with acute symptoms both cerebrospinal fluid (CSF) and serum are taken. For patients in convalescence phase, a second serum sample is taken 14–21 days later. A probable or confirmed case of WNV infection is defined as a person who met the relevant clinical and the laboratory criteria for probable or confirmed cases described in the European Union case definition [4]. A suspected case is considered not to be a case if WNV-specific IgM was not detected in CSF and serum.

Data were obtained from infectious disease hospitals and reported using a standardised form containing information on symptoms, onset date and possible risk factors.

Following an outbreak of WNV infection in Greece in July to August 2010 [5], surveillance for WNND was enhanced in all districts in Romania from 12 August 2010. All districts were asked to increase their vigilance. In addition, the case definition for suspected cases used for routine surveillance was modified: a history of travel in the Danube Delta and/or in Greece was added.

Outbreak description

On 30 August 2010, after the 10 WNND cases had been reported on 28 August, the NCSCCD further reinforced the WNV surveillance activities in humans at national level and the case definition was modified once more: all persons aged over 15 years presenting with fever and meningitis or encephalitis or meningoencephalitis and clear CSF were considered suspected cases and were tested for WNV-specific antibodies.

After a cluster of five cases was recorded on 28 August 2010 in a newly affected area in Central Transylvania (Alba district – Blaj city, Mures and Sibiu districts), active perifocal surveillance was set up locally for WNF cases as part of the enhanced surveillance: epidemiologists were involved in retroactively identifying persons who presented to general practitioners with fever and rash during August 2010. Samples from these patients were tested for the presence of WNV.

The WNV surveillance season starts every year from early May and ends on 30 October. In 2010, the surveillance season was exceptionally extended by two weeks in two places that were most affected by the outbreak (Bucharest city and Constanta district). From 10 May to 15 November 2010, a total of 170 suspected cases with WNV infection were reported in Romania. Of these, 52 were confirmed cases (49 with WNND and three with WNF), five were probable cases and 113 were negative for WNV.

The first confirmed WNND case had symptom onset on 4 July 2010 and the last on 11 October 2010. The distribution of the probable and confirmed cases of WNV infection by date of symptom onset is presented in Figure 1.

The first case was diagnosed retroactively, during the investigation of a cluster of unexpected deaths in Constanta district, thought to be caused by hyperthermia due to high temperatures (39 °C) in early July 2010. For the rest of the cases, most (n=28) had symptom onset during the second half of August, 19 in September and only three cases had symptom onset in October (Figure 1).

Among the 57 cases, the sex ratio (male:female) was 1.7:1 (36 male:21 female). The median age was 53.4 years (age range: 12–81 years). The highest number of cases (n=15) belonged to the age group 60–69 years (Figure 2).

The incidence per age group ranged from 0.1 to 0.8 per 100,000 population, with the highest values being for the age groups 60–69 years (0.8 per 100,000 population) and 70 years and above (0.5 per 100,000 population). The lowest incidence was in the age groups under 20 years and 20–29 years (0.1 per 100,000 population).

All confirmed and probable cases were hospitalised with WNV infection (31 with meningitis, 19 with meningoencephalitis and four with encephalitis). Three had non-neuroinvasive symptoms: two had fever and maculopapular exanthema and one had prolonged febrile syndrome. Among the severe cases, eight entered into a coma. Clinical symptoms included: fever (n=53), headache (n=50), stiff neck (n=42), shivering (n=26), confusion (n=21) vomiting (n=22), myalgia (n=25), disorientation (n=17), photophobia (n=12), Kernig sign (n=14), Brudzinski sign (n=8), memory loss (n=3), maculopapular exanthema (n=2).

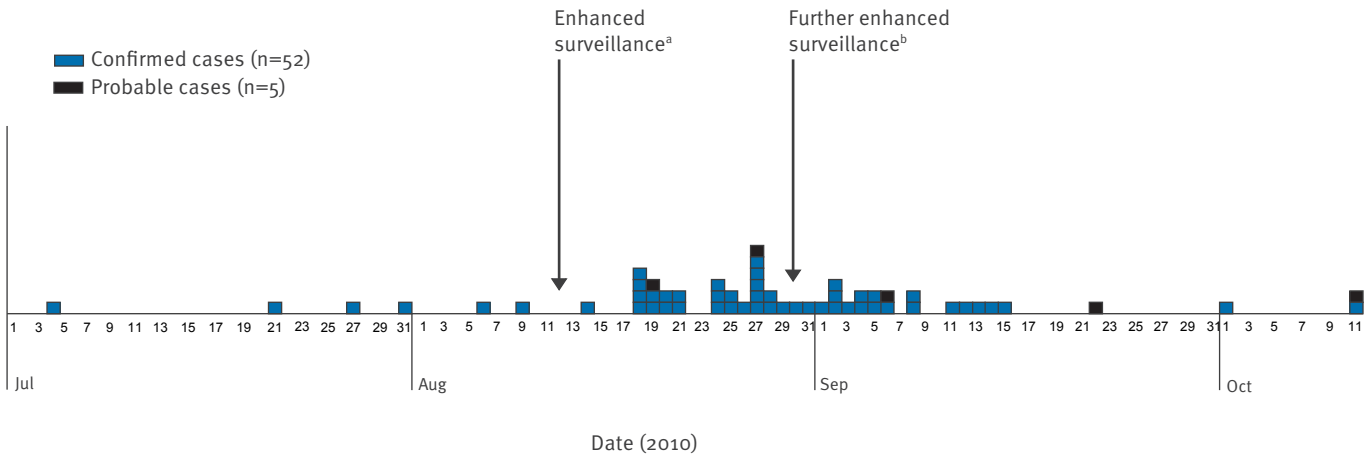
Five deaths were recorded among the 57 identified cases, giving a case fatality rate of 8.8%. All deceased patients were aged over 65 years, and had underlying conditions (hypertension, diabetes).

Of the 57 cases, 30 lived in urban settings and 27 in rural areas, giving an urban: rural ratio of 1.1:1.

Most cases (n=35) were recorded in the southern part of the country, an area known to be endemic for WNV from previous years. However, WNV infections were reported in humans in previously unaffected areas, such as districts in central Transylvania, and in the Moldavian Plateau (Figure 3).

Figure 1

Distribution of cases of West Nile virus infection (probable and confirmed) by date of symptom onset, Romania, July – October 2010 (n=57)



[a] Increased vigilance and amendment of case definition.

[b] Reinforced surveillance activities and second amendment of the case definition.

Laboratory investigation

Serum and CSF samples were tested for the presence of IgM and IgG antibodies specific for WNV, using a commercial enzyme-linked immunosorbent assay (ELISA) kits (Focus Technologies, USA). A total of 45 WNV neuroinvasive cases were confirmed by IgM-capture ELISA, based on the presence of WNV-specific IgM antibodies in CSF.

In nine cases with neurological clinical picture, CSF samples were either not available or were negative or borderline positive for WNV-specific IgM by ELISA. Serum samples from these cases were tested also by seroneutralisation assay using a lineage 1 WNV strain from Israel: four additional neuroinvasive cases were confirmed by the presence of WNV neutralising antibodies in serum, while in the other five cases, the seroneutralisation assay was negative. In the three cases with non-neuroinvasive WNV infection, the infection was confirmed by the presence of WNV neutralising antibodies in serum.

Cases from Transylvania were also tested for the presence of tick-borne encephalitis virus-specific antibodies because this virus had previously been found to be circulating in this area.

Serum and CSF samples were collected within five days from symptom onset from 16 of the 49 confirmed neuroinvasive cases; tissue samples were collected from one fatal case at the autopsy. Reverse transcription (RT) and real-time polymerase chain reaction (PCR) was used to detect the WNV genome in these samples.

Figure 2

Incidence rate of cases of West Nile Virus infection (probable and confirmed) by age group, Romania, July – October 2010 (n=57)

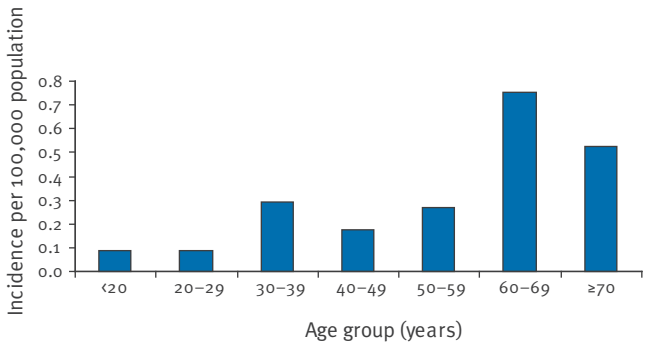
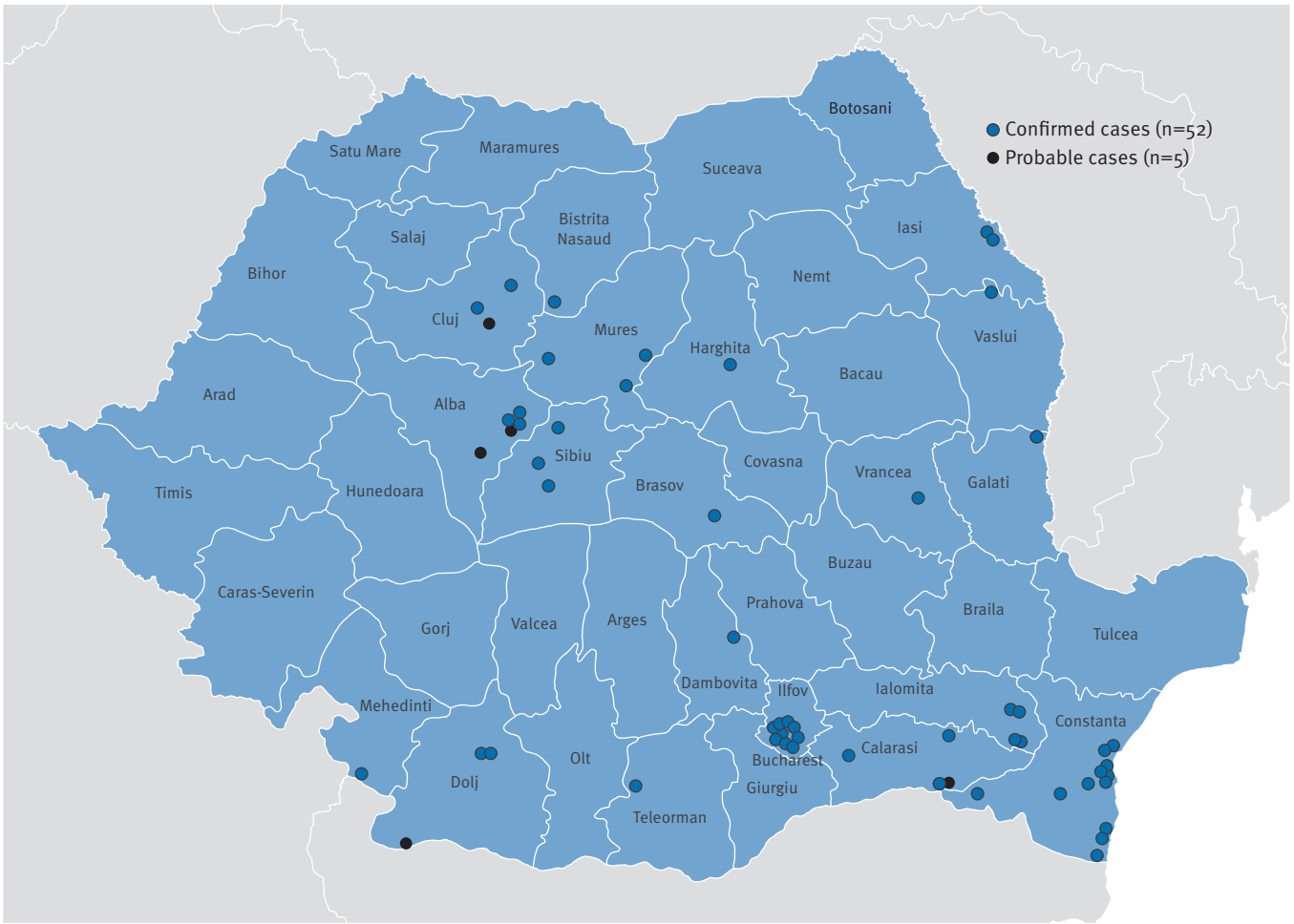


Figure 3

Distribution of cases of West Nile virus infection (probable and confirmed) by place of exposure, Romania, July – October 2010 (n=57)



One dot represents one case.

The target sequence was a conserved region of the 3´ non-coding region of WNV (Vázquez *et al.*, unpublished data). In addition, partial sequence of the flavivirus NS5 gene was obtained following a generic RT-nested-PCR to detect flaviviruses [6]. New degenerate internal primers were designed for sequencing. Virus culture was performed for the same cases using Vero and C6-36 cells, and gave negative results after three blind passages.

Molecular investigation detected the WNV genome in the brain tissue of the fatal case, and in the serum and/or CSF of four of the 16 cases tested. Partial sequencing of the NS5 gene was performed for only one positive (approximately 1,200 genome equivalents/ml)) serum sample: analysis of 780 nucleotides of the NS5 gene demonstrated that the virus was a WNV lineage 2 strain, with 99.3% sequence identity to the virus circulating in Volgograd in 2007 (GenBank: FJ425721.1).

Public health measures

Surveillance has been gradually increased following reports of the outbreak of WNV infection in Greece and the detection of the first cases in Romania. The Ministry of Health and the regional public health authorities informed the local authorities about recommended measures for mosquito control and communicated data on the evolution of the outbreak to the general population on a weekly basis. The population was also informed about measures to reduce exposure to mosquitoes and to prevent mosquito bites.

The Ministry of Health informed the National Institute of Haematology on a daily basis about the situation of the confirmed human cases of WNV infection and about the places where they have been identified. The National Institute of Haematology deferred donations from blood donors in rural areas until 1 December 2010. Initially, donations from affected urban areas were also deferred, but at a later stage, in order to maintain a sufficient blood supply, only donors from these areas presenting with a history of fever were excluded. In addition, those who donated blood were required to report to the Blood Donation Centre any symptoms of fever in the 15 days after giving blood. Donated blood was stored and not used before the five-day period had elapsed. Donors who had spent at least one night in areas with human cases of WNV infection were excluded from donation for a period of 28 days after having left the affected area.

Veterinary doctors were informed about the occurrence of WNV infection in humans and were requested to provide information on WNV infection in animals. According to the information received from the national veterinary authority, no dead birds infected with WNV and no cases of encephalomyelitis or recent WNV infection in horses have been recorded during the outbreak in humans. Seroprevalence studies found WNV-specific antibodies in poultry from two districts in the eastern and western parts of the country. WNV-specific IgG antibodies were detected in horses from 22 districts across the country, including nine districts in which human cases of WNV infection occurred in 2010.

Discussion

With 52 confirmed cases of WNV infection widely distributed in the country, the 2010 transmission season was associated with the most important WNV infection outbreak since 1997, when the WNV surveillance system was implemented in Romania.

Weather conditions (rainfalls, high temperatures) in 2010 were favourable to the increase of mosquito populations. Culex pipiens had already been identified as the vector of WNV in the 1996 outbreak [7]. In late summer, at least in urban areas, Cx. pipiens is the main mosquito biting humans, and we may assume that in this type of environment, this species was the WNV vector in 2010 also.

A specific feature of this outbreak was its extended area in the country: cases were distributed in 19 districts, with some concentration of cases in the south-eastern district of Constanta and in urban areas such as Blaj (western part) and Bucharest. Although most cases occurred in the already known endemic area in the south (in the Danube lowland and Delta neighbouring counties), in the 2010 transmission season, cases were also recorded in previously unaffected areas, from the valleys of other major rivers, known to be bird migration pathways.

Partial sequencing of the NS5 gene from a WNV-positive serum of a Bucharest resident revealed a virus strain belonging to the genetic lineage 2, highly similar (99.3%) to the Volgograd strain involved in the 2007 WNV outbreak in the Volga Delta area [8]. It is unsure whether the same WNV strain was involved in the outbreak beyond the Carpathian Mountains in Transylvania in 2010. Lineage 2 WNV strains were previously thought to be of low virulence. Nevertheless recent studies in South Africa suggest that lineage 2 WNV strains are a cause of neurological disease in horses and humans [9]. The WNV strain circulating in Romania from the 1996 epidemic belonged to the genetic lineage 1 [7] and was associated with a case fatality rate of 4.3% (an 8.8% rate was recorded in 2010) [1]. In conclusion, a change in the epidemiological profile of WNV infection was recorded in 2010 in Romania, with emergence of cases in previously unaffected areas in western and eastern parts of the country, and the emergence of a neuroinvasive lineage 2 WNV strain.

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Increasing West Nile virus antibody titres in central European plasma donors from 2006 to 2010

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We analysed by neutralisation assay 55 intravenous immunoglobulin preparations produced from human plasma collected in three central European countries, specifically Austria, Germany and the Czech Republic, from 2006 to 2010. The preparations from 2009 and 2010 contained increasing titres of neutralising antibodies against West Nile virus (WNV) in the absence of reported human WNV cases in these countries.

Introduction

Clinical cases of West Nile virus (WNV) infection in the European Union (EU) have so far been limited to sporadic outbreaks (Table), while serological studies in sentinel horses and birds, as well as humans, have shown WNV circulation for decades, particularly in the southern EU Member States [1]. However, as exemplified by the WNV outbreak in Greece in summer 2010, the pathogenicity of this virus for humans can change. A study in 2007 detected 1% (4 of 392) WNV seropositivity in human serum samples collected from asymptomatic individuals living in Greece [2], whereas in 2010, WNV caused the second largest outbreak of human infections recorded in the EU since the outbreak in Romania in 1996 [3].

In order to assess how many people in central European countries may have been exposed to the virus, including individuals with subclinical disease, we analysed the WNV neutralising antibody titre of intravenous immunoglobulins (IVIG), a blood product containing pooled immunoglobulins from the plasma of more than a thousand donors from Austria, Germany and the Czech Republic. The suitability of this approach for assessing the cumulative number of WNV infections in a large donor population has recently been demonstrated through the use of IVIG to follow the spread of WNV in the United States [35].

Methods and results

Fully validated neutralisation assays were used to determine WNV and tick-borne encephalitis virus (TBEV) neutralisation titres (NT_{50}) in 55 individual lots of IVIG manufactured from plasma pools (≥1,000 donors) collected in three EU countries (KIOVIG, Baxter), Austria (ca. 40-45%), Germany (ca. 40-45%) and to a lesser extent the Czech Republic (ca. 15%). The IVIG lots tested were produced in the years 2006 (n=10), 2007 (n=9), 2009 (n=20) and 2010 (n=16). For the year 2008, IVIG lots produced exclusively from EU plasma were not available for testing. The NT_{50} were determined, at least in duplicate, as described [35], using WNV strain 385-99, lineage 1 or TBEV strain Neudoerfl. When tested against WNV, IVIG samples were used undiluted and titrated on Vero cells (ECACC 84113001); against TBEV, samples were initially diluted 1:5 and titrated on A549 (ATCC CCL-185) cells. After seven days, each well was assessed for virus-induced cytopathic effect, and the NT_{50}, i.e. the reciprocal dilution resulting in 50% virus neutralisation, is reported as mean±standard deviation of two or more replicates (Figure 1B). Plotting the WNV neutralisation titres of the respective IVIG lots against the year of product release, a statistically significant increase over the years 2006 to 2010 was observed ($p=0.0269$ one way ANOVA and $p=0.004$, post test for linear trend; Figure 1A).

To specifically discriminate antibodies induced by WNV infections of EU plasma donors from possibly cross-reactive antibodies against TBEV, a Flavivirus related to WNV and widely endemic in central Europe, the TBEV neutralisation capacity of the IVIG lots was also determined, after the first lots with higher WNV activity had been detected. This was done for 30 of the 55 IVIG lots (Figure 1B). We assessed a potential contribution of TBEV neutralising antibody titres to the measured WNV antibody titres by correlation analysis and found a significant correlation ($r^2=0.5$, $p=0.002$) between the TBEV and WNV neutralisation titres for 19 of 30 IVIG lots analysed (Figure 1B, open circles). The apparent WNV neutralisation capacity of the majority of IVIG lots produced from plasma collected in Austria, Germany and the Czech Republic therefore resulted, at least partially, from cross reactive antibodies as induced by the wide-spread use of TBEV vaccines or TBEV infections. The IVIG lots produced from plasma collected more recently, six lots in 2009 and five lots released for 2010 until April, did not fit this correlation. In general, plasma collection pre-dates release of IVIG lots

Table

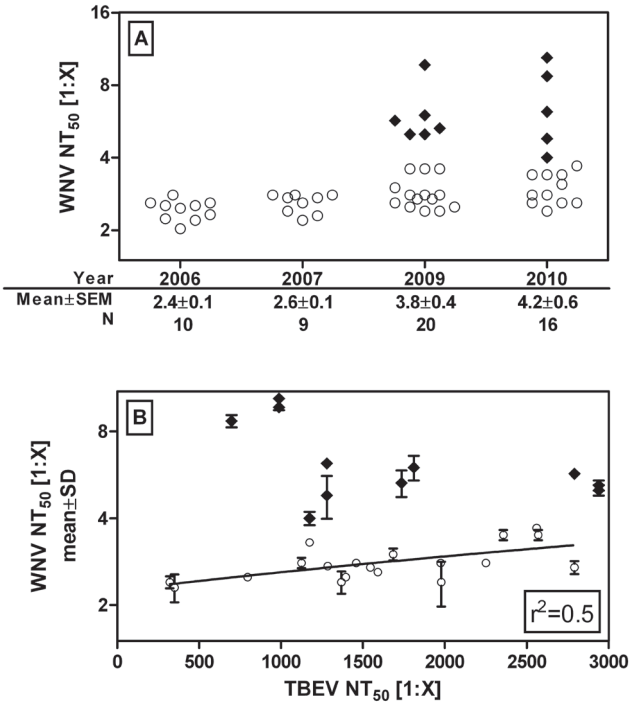
Cases of West Nile virus infection and seropositivity in EU countries, Russia and Israel, by year of occurrence, 1962–2010

Year	Country	Cases	WNND	Fatalities	Seropositivity	CFR [%]	Lineage	Reference
1962–1964	France	13	NI	0	NI	NI	NI	[4]
1973	Portugal	NI	NI	NI	0.5% (1,649 sera anti-WNV-positive)	NI	NI	[5]
1973	Spain	NI	NI	NI	17% (701 sera anti-Flavivirus-positive)	NI	NI	[6]
1975–1979	France	NI	NI	NI	5% (235 sera anti-WNV-positive)	NI	NI	[7]
1975–1976	Spain	NI	NI	NI	8% (1,037 sera anti-WNV-positive)	NI	NI	[8]
1980	Spain	NI	NI	NI	8% (130 sera anti-Flav-positive)	NI	NI	[9]
1982	France	1	1	NI	NI	NI	NI	[10]
1996	Romania	393	352	17	NI	4	1	[11] [12] [13] [14]
1997	Czech Republic	5	0	0	2.1% (619 sera anti-WNV-positive)	NI	NI	[15]
1997–1998	Romania	NI	13	1	4% (959 sera anti-WNV-positive)	NI	NI	[12] [16]
1999	Czech Republic	4	NI	NI	NI	NI	NI	[15]
1999	Czech Republic	NI	NI	NI	2% (619 sera anti-WNV-positive)	NI	NI	[15]
1999	Russia	318	84	40	NI	13	1	[11] [17] [18]
2000	Russia	56	20	0	NI	NI	NI	[12] [17]
2000	Israel	417	307	35	NI	8	NI	[19] [20]
2001	Russia	64	NI	NI	NI	5-10	NI	[12] [17]
2002	Czech Republic	1	0	0	NI	NI	NI	[21]
2002	Spain	NI	NI	NI	1% (797 sera anti-Flavivirus-positive)	NI	NI	[22]
2003	France	4	2	0	NI	NI	NI	[23]
2003	Hungary	NI	14	0	NI	NI	NI	[24]
2007	Greece	NI	NI	NI	1.02% (4 sera anti-WNV-positive)	NI	NI	[2]
2007	Russia	54	NI	2	NI	4	2	[25]
2008	Hungary	NI	14	0	NI	NI	NI	[26]
2008	Italy	13	8	0	NI	NI	NI	[27] [28]
2009	Italy	NI	17	3	NI	NI	1	[29] [30]
2010	Hungary	3	NI	NI	NI	NI	NI	[31]
2010	Russia	448	26	6	NI	1	NI	[25] [32]
2010	Romania	41	NI	4	NI	10	NI	[25] [31]
2010	Greece	261	191	35	1.5% (392 sera anti-WNV-positive)	18	2	[31] [33] [34]

CFR: case fatality rate; NI: no information available; WNND: case with West Nile neuroinvasive disease; WNV: West Nile virus.

Figure 1

West Nile virus and tick-borne encephalitis virus neutralisation by intravenous immunoglobulin lots produced from plasma collected in Austria, Germany and the Czech Republic (N=55)



IVIG: intravenous immunoglobulins; NT_{50}: the reciprocal dilution resulting in 50% virus neutralisation; TBEV: tick-borne encephalitis virus; SD: standard deviation; SEM: standard error of the means; WNV: West Nile virus.

A: WNV neutralisation titres determined in IVIG lots produced between 2006 and 2010. WNV neutralisation titres within (open circles) and out of (black diamonds) the correlation slope as shown in B. Oneway ANOVA analysis revealed a systematic (p=0.0269) mean increase in NT_{50} of IVIG sorted by production year.
B: Correlation analysis (r^2=0.5, p=0.002) of EU plasma-derived IVIG lots (N=30) tested at least in duplicate for WNV and TBEV neutralisation. Results are given as mean NT_{50}±SD.

IVIG lots exclusively collected from EU plasma were not available for the year 2008.

by approximately 6–8 months. The IVIG lots containing significantly higher WNV-neutralising capacity (Figure 1, black diamonds) did not follow a seasonal pattern.

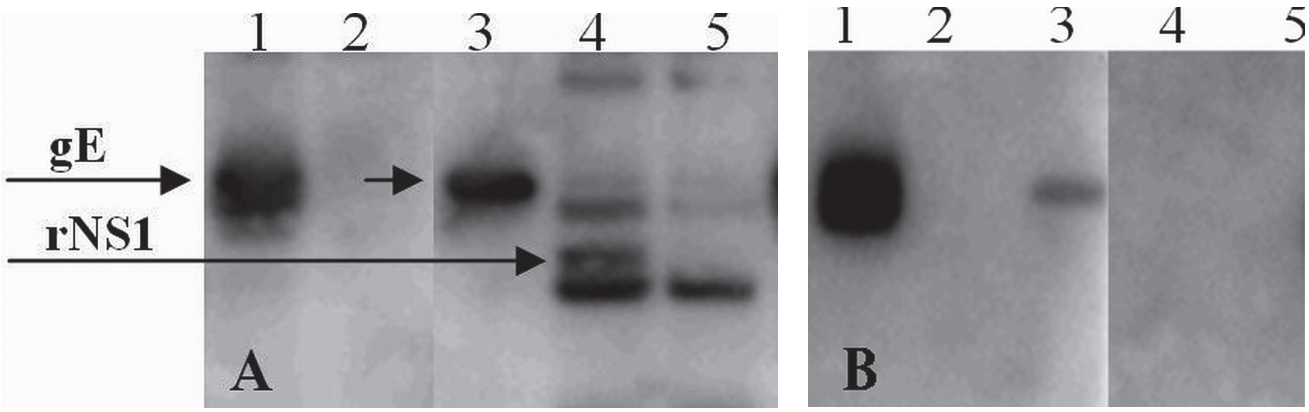
The non-structural flavivirus protein NS1 is only expressed during infection, but not present in the inactivated whole virus vaccines that are used in the countries analysed here. In addition, reactivity of antibodies with the NS1 protein is specific to the flavivirus serotype, and thus infections with either WNV or TBEV can be differentiated [36]. We therefore analysed the antibody specificity of six randomly selected IVIG lots, produced in 2007 (n=1) and 2009 (n=5), by Western blot. TBEV- or WNV-infected Vero cells as well as recombinant WNV NS1 antigen were used as a positive and uninfected Vero cells as a negative control [37, 38]. The blots were incubated with IVIG produced from plasma collected in Austria, Germany and the Czech Republic (Figure 2A), or with a control serum from TBEV-infected mice (Figure 2B). IVIG interacted with the flavivirus structural envelope protein E as well as the WNV-specific NS1 (Figure 2A). In contrast, the control mouse serum reacted strongly only with the E protein of TBEV-infected cells and weakly with WNV-infected cells (Figure 2B).

Discussion and conclusion

We found that IVIG preparations manufactured from plasma collected in Austria, Germany and the Czech Republic, contained neutralising antibodies against WNV at titres which have increased significantly since 2009. As WNV and TBEV are related flaviviruses, albeit distantly, we quantified neutralising antibody titres against TBEV in these IVIG lots, to investigate cross-reactivity between these two viruses as a potentially confounding variable. Indeed, very high TBEV NT_{50} titres of between 400 and 3,000 were observed in all

Figure 2

Reactivity of intravenous immunoglobulin lots produced from plasma collected in Austria, Germany and the Czech Republic with viral proteins



HRP: horseradish peroxidise; IVIG: intravenous immunoglobulins; NT_{50}: the reciprocal dilution resulting in 50% virus neutralisation; TBEV: tick-borne encephalitis virus; PVDF: polyvinylidene fluoride; SD: standard deviation; SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; WNV: West Nile virus.

SDS PAGE transferred to Immobilon PVDF membranes and probed with (A) EU IVIG (1:10; WNV NT_{50} mean±SD: 1.6±1.2, n=4) and HRP-coupled anti-human IgG (1:10,000) or (B) with TBEV-infected mouse serum (1:200) and HRP-conjugated anti-mouse IgG (1:10,000).

Lanes 1: Supernatants of TBEV-infected Vero cells; lanes 2: uninfected Vero cells (negative control); lanes 3: WNV-infected Vero cells, lanes 4: *E.coli*-expressed recombinant NS1 (positive control).

The figure shows one representative result of six IVIG lots tested.

investigated years, a result of vaccination and possibly subclinical TBEV infections. Despite a significant correlation of TBEV and WNV neutralisation titres in approximately 60% of the IVIG lots, 11 of the 30 lots contained significantly higher ($p<0.0001$) neutralisation titres against WNV (mean±standard error of the means (SEM): 6.5±0.6, n=11, compared with 2.8±0.1, n=19) that did not correlate with the TBEV-neutralising capacity. IVIG lots produced from plasma collected in Austria, Germany and the Czech Republic were shown by Western Blot to contain specific antibodies against WNV NS1, the most useful differentiation marker for flavivirus infections in humans [36], which provided further evidence for past WNV infections in plasma donors from the central part of the EU. As the detection of antibodies to the WNV NS1 protein is serotype-specific, a possible contribution of antibodies against dengue virus can be excluded. The theoretical possibility of a contribution of neutralising antibodies to Usutu virus (USUV) [39], a virus that belongs to the same serocomplex as WNV, was not evaluated in vitro, as even the highest USUV activity in Austria as observed during the summer of 2003 [40] had no impact on the WNV-specific neutralisation titres in IVIG lots [41]. Moreover, despite a comprehensive surveillance programme for dead birds and mosquitoes in Austria [42], where around 45% of the plasma is collected for production of the IVIG lots tested in this study, no evidence of human exposure to USUV could be found [43], which makes a significant contribution of USUV antibodies to WNV neutralisation titres determined in the present study unlikely. However, additional studies would be relevant to describe this issue more precisely.

These results demonstrate WNV seropositivity in asymptomatic plasma donors from Austria, Germany and the Czech Republic, although some of the seropositivity could be due to travel-related infections. The present epidemiological situation in these countries is thus similar to the one in Greece before the recent epidemic. The causative agent of the outbreak in Greece, WNV lineage 2, has only once before been isolated from humans with severe clinical progression, in an outbreak in Volgograd, Russia in 2007 [18]. Before those two outbreaks, this lineage was considered to be less virulent in humans compared to isolates belonging to the lineage 1 [44]. It has been suggested that the evolution from low to high human pathogenicity is associated with mutations of only a few amino acids, most likely in the non structural proteins [45].

The WNV neutralisation capacity of IVIG lots produced from Austria, Germany and the Czech Republic has increased from a mean NT_{50} of 2.5 in 2006 and 2007 to 4.2 at the beginning of 2010, which accounts for an increase of 1.7 in the WNV neutralising capacity (Figure 1). The TBEV neutralisation titres of IVIG have not changed over time, and thus the increase in WNV neutralisation titres is most likely a true reflection of increased virus circulation. Using the same, fully validated assay, reconvalescent sera from human WNV cases in North America have earlier been shown to have a mean NT_{50} of 208 [41], 120-fold higher than the increase now observed. This would indicate past WNV exposure of just under 1% of the population of Austria, Germany and the Czech Republic. The increasing WNV seropositivity in these countries marks this virus as a potential public health concern in this area, and a future epidemic associated with human morbidity and mortality similar to that observed in summer 2010 in Greece cannot be excluded.

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Conflict of interest

The authors of this manuscript have conflicts of interest to disclose: All authors were employees of Baxter AG and the work reported in this article was funded by Baxter AG.

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Molecular detection and phylogenetic analysis of West Nile virus lineage 2 in sedentary wild birds (Eurasian magpie), Greece, 2010

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A West Nile virus (WNV) lineage 2 strain was molecularly identified and characterised in a Eurasian magpie hunted in Greece in 2010, during a WNV outbreak in humans. Phylogenetic analysis revealed the highest sequence similarity (>99%) with other WNV lineage 2 strains derived from birds of prey in Austria and Hungary (2004–2009). This first molecular detection of WNV in sedentary wild birds in Greece, which are possible reservoirs of the virus, is a public health concern.

Introduction

West Nile virus (WNV) is a mosquito-transmissible *Flavivirus* with zoonotic potential. The virus has been present in Europe for decades; however, only recently were strains of lineage 2 (L2) identified outside of Africa: in 2004 and 2005 in goshawks in Hungary, in 2007 in Volgograd, Russia, and in 2008 and 2009 in goshawks and a falcon in Austria [1-3]. From early July through October 2010, 261 laboratory-confirmed cases of WNV infection in humans were reported in northern Greece as part of an outbreak. Of these, 191 patients presented with neuro-invasive symptoms, and 34 deaths were reported [4]. Most cases were observed in central Macedonia, in areas located between four major rivers (Axios, Loudias, Aliakmon and Gallikos) which converge into a common delta, a well-known resting and breeding ground for migratory birds.

Methods and results

The objective of our study was to detect possible infection of wild birds with WNV during the outbreak in Greece, and to molecularly characterise and define the WNV strain geographical origin in positive samples.

Our first focus was on members of the *Corvidae* family. Many corvid species are sedentary and territorial, having a wide daily dispersal range of up to 20 km, social, roosting in large colonies and abundant in both wetlands and urban areas [5]. Hence, introduction of the virus in an area (i.e. via migratory birds) may result in its transmission, circulation and maintenance in local corvid populations. Samples from hunter-harvested corvids (Eurasian magpies and carrion crows, hunted species according to Greek law) were collected during the hunting season (from 20 August until 28 February the following year) of 2009/10 and of 2010/11. Sampling was carried out in the municipalities of Thermi and Axios (prefecture of Thessaloniki, central Macedonia, Greece) by members of the Hunting Federation of Macedonia and Thrace, locating corvid roosting sites in nearby wetlands. Hunters were briefed on signs of encephalitis in birds, and were instructed to report any such observations. No findings of birds with signs of encephalitis or dead birds were reported from any of the hunters.

Of 96 corvids collected, 36 were tested, including 28 Eurasian magpies (*Pica pica*) and eight carrion crows (*Corvus corone*). A pool of selected tissues (kidney, heart, liver) was created from each bird. RNA was extracted from each pool, which constituted a single sample, using the PureLink RNA Mini Kit (Invitrogen). An -RT-PCR specific for Japanese encephalitis virus complex was performed for all extracts resulting in a 1,084-bp amplification product covering part of the nonstructural protein 5 (NS5) gene, as described earlier [6]. A band of expected size was obtained from one PCR product derived from a magpie harvested near the village of Trilofos (40°28'25.57"N, 22°58'28.62"E) in September 2010 (Figure 1). A serum sample from the magpie in question was tested for the presence of WNV IgG antibodies by indirect immunofluorescence test using a commercial kit (EUROIMMUN) [7]; the serum sample was positive at a dilution of 1/30.

The positive PCR product was purified using the PureLink PCR Purification Kit (Invitrogen) and was

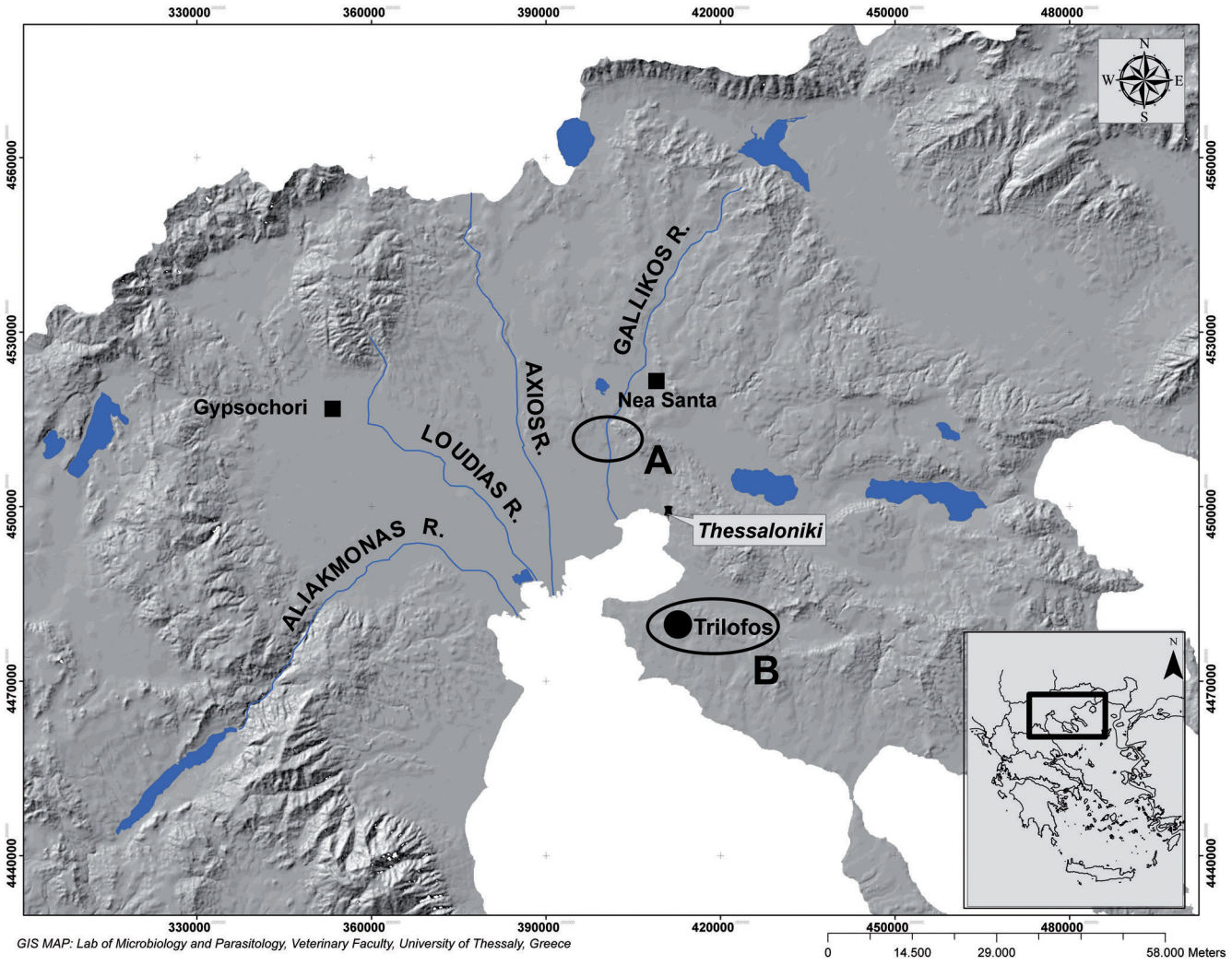
bidirectionally sequenced using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems), followed by fragment separation with a 3,730xl DNA Analyzer (Applied Biosystems).

Phylogenetic analysis was conducted using MEGA 3.1 [9]. Nucleotide sequences from other WNV strains were retrieved from Genbank (NCBI). Phylogenetic analysis of 797 nucleotide-long partial NS5 sequences was performed. A neighbour-joining phylogenetic tree using Kimura-2 parameter distance matrix was inferred from 26 WNV strain sequences (including that derived from the magpie in our study) and two sequences of the Japanese Encephalitis virus complex as outgroups (Figure 2). Node support was assessed with 1,000 bootstrap pseudo-replicates.

The WNV sequence derived from the Greek magpie clustered with WNV L2 strain sequences and presented highest (99.9%) sequence similarity to L2 strain sequences derived from birds of prey in Austria obtained in 2008 and 2009 [2]. A 99.6% similarity was also observed with the corresponding region of an L2 strain derived from a dead goshawk in Hungary in 2004 [1]. No amino acid changes were observed in the genomic region of the magpie derived WNV strain compared to Austrian and Hungarian strains. According to our analysis, all these strains as well as two strains from South Africa belong to the same sub-cluster. A lower sequence similarity (96.8%) was observed with a WNV L2 strain isolated during an outbreak in Russia in 2007. The Russian strain sequence groups with other African strains (including other South African strains) in

Figure 1

Bird sampling area for West Nile Virus, Central Macedonia, Northern Greece, 2009–2011



WNV: West Nile Virus.
The study area corresponds to the areas where most human cases occurred during the WNV outbreak.
Black square boxes indicate where WNV was detected in mosquitoes [8].
A and B indicate areas, where tested corvids were harvested.
The black circle indicates where the WNV-positive Eurasian magpie was hunted.

a separate sub-cluster, suggesting a different reintroduction of WNV L2 in Europe [3]. The sequence from the Greek magpie isolate was deposited in GenBank under accession no. JF719073.

Discussion

From early July through October 2010, a WNV outbreak in humans occurred in northern Greece, as confirmed by serologic evidence. To date, no WNV genomic sequences are available from the human cases during this outbreak. A WNV strain sequence derived from a magpie hunted during the outbreak of the human disease was found in this study. The sequence has highest sequence similarity to L2 strain sequences from birds of prey in Austria obtained in 2008 and 2009. WNV RNA fragments, though limited in size, (146 nt NS5 genomic region) with 100% sequence similarity to Hungarian and Austrian L2 strains, were also detected in two pools of mosquitoes caught during the time of the Greek outbreak and in the same area [8]. The mosquito WNV sequence was not included in our analysis because it did not overlap with the magpie WNV sequence. However, the similarity of both to the Austrian L2 strain sequences suggests that the same WNV strain is implicated in the magpie and mosquito infections and associated with the human outbreak. The evidence may implicate this corvid species in local virus maintenance and generates concerns about possible overwintering and expansion of the virus in neighbouring areas. To test this hypothesis, research must be extended in non-epidemic periods, by performing molecular and serologic surveillance in wild birds and focusing efforts on the isolation of infectious WNV from avian samples.

Phylogenetic analysis of our strain revealed a high sequence similarity with Austrian and Hungarian WNV strains detected in previous years in birds of prey (2004–2009). According to these findings, it can be hypothesised that the virus expanded from northern Europe southwards. The area of the recent outbreak is a well-known resting and breeding ground for migratory birds passing on the way from nesting grounds in Europe to wintering areas in Africa. Re-introduction of the virus in the future by birds migrating along the south-eastern migration route that leads from Europe and western Asia to Africa should also be considered possible and needs further investigation.

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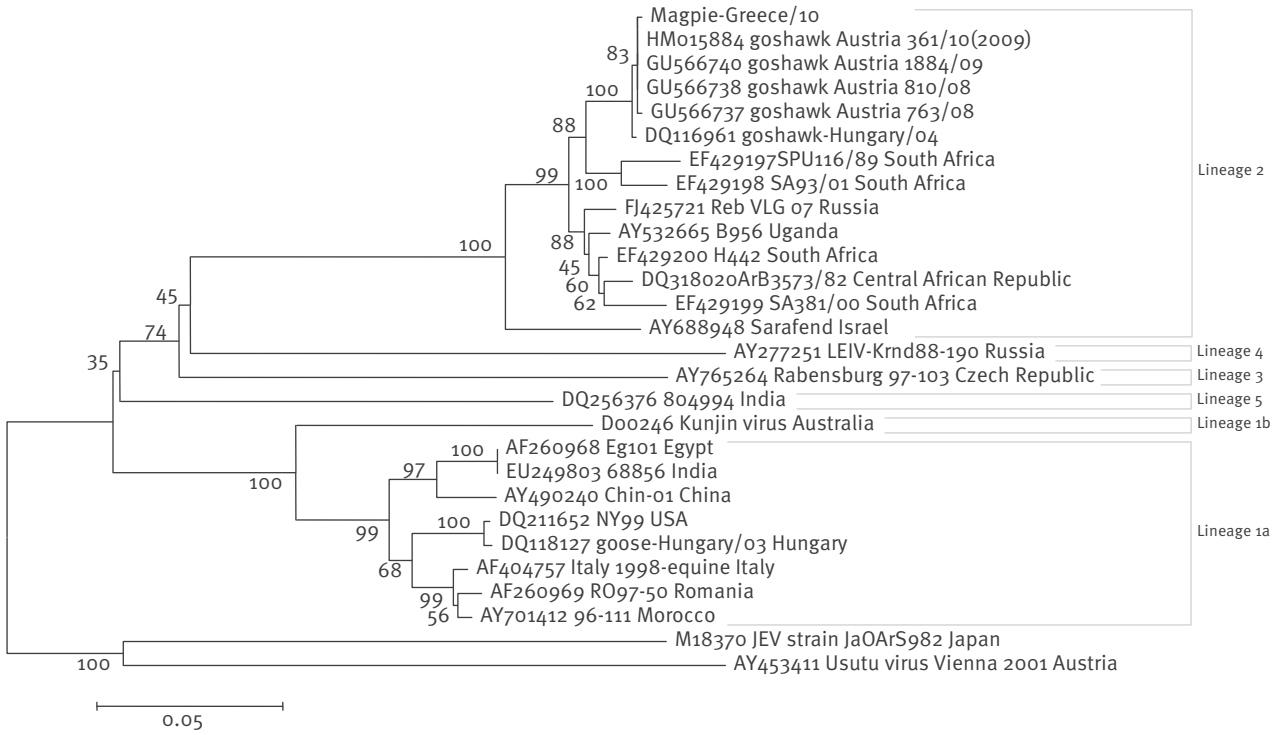
Figure 2

Phylogenetic tree of West Nile Virus strains based on nt sequences of the NS5 genomic region



The sequence from the present study is shown in bold.
The sequences used to derive the phylogenetic tree were 797 nt long.
GenBank accession numbers and geographic origins of strains are shown.
Bootstrap values (in per cent) are represented at each tree node.

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Evidence of enzootic circulation of West Nile virus (Nea Santa-Greece-2010, lineage 2), Greece, May to July 2011

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A West Nile virus (WNV) surveillance network including sentinel chickens was deployed in Thessaloniki county, Greece, from May to July 2011. For the first time in summer 2011, a chicken WNV isolate from 6 July was molecularly identified. The partial NS3 sequence was identical to that of the Nea Santa-Greece-2010 WNV lineage 2, detected in central Macedonia in 2010. This suggests that WNV is actively circulating in central Macedonia and that it may have overwintered in northern Greece.

During 2010, Greece underwent the second largest West Nile virus (WNV) epidemic in Europe in the last two decades with 262 clinical human cases and 35 fatalities [1]. WNV lineage 2 was identified in two pools of *Culex* mosquitoes (Nea Santa-Greece-2010 virus) [2] and in wild birds [3] that were sampled during the epidemic season of 2010 from areas in close proximity to human cases.

No active vector and arbovirus surveillance system was in place in Greece before the epidemic in 2010. We initiated a monitoring programme in 2011, from May to November, in order to understand subsequent transmission, to document virus activity, and to better assess the relative importance of vector species. A small scale mosquito and animal surveillance network was established in the county of Thessaloniki, one of the areas with the highest number of human cases during the epidemic of 2010 [1]. The long term objective of this project is to design within the following years an optimum, large scale arbovirus surveillance programme for Thessaloniki. We report here preliminary findings of the study that will have interest for public health authorities.

Methods

Sentinel chickens

Six chicken flocks (six chickens per flock) were placed in stationary cages along the western and eastern edges of Thessaloniki (three flocks on each side) to monitor WNV activity in areas suitable for potential enzootic transmission [4] (Figure 1). These areas combine abundance of mosquito larval sites (e.g. rice fields) and potential habitat for migratory birds (e.g. Axios River delta) that may serve as reservoir populations for WNV. The flocks were placed within or in close proximity to residential communities that experienced abundant mosquito activity. All chickens were confirmed WNV antibody negative prior to placement in the field. For each flock, the chicken cage was divided in six compartments so that each chicken would be kept separate from the others. Chickens were bled through the ulnar vein weekly (about 1ml of blood sample per chicken).

Mosquito population monitoring

Carbon-dioxide (dry ice) baited Centers for Disease Control (CDC) light traps (John W. Hock, Gainesville, United States (USA)) were deployed once a week at 28 sites in the Thessaloniki area beginning 20 May 2011 (Figure 1). Traps were located at approximately equal intervals in order to provide a geographically representative sampling.

Laboratory analysis

Chicken plasma (0.5 ml) and sera (0.25 ml) were collected for virus detection and serology, respectively. Serum samples were tested by ELISA for the detection of WNV-specific antibodies using a commercial ELISA kit (ID Screen West Nile Competition, IDVET, France). After the detection of seroconversion, RNA was extracted from selected plasma samples previously taken from the seroconverted birds. RNA extracts were examined using a one tube RT-PCR screening protocol employing a primer pair (WNPolUp: 5'-TTTTGGGAGATGGTGGATGARGA-3' and WNPolDo2: 5'-CCACATGAACCAWATGGCTCTGC-3') designed for the specific detection of WNV and targeting a 144 bp part of the nonstructural protein 5 (NS5) gene. Samples found positive by the RT-PCR screening protocol, were additionally subjected to RNA reverse transcription using random hexamers, followed by two PCR assays employing a primer pair (WN-NS3up1: 5'-GCTGGCTTCGAACCTGAAATGTTG-3' and WN-NS3do1: 5'-CAATGATGGTGGGTTTCACGCT-3')

targeting a 778 bp part of the nonstructural protein 3 (NS3) gene, and a nested primer pair (WN-NS3up2: 5'-GCAAGATACTTCCCCAAATCATCAAGG-3' and WN-NS3do2: 5'-TGTCTGGGATCTCTGTTTGCATGTC-3') targeting a respective 423 bp part. The nested PCR products were bidirectionally sequenced. The NS3 gene was selected for molecular characterisation because it is phylogenetically informative [5] and it encodes a protein residue (NS3-249) subject to adaptive evolution leading to increased viremia potential and virulence [6].

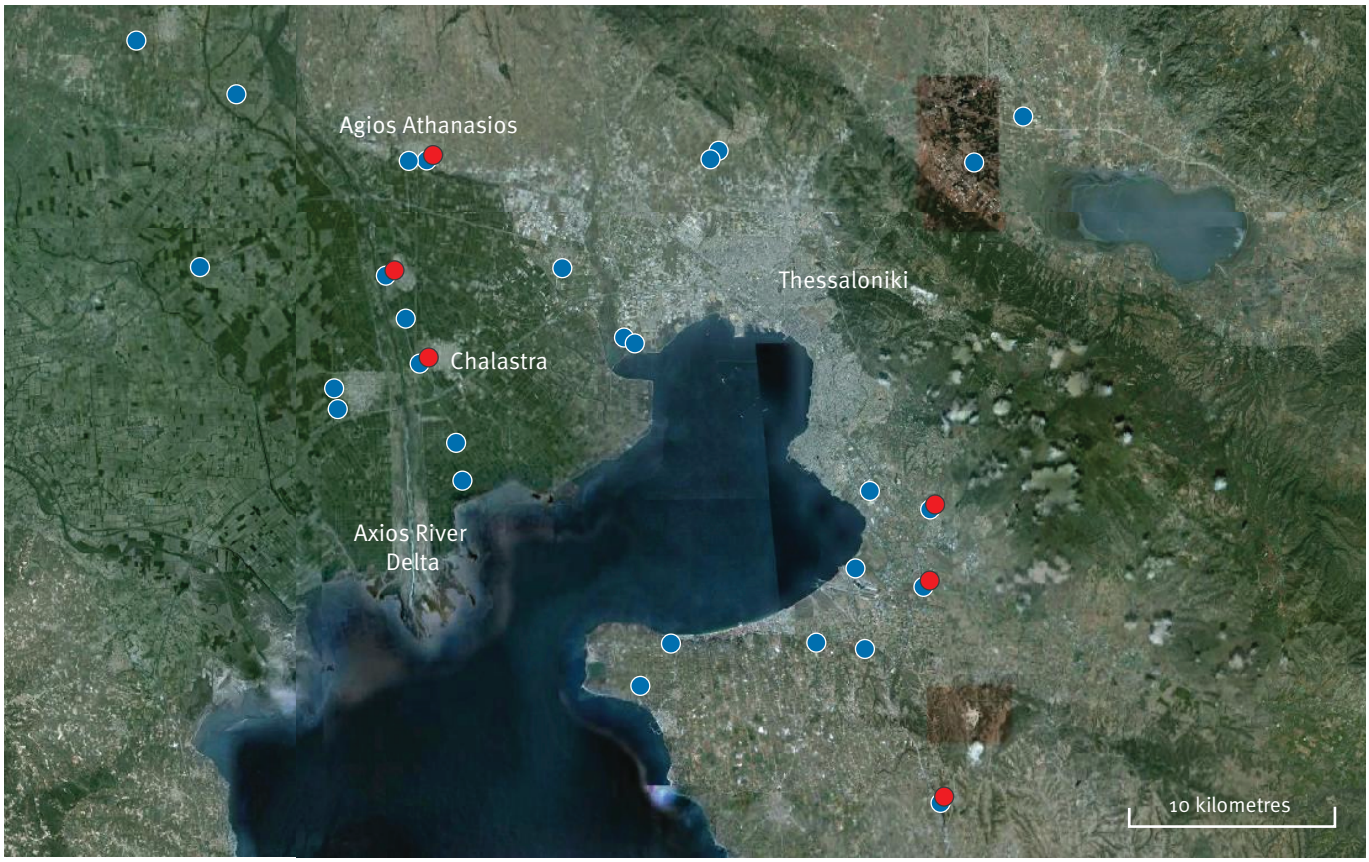
Results

Seroconversion of the first sentinel chicken was detected in the agricultural area of west Thessaloniki in the city of Chalastra (40°37'37.27"N, 22°43'45.05"E) (Figure 1) on 29 June. On 13 July a second chicken seroconversion was detected in the city of Agios Athanasios (40°43'0.59"N, 22°44'7.04"E), followed by a third chicken seroconversion on 20 July in the same area.

All prior samples collected from the three seroconverted chickens were tested using the one tube RT-PCR screening protocol targeting NS5. All RNA samples were negative except in the case of the sentinel chicken in the city of Agios Athanasios (40°43'0.59"N, 22°44'7.04"E) which seroconverted on 13 July. More specifically, a band of expected size was obtained from one PCR product derived from a sample taken from that respective chicken, one week before seroconversion (6 July). The specific RNA extract was subjected to nested PCR, targeting the partial NS3 gene sequence, which was subsequently determined. The sequence was deposited in GenBank database under accession number JN398476 and according to BLAST algorithm, it presented highest nucleotide sequence identity (99.73%) to that from Nea Santa-Greece-2010 virus derived from a *Culex* mosquito pool tested during the 2010 epidemic in Central Macedonia [7]. The inferred partial NS3 amino acid sequence was 100% identical to that of the Nea Santa-Greece-2010 WNV lineage 2. As in the Nea Santa-Greece-2010 virus NS3 sequence, the inferred NS3 residue 249 was determined to be proline, similar to several neuroinvasive lineage 1 WNV strains [6]. In contrast, all other investigated lineage 2 viruses have a NS3 protein with a histidine at this position [7].

Figure 1

Location of mosquito traps (n=28) and sentinel chicken flocks (n=6) for West Nile virus surveillance, Thessaloniki county, Greece, 2011



Mosquito trap location
Chicken flock location (collocated with mosquito trap)

Agios Athanasios and Chlalastra are the two cities where enzootic circulation of the virus was detected.

The cumulative number of *Culex* mosquitoes trapped weekly in the agricultural area of Thessaloniki (Figure 2) was low (n=142) during the last week of May and the first two weeks of June. The population rapidly increased during the second half of June, with a peak (n=23,867) at the end of the month. During the following two weeks, the population decreased and then started building up again during the third week of July. So far, the most prevalent mosquito species in both residential and agricultural areas (rice-fields) were *Culex pipiens* followed by *Culex modestus* Ficalbi. It should be noted that *C. modestus* populations started building up significantly in early July. Testing for WNV in mosquitoes is in progress but no results are available at this stage of the surveillance programme.

Discussion

This is the first report of enzootic circulation of WNV Nea Santa-Greece-2010 in Greece during 2011, one year after the WNV epidemic in Greece. The virus in 2010 was detected in Nea Santa from a *Culex* mosquito pool [2], and in 2011 we detected an identical isolate (molecular characterization based on NS3 gene) in the agricultural area of west Thessaloniki in the city of Agios Athanasios, approximately 21 km southwest of Nea Santa. The 2011 Greek WNV isolate shows close genetic relationship to the lineage 2 goshhawk-Hungary-2004 strain that emerged in Hungary in 2004 but differs from the latter in that it maintains the amino acid substitution H249P found in the Nea Santa-Greece-2010 isolate, which may be associated with increased virulence [7]. WNV lineage 1 strains are distributed in north Africa, Europe, America, Asia and Australia, whereas lineage 2 are mostly distributed in south Africa and Madagascar. Due to increased illness and death caused by WNV lineage 1 compared to lineage 2 in the past, lineage 2 strains were previously considered to be less virulent. However, recent evidence from Africa and Hungary demonstrated that lineage 2 strains may also result in severe disease [8,9].

Up to now, no WNV genomic sequences have been published from the human cases during the 2010 epidemic and there is no direct evidence to incriminate the WNV Nea Santa-Greece-2010 strain as the cause of the 2010 human epidemic. The discovery of the same strain in sentinel chickens in 2011 suggests that the virus was able to overwinter in this region, consistent with current opinion on the endemicity of WNV in Europe [10]. Specifically, the reoccurrence of WNV in continuous years in the same places in Romania and Italy, involving humans and equines, is likely linked to the endemicity of the infection in the areas rather than to a new introduction of the virus [10]. This situation appears to parallel that experienced in California, USA, which has a similar climate (warm temperate, seasonal winter rainfall) [11], where WNV was introduced in 2003, quickly spread throughout the state, and became endemic with the ability to overwinter in a cycle between winter mosquitoes and birds.

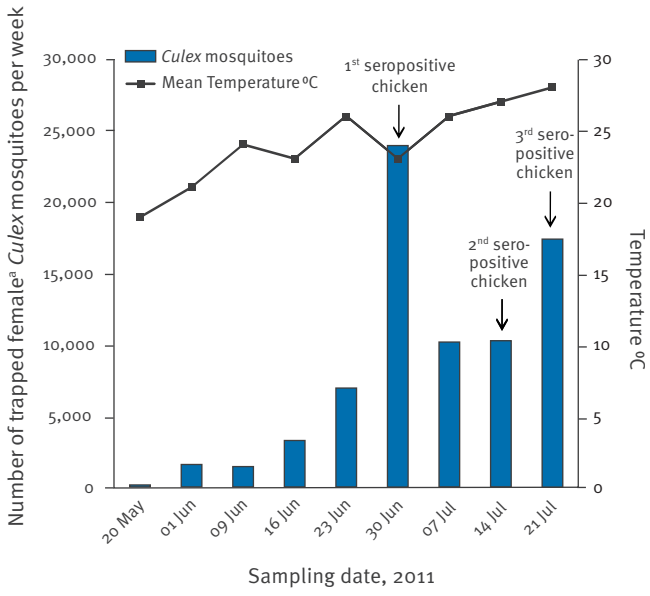
Transmission in the sentinel chickens occurred immediately after the first significant *Culex* population peak, as has been observed in WNV outbreaks [11,12]. Two of the principal WNV vectors in Europe, *C. pipiens* and *C. modestus* [13], are highly abundant in the agricultural area of Thessaloniki and both species may be associated with the transmission of the virus. In Greece, so far, the virus has been isolated from two pools of *C. pipiens* mosquitoes during the epidemic of 2010 [2]. More studies are needed to increase our knowledge on the role of the aforementioned species in the enzootic, epizootic and tangential (e.g. to humans) transmission of WNV in Greece.

Monitoring disease activity by using sentinel animals can provide critical information regarding periods of increased transmission. Surveillance networks involving sentinel animals and mosquitoes have been used in many parts of the world as an early warning system aiming to identify periods and locations of elevated risk of WNV disease transmission [14,15,16]. The rationale behind these surveillance networks is (i) to increase our understanding of the epidemiology of arboviruses, (ii) to identify the circumstances favourable to the appearance of the disease in humans before this occurs, and (iii) to guide mosquito control efforts in time and space to reduce the impact or likelihood of an epidemic.

Arbovirus surveillance systems can be expensive and labour intensive, with weekly monitoring of chickens. This is nevertheless feasible and these systems have been successfully established in some regions [16]. In urban centres of increased vulnerability to mosquito

Figure 2

Culex female mosquito abundance in the agricultural area of west Thessaloniki, Greece, 2011 (n=74,909)



[a] Female mosquitoes were targeted by mosquito traps and accidental male mosquito collection was negligible (<0.1%).

borne epidemics, such as Thessaloniki (approximately 1 million inhabitants, in close proximity to prolific mosquito breeding environments), there is a need and demand for such systems and the benefits associated with their successful deployment outweigh the associated costs. This is the first active arbovirus surveillance system in place in Thessaloniki and in order to optimise its use, extensive data are required in the following years. These data could help create a useful disease surveillance tool that may increase our understanding of the disease transmission cycle and help the local authorities to design a local WNV response plan based on the disease transmission levels. It is encouraging, that through a small scale surveillance system, like the one described in this paper, we were able to detect WNV enzootic circulation in Greece before the onset of any human cases for the year of 2011.

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Ongoing outbreak of West Nile virus infection in humans, Greece, July to August 2011

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Between 16 July and 21 August 2011, 31 cases of West Nile neuroinvasive disease were reported from four regions in Greece. Of these, 17 occurred in districts that had not been affected in 2010. The reoccurrence of human cases in two consecutive years (following the large 2010 outbreak) and the spread of the virus in new areas suggest that West Nile virus is established in Greece, and its transmission may continue to occur in the future.

Since July 2011, an outbreak of West Nile virus (WNV) infection has been ongoing in regions in Greece that had already been affected in 2010, and in regions that had never reported human cases before. Here we present information on the extent of the ongoing outbreak and describe the geographical and temporal distribution of West Nile neuroinvasive disease (WNND) cases.

During 2010, Greece experienced the second largest outbreak of WNV infections in Europe since the one that had occurred in Romania in 1996 [1-3]. Overall, 262 cases of WNV infection in humans were notified mainly in northern Greece. In the Central Macedonia region all seven districts had reported cases and in the adjacent Thessalia region one of the four districts was affected. Among reported cases, 197 presented with WNND and 33 of these died, indicating a case fatality rate of 17% among WNND cases [1]. WNV lineage 2 sequences were obtained from three pools of *Culex* mosquitoes (strain Nea Santa-Greece-2010), from two blood donors and from wild birds [4-6]. This was the first time that WNV infection was documented in humans in Greece, although serosurveys in the early 1960's, 1980's and 2007 identified WNV antibodies in approximately 1% of the human population, suggesting that WNV, or a related flavivirus, was circulating in Greece [7-9].

Surveillance methods

As part of the general surveillance system, physicians in Greece are asked to notify the Hellenic Centre for Disease Control and Prevention (HCDCP) of all cases of WNV infection, using a slightly modified 2008 European Union (EU) case definition (the same as the one used in 2010) [2,10]. A confirmed case is defined as a person meeting any of the following clinical criteria: encephalitis, meningitis, fever without specific diagnosis and at least one of the four laboratory criteria: (i) isolation of WNV from blood or cerebrospinal fluid (CSF), (ii) detection of WNV nucleic acid in blood or CSF, (iii) WNV-specific antibody response (IgM) in CSF, and (iv) WNV IgM high titre, and detection of WNV IgG, and confirmation by neutralisation. A case is considered probable if the patient meets the above clinical criteria and a WNV-specific antibody response is demonstrated in his or her serum sample.

A standardised reporting form is used to collect information regarding the demographic characteristics, clinical manifestations, underlying chronic medical conditions, potential risk factors and laboratory results of the reported cases. Regular telephone inquiries to hospitals in the affected areas are conducted for case finding, follow-up and data validation. In addition, in-depth telephone interviews are conducted using a semi-structured questionnaire to obtain a detailed exposure history of all cases. Cases reported as encephalitis (including meningoencephalitis), meningitis, or acute flaccid paralysis, are classified as WNND cases. All other cases are considered non-neuroinvasive.

Laboratory methods

Serum and CSF specimens were tested for the presence of WNV-specific IgM and IgG antibodies using commercial ELISA kits (WNV IgM capture DxSelect and WNV IgG DxSelect, Focus Diagnostics Inc, Cypress, CA, USA). WNV positive specimens were also tested for the presence of other flaviviruses: tick-borne encephalitis virus (TBEV) and dengue virus (DENV).

Data analysis

Incidence rates were calculated using as denominator the 2007 mid-year population estimates of the Hellenic

Statistical Authority (HSA) [11]. Comparison of categorical variables was assessed using the chi-squared test. Risk ratios (RR) were calculated to compare incidence rates. The analysis was carried out using STATA version 10 software (Stata Corporation LP, Texas, USA). Data were mapped using the GNU R software (www.gnu.org/s/r/).

Results

By 21 August (week 33), 37 laboratory-diagnosed cases of WNV infection were reported to the HCDCP; 31 of these (24 confirmed and seven probable) presented as WNND cases and six of them (all probable) as non-neuroinvasive. This report focuses mainly on the 31 WNND cases, which were identified and reported more consistently, because of the disease severity. The overall incidence of WNND in the country was 0.28 cases per 100,000 population (Table).

For the 37 laboratory confirmed cases, 31 serum samples and 25 CSF specimens were available; for 19 patients both CSF and serum specimens were provided, while for six patients only CSF was available. WNV-specific IgM antibodies were detected in all 31 serum samples and in 24 CSF specimens, while WNV-specific IgG antibodies were detected in 15 serum and eight CSF specimens. In all 19 patients for whom both types of specimen were available, WNV-specific IgM antibodies were detected in both CSF and serum. As was the case in 2010, all specimens were negative for TBEV, while low level of cross-reactivity was seen in IgM with DENV [12]. None of the patients had been vaccinated for yellow fever.

The first case of WNND reported onset of symptoms on 16 July 2011 (week 28) (Figure 1). An increased number of cases was observed during weeks 30 and 32.

The median age of patients with WNND was 70 years (range 21–87) with the age-specific attack rate of WNND increasing significantly (p=0.009) with increasing age. The incidence in persons aged 70 years or older was approximately 23 times higher compared to that of individuals younger than 30 years (Table). Of all WNND cases, 19 were male. The place of residence of WNND cases is presented in Figure 2.

None of the cases reported travel abroad during the incubation period. The first cases occurred in northern Greece in Central Macedonia and Thessalia region, whereas approximately 10 days later, cases were reported for the first time from Eastern Attiki (in close proximity – approximately 43 km – to the metropolitan area of Athens). Overall, cases were distributed throughout nine of 54 Greek districts in four of 13 Greek regions. Of all WNND cases, 17 occurred in areas where cases had not been documented in 2010 (namely Karditsa and Trikala in Thessalia region, and Eastern Attiki and Viotia, in Central Greece). None of the cases had a history of recent blood transfusion or tissue/organ transplantation.

Table

Basic characteristics of reported cases of West Nile neuroinvasive disease, Greece, 16 July – 21 August 2011 (n=31)

Characteristic	Number of cases	Incidence rate[a] (per 100,000 population)	Risk Ratio (95% confidence interval)
Age group (years)			
<30	2	0.05	reference
30-49	2	0.06	1.10 (0.15–7.78)
50-59	3	0.21	3.91 (0.65–23.41)
60-69	5	0.42	7.77 (1.51–40.03)
≥70	19	1.26	23.30 (5.43–100.04)
Sex			
Female	12	0.21	reference
Male	19	0.34	1.61 (0.78–3.33)
District/prefecture (region) of residence			
Karditsa (Thessalia)	5	4.30	12.26 (3.29–45.66)
Eastern Attiki (Attiki)	10	2.48	7.05 (2.21–22.49)
Serres (Central Macedonia)	3	1.59	4.54 (1.02–20.27)
Larissa (Thessalia)	4	1.40	3.99 (1.00–15.95)
Imathia (Central Macedonia)	2	1.39	3.95 (0.72–21.58)
Viotia (Sterea Ellada)	1	0.80	2.27 (0.25–20.32)
Trikala (Thessalia)	1	0.77	2.18 (0.24–19.50)
Pella (Central Macedonia)	1	0.69	1.96 (0.22–17.57)
Thessaloniki (Central Macedonia)	4	0.35	reference
Total	**31**	**0.28**	

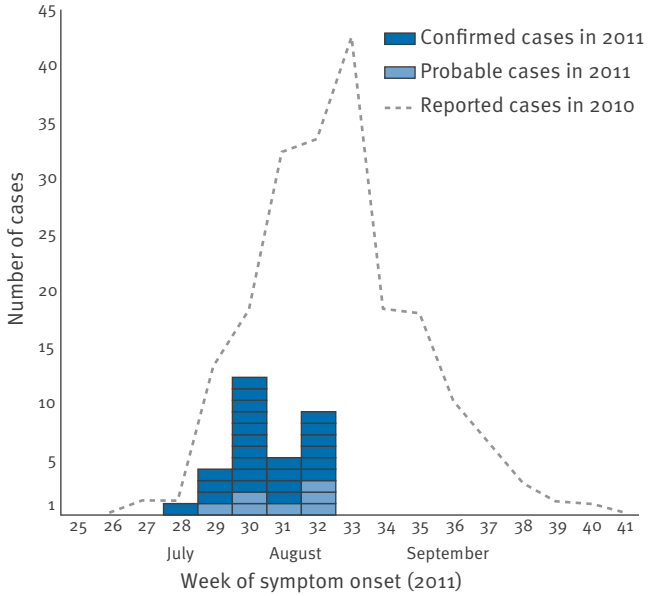
[a] Incidence rates were calculated using as denominator the 2007 mid-year population estimates of the respective groups available from the Hellenic Statistical Authority.

Of all WNND cases, 22 presented with meningoencephalitis and nine with meningitis alone. Two patients presented with a combination of meningoencephalitis and acute flaccid paralysis. Information on underlying chronic medical conditions was available for 23 of the WNND cases: 17 had at least one underlying disease, with the most common being hypertension (n=11), diabetes mellitus (n=7) and coronary artery disease (n=6). All WNND cases were hospitalised and six were admitted to an intensive care unit (ICU). As of 21 August 2011, one case (aged over 70 years) who had several underlying conditions, had a fatal outcome.

There were also six non-WNND cases reported, who probably represent a very small fraction of all non-WNND, as mild WNV cases are less likely to seek medical care and be identified. In depth interviews were conducted with all of them. The median age of the reported non-WNND cases was 44 years (range 10-78) and was significantly different (p=0.009) from that of non-WNND ones (median age 70 years; range 21-87). Of those, five were hospitalised but none in an ICU. With regard to specific symptoms, fever was reported by all of them, followed by headache (n=3), rash (n=2), weakness (n=2) and nausea/vomiting or diarrhoea (n=1).

Figure 1

Reported cases of West Nile neuroinvasive disease by week of symptom onset, Greece, 16 July – 21 August 2011 (n=31)

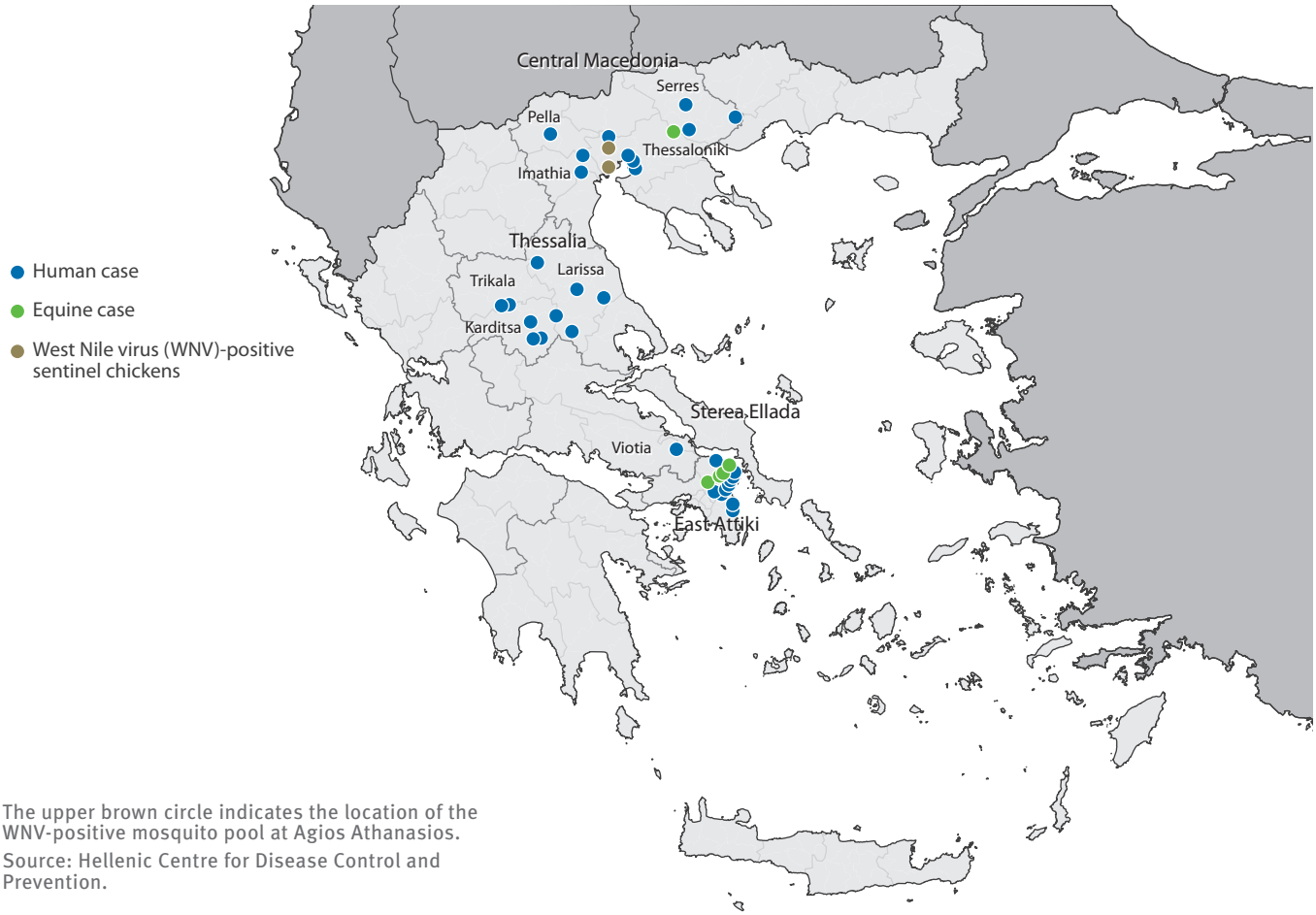


As this is an ongoing outbreak, the number of 2011 cases corresponding to previous weeks may increase as more cases are confirmed retrospectively.

Source: Hellenic Centre for Disease Control and Prevention.

Figure 2

Place of residence of reported cases of West Nile neuroinvasive disease, Greece, 16 July – 21 August 2011 (n=31)



The upper brown circle indicates the location of the WNV-positive mosquito pool at Agios Athanasios.

Source: Hellenic Centre for Disease Control and Prevention.

Discussion and conclusions

Following the large WNV outbreak in 2010, WNV human infections are notified in Greece for a second consecutive year. As of 21 August, 31 WNND were reported from four regions in Greece. Human cases of WNV infection were also detected in other European and Mediterranean countries during this season (as of 18 August, two cases had been reported in Romania, 21 in the Russian Federation, two in Albania, and five in Israel) [13].

The 2010 outbreak in Greece was mainly localised in Central Macedonia. Although cases seem to reoccur in the same districts as in 2010, a new geographic pattern of WNV spread is being observed in 2011. Following the intense WNV amplification and transmission in Central Macedonia in the previous year, the virus seems to disperse southward to the newly affected areas of Thessalia region (Karditsa) and further south to East Attiki (approximately 500 km from Central Macedonia), in proximity to the metropolitan area of Athens. Similar dispersal patterns have been observed in California, which has a similar climate to Greece, where WNV was introduced in 2003 and quickly spread throughout the state [14,15].

The temporal distribution of cases shows that the first human cases in 2011 occurred approximately two weeks later compared to 2010. Comparing the magnitude of the current outbreak in Central Macedonia to the outbreak in the previous year, the current one remains lower to date, suggesting decreased or delayed WNV transmission in humans. Due to the high visibility of the 2010 outbreak and the subsequent raised awareness to the infection among physicians, it is unlikely to be due to delayed recognition of the disease or under-reporting. However, as this is an ongoing outbreak, the number of 2011 cases corresponding to previous weeks may increase as more cases are confirmed retrospectively.

In early May and June 2011, WNV transmission was detected by seroconversions of sentinel chickens and domestic pigeons in Central Macedonia [15]. Lineage 2 WNV sequences were obtained from one pool of *Culex pipiens* mosquitoes trapped in the city of Agios Athanasios (40º43'0.59''N, 22º44'7.04''E) west of Thessaloniki on 23 June 2011. This strain showed the highest homology (99.4%) to the Nea Santa/Greece/2010 WNV strain detected in *Culex pipiens* in 2010. An identical strain was also detected in seroconverted sentinel chickens in the same city on 13 July 2011 [15]. Genetic characterisation of WNV strain(s) of 2011 circulating in other areas will elucidate whether it is an identical strain or a newly introduced one.

Regarding equidae, five horses from East Attiki presented with clinical WNV disease manifestation, as well as one horse from Serres (Central Macedonia) with antibodies against WNV that demonstrated a recent infection (IgM positive) (Figure 2) [16]. The first two cases in horses were identified before human cases had been reported in Attiki and thus functioned as an early warning signal.

Following the large 2010 WNV infection outbreak, a number of public health measures were implemented in 2011:

- guidelines for healthcare professionals for the recognition, management and diagnosis of encephalitis and WNV infection in order to improve their awareness regarding the disease;
- enhanced surveillance of encephalitis and WNV infection in humans;
- a project on mosquito mapping across the country;
- a project on mosquito surveillance;
- a seroprevalence study of WNV infection among humans in the epicentre of the 2010 WNV infection outbreak;
- a WNV seroepidemiological study in domestic pigeons and poultry;
- multi-sectoral collaboration and exchange of information between human health, veterinary health and entomological sectors;
- guidance for blood and blood product safety according to the EU directives;
- communication and health promotion activities encouraging personal protection against mosquito bites in the general population;
- vector control activities.

In conclusion, the reoccurrence of human WNV infection cases in two consecutive years and the spread of the virus in newly affected areas, suggest that WNV is established in Greece and transmission may continue in the future. Intensified vector mosquito control programmes, along with ongoing public health education, integrated human and animal WNV surveillance to monitor the spread of the virus and implementation of blood transfusion measures are necessary to prevent transmission and control the disease.

Updates on reported WNV cases in Greece are published in the Weekly Surveillance Reports available in English on the HCDCP website [17].

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West Nile virus: the Italian national transplant network reaction to an alert in the north-eastern region, Italy 2011

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We report four cases of West Nile virus (WNV) transmission following a single multiorgan donation in north-eastern Italy. The transmissions were promptly detected by local transplant centres. The donor had been tested for WNV by nucleic acid amplification test (NAT) prior to transplantation and was negative. There were no detected errors in the nationally implemented WNV safety protocols.

Case reports

In August 2011, a multiorgan and tissue retrieval was carried out in north-eastern Italy from a donor who was a resident in the same area. The donor's organs (kidneys, lungs, heart and liver) were successfully transplanted to recipients in other Italian regions, including the north-eastern region. The donor's health status was confirmed prior to donation, by blood- and instrumental-tests and detection of markers for transmissible diseases (hepatitis B surface antigen, hepatitis C virus antibodies, human immunodeficiency virus 1/2 antibody, *Treponema pallidum* Particle Agglutination Assay), in addition to interviews with relatives. In line with transplant procedures, the donor cause of death was not related to any transmissible disease. Moreover, due to special procedures in place for prevention of West Nile virus (WNV) in this part of Italy, a donor blood sample had tested negative for WNV by nucleic acid amplification test (NAT).

Ten days after transplantation, two patients who had each received a respective kidney, developed fever and neurological symptoms, suggestive of West Nile neuroinvasive disease. The purpose of this rapid communication is to describe how, despite testing strategies in place for WNV, transmissions occurred and how the Italian National Transplant Network responded to the WNV transmissions associated with a multiorgan transplant, in the context of negative nucleic acid amplification test (NAT) results in the donor.

Background

Due to WNV circulation and documented infections in humans in north-eastern Italy [1], several preventive measures related to WNV transmission to humans have been implemented. Since 2008, the Italian National Transplant Network, in collaboration with the regional health authorities, started an epidemiological surveillance programme in order to detect WNV in organ donors in north-eastern Italy [1-3]. Moreover, in the same area, plans are in place in the medical and veterinary fields for active surveillance and monitoring of WNV infection in animals and humans [4-7]. In addition to this epidemiological monitoring, the Italian National Transplant Network decided to perform NAT within 72 hours of donation on all donors living in areas where WNV had been demonstrated to be endemic [1,7,8]. These measures are carried out from 15 July to 15 November 2011 in order to prevent WNV transmission from organ and/or tissue donations to recipient patients.

Laboratory investigations and control measures

On the basis of time schedules foreseen by rules and protocols issued for prevention of WNV (within 72 hours from donation) [1], virological testing was carried out on the blood sample collected before donor death by the virology laboratory of Padua University, using a NAT technique (cobas TaqScreen West Nile Virus Test – Roche). No signs of fever or malaise had been documented in the week prior to donation. The result of the test on the donor was negative. About ten days after transplant, two transplant centres reported to the Italian National Transplant Centre suspected neurological symptoms in patients who had received a kidney

transplant from this donor. Between four and five days after transplantation, both kidney recipients had developed fever and ongoing encephalitis, symptoms compatible with WNV neuroinvasive disease [9,10]. The WNV NAT test performed with the same technique as with the donor, on both patients resulted positive (blood and urine samples). Following a protocol that had been successfully used in similar situations [11,12], high titre West Nile intravenous immunoglobulin was only administrated to one of the two kidney recipient, since the other one had already produced anti-WNV antibodies. After the reports of suspect symptoms in the two kidney recipients, virological tests on donor materials were repeated again using the NAT technique (cobas TaqScreen West Nile Virus Test – Roche) by the virology laboratory at the National Institute for Infectious Disease "L. Spallanzani" in Rome. The negative initial test result was confirmed, whereas serological tests showed the presence of anti-WNV antibodies (immunofluorescence assay – Euroimmun Italia) (Table). After this, NAT and serological tests were performed on the further three organ recipients who had received heart, lung and liver from the same donor. The NAT results for the heart and liver recipients were negative. The NAT result of the lung recipient was positive.

Thirty seven days after transplantation, one of the kidney recipients was more critically ill than the other kidney recipient; investigations on a possible link between the severity of the clinical condition and a genetic disease affecting the first patient are ongoing. Also at 37 days after transplantation, the NAT test- negative liver and heart recipients were in good health, while the lung recipient, who tested positive for WNV, presented neurological symptoms that can possibly be ascribed to immunosuppressive therapy toxicity.

As soon as it was suspected that WNV transmission from the donor could have occurred in the organ recipients, further use of all remaining tissues from the donor was stopped.

Conlusion

When the first report of symptoms indicating suspected transmission of WNV from donor to recipient was detected ten days after the transplantation, the Italian National Transplant Network promptly followed all communication and clinical protocols. First, the other transplant centres where the three recipients of heart, lungs and liver had been operated were alerted. At the same time, the National Transplant Centre and the Interregional Centre of competence, in cooperation with a national expert on infectious diseases (in charge of giving a "Second Opinion" on particular donation case) agreed and coordinated the clinical measures to be put in place to prevent further transmission and to insure adequate managing and care of the organ recipients. In particular, we took all therapeutic measures currently available for WNV, using stocks of plasma collected from donors positive for antibodies to WNV as a result of infections recorded in 2008 and 2009 in the north-east of our country. As no errors in safety protocols pre-donation occurred, it is assumed that virus concentration in the donor was not sufficient to be detected by the NAT technique.

The rapidly available test results and traceability of materials allowed prevention of further use of all remaining tissues from the donor. Testing the donor sample, earlier than within the required 72 hours post-donation, would not have been useful because of the likely low-level viraemia in the donor. It is however necessary to follow recommendations given in 2010 by the Italian Higher Health Council [13], that advised to screen donors by testing for viral RNA by the NAT technique within 72 hours of donation. This measure should be enhanced by the search for antibodies which should be carried out in a limited number of references laboratories, so as to ensure high quality standards. Clearly, traceability of donor organs through a national transplant network is crucial to facilitate tracing back to the donor also to other recipients of the latter, and to allow the study of suspected transmissions. In our case, rapid detection of the viral transmission facilitated the prevention of further transmissions to other tissue recipients.

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Table

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	NAT test result	Antibodies determination
Donor	Negative on blood	Positive (IgG and IgM) on blood
First kidney recipient	Positive on blood and spinal fluid	Positive (IgG and IgM) on blood and spinal fluid
Second kidney recipient	Positive on blood and spinal fluid	Positive (IgG and IgM) on blood and spinal fluid
Heart recipient	Negative on blood	Negative on blood
Liver recipient	Negative on blood	Positive (IgG and IgM) on blood
Lung recipient	Positive on blood	Positive (IgG and IgM) on blood

NAT: Nucleic acid amplification test.

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Human case of autochthonous West Nile virus lineage 2 infection in Italy, September 2011

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On 10 September 2011, a patient in his 50s was admitted to hospital in Ancona, Italy, after six days of high fever and no response to antibiotics. West Nile virus (WNV) infection was suspected after tests to determine the aetiology of the fever were inconclusive. On 20 September, WNV-specific IgM and IgG antibodies were detected in the patient's serum. Genomic sequencing of the viral isolate showed that the virus belonged to WNV lineage 2.

Case report

On 4 September 2011, a man in his late 50s in Ancona, Italy, first became unwell, with general malaise and fever (body temperature higher than 39.0 °C). For these reasons, his general practitioner (GP) prescribed antibiotics, but as the patient's fever persisted after six days of treatment, he was admitted to hospital (Infectious Disease Unit of the Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona). On 10 September, the ward physician reported that the origin of the fever was unknown and that there were no pulmonary or other organ-specific symptoms, except the persistence of a general malaise. A chest examination was normal, as were the laboratory tests (haemocultures, urine cultures, haemocytometer analysis, liver and renal biochemical tests, erythrosedimentation rate, C-reactive protein and blood electrolytes). He did not have neuroinvasive disease. He was discharged on 27 September and has completely recovered.

The patient was a local fisherman who lived in Ancona, close to the harbour on the Adriatic coast. In the month before his symptoms began, he had neither travelled outside the Marche region (where Ancona is the main port) in central Italy, nor had he been at sea.

Virological analysis

Tests on a blood sample taken from the patient on 13 September (tested at the Ancona Virology Laboratory) excluded cytomegalovirus, Epstein–Barr virus, human immunodeficiency virus and Toscana virus as the cause of the fever.

On 20 September, the same sample was also tested for West Nile virus (WNV), based on a recent protocol adopted by the laboratory for differential diagnosis of meningoencephalitis and for patients with fever of unknown origin in the summer months: WNV serological tests are performed in all patients negative for Toscana virus infection. The sample was found to be positive for WNV-specific IgM and IgG (with index values (signal/cut-off ratio) of ›5.4 and 2.0, respectively) using the IgM capture DxSelect ELISA and IgG DxSelect ELISA kits (Focus Diagnostics, United States) (Table).

Following the positive ELISA results for WNV, RNA extracted from serum (collected on 13 September) and urine (collected on 21 September) was reverse transcribed and amplified by PCR using an in-house assay that uses degenerate primers designed to recognise a region in the NS5 gene that is conserved (on the basis of an alignment of database sequences) in most animal and human *flaviviruses*. In the case of WNV, the resulting PCR product is 273 bp long using the forward primer (5'-TGCATITWCAACATGITGGG-3') and reverse primer (5'-GTRTCCCAICCIGCIGTGTCATC-3').

The patient's serum sample tested negative, while the urine sample was positive. Sequencing the amplified product – in both forward and reverse directions with the same primers used for amplification – showed that the WNV NS5 gene sequence had been amplified.

BLAST analysis of the 273 bp sequence showed highest homology (100%) to the Nea Santa/Greece/2010 WNV strain detected in *Culex pipiens* in 2010 [1] as well as to other strains belonging to WNV lineage 2.

The full genomic sequence of the virus was subsequently obtained from RNA extracted from the patient's

urine sample of 21 September: this confirmed that the virus was lineage 2. It also showed 99% identity to the complete genome of isolate goshawk-Hungary/04 (10, 380 of 10, 423 nucleotides identical and no gaps) and to the more recent Nea Santa-Greece-2010 (10, 374 of 10,423 nucleotides identical and no gaps) [1-2]. The sequences obtained were submitted to GenBank: the accession numbers are JN797253 (NS5 fragment) and JN858070 (complete genome). The case was notified to the regional health authorities on 23 September and to the Istituto Superiore di Sanità on 26 September.

Another serum sample collected from the patient on 26 September showed an increased WNV-specific IgG antibody titre and a high level of WNV-specific IgM. Plasma collected on the same day was negative for WNV by RT-PCR.

The serum samples collected on 13 and 26 September and the urine sample collected on 21 September were tested at the National Reference Laboratory for WNV surveillance at the Istituto Superiore di Sanità, which confirmed the diagnosis by amplification of a different region of the NS5 gene [3].

Urine and plasma samples collected on 28 September were also tested at the National Institute of Infectious Diseases L.Spallanzani, which further confirmed the diagnosis by amplification of WNV sequences in urine but not in plasma using the cobas TaqScreen West Nile Virus Test (Roche Molecular Diagnostics, United States). The plasma sample was also tested at the National Institute of Infectious Diseases L.Spallanzani for the presence of WNV-specific IgG and IgM by an indirect immunofluorescence assay (Euroimmun, Italy), which indicated high antibody titres. In addition, a microneutralisation assay against both lineage 1 and 2 strains [4] revealed cross-neutralising activity. This does not demonstrate co-circulation of the two lineages; antibodies elicited by one WNV lineage are not expected to be highly lineage-specific, because of extensive antigenic similarity between the lineages.

WNV RNA was still detectable by the in-house RT-PCR analysis at the Ancona Virology Laboratory in the urine sample collected on 29 September, 25 days after symptom onset.

The results of all serological and molecular investigations performed on the patient's samples are shown in the Table.

WNV infection in Italy

WNV infections have been reported in both humans and horses since the summer of 2008 in north-eastern Italy [5-8] but until now, as far as we are aware, only WNV lineage 1 infections have been described in the country.

To the best of our knowledge, WNV infection has never been reported before in horses or other sentinel animals in the Marche region. A possible arthropod reservoir has never been investigated, but given the absence of infection in sentinel animals and the absence of diagnosed cases of WNV meningoencephalitis, the region was considered to be at lower risk than the WNV-affected areas of north-east of the country [9].

In contrast, Toscana virus is endemic in the Marche, as well as in the rest of central Italy, and is routinely investigated in all cases of meningoencephalitis reported in the summer and in patients with fever of unknown origin. Due to the circulation of WNV in the north-east of the country, our laboratory testing algorithm was revised, introducing molecular (in summer 2010) and serological (in summer 2011) assays for the diagnosis of WNV infection. These assays are performed for

Table

Serological and molecular test results on samples from the patient with West Nile virus lineage 2 infection, Italy, September 2011

Date of sample collection (2011)	Sample type	ELISA IgG (index)[a]	ELISA IgM (index)[b]	IFA IgG (dilution)	IFA IgM (dilution)	MNTA titre lineage 1	MNTA titre lineage 2	RT-PCR[c]
13 Sep	Serum	2.00	>5.40	ND	ND	ND	ND	Negative
21 Sep	Urine	NA	NA	NA	NA	NA	NA	Positive
26 Sep	Serum (ELISA) Plasma (RT-PCR)	3.01	>5.40	ND	ND	ND	ND	Negative
28 Sep	Plasma (IFA and RT-PCR) Urine (RT-PCR)	ND	ND	>1:320	>1:320	1:20	1:40	Plasma negative Urine positive
29 Sep	Urine	NA	NA	NA	NA	NA	NA	Positive

ELISA: enzyme-linked immonosorbent assay; IFA: immunofluorescence assay; MNTA: microneutralisation assay; NA: not applicable; ND: not done; RT-PCR: reverse-transcription polymerase chain reaction; WNV: West Nile virus.

[a] An index (signal/cut-off ratio) value of >1.50 indicates the presence of IgG antibodies to WNV.

[b] An index (signal/cut-off ratio) value of >1.10 indicates the presence of IgM antibodies to WNV.

[c] Carried out using an in-house protocol that uses degenerate primers designed to recognise a region in the NS5 gene that is conserved (on the basis of an alignment of database sequences) in most animal and human *flaviviruses* or, for the samples collected on 28 September 2011, using the cobas TaqScreen West Nile Virus Test, a real-time RT-PCR (Roche Molecular Diagnostics).

hospitalised patients in the Marche region during the months when mosquitoes and other insect vectors are active, generally from early June to late October. Although tests, carried out at the Ancona Virology Laboratory, are usually performed on blood and cerebrospinal fluid (CSF), we recently detected WNV from urine from a kidney transplant patient with encephalitis in the context of an investigation into WNV transmission through organ transplants (unpublished data): in this transplant patient, the virus was detectable in urine by molecular tests for a longer period than in serum, plasma or CSF, consistent with the fact that the kidney is a well-established site of active WNV replication in animals such as birds, dogs and rodents [10-12]. Persistent replication of the virus in kidneys in humans is supported by studies reporting WNV shedding in urine, not only early post-infection [13], but even years after the initial infection [14], although the issue is still debated [15]. In our modified algorithm, a urine sample – the preferred sample for virus detection – is currently requested from patients whose serological tests for WNV are positive in order to confirm the serological results by detecting WNV RNA.

Discussion

A number of cases of human WNV infection have been reported over the past few years in Italy [16], but never in or close to the Marche region, with the exception of one infection acquired through a kidney transplant from a donor from an affected region [17]. In the Marche, since the summer of 2010, tests for WNV infection have been performed exclusively for the differential diagnosis of meningoencephalitis cases in the summer months . Since the summer of 2011, WNV serological tests are carried also out for patients with fever of unknown origin who are negative for Toscana virus. Had this diagnostic algorithm not been adopted, the cause of the patient's febrile illness would not have been determined and the WNV lineage 2 strain would not have been identified.

This case report suggests that screening for human cases of WNV infection should be further strengthened in the summer, for cases with neuroinvasive disease and for patients with fever of unknown origin, in regions of the country not previously affected by WNV. It is well known that there is an extensive cross-reactive antibody response to members of the *Flavivirus* genus, thus molecular tests should be performed to confirm the clinical diagnosis and identify the causative virus.

Our data show that tests to detect WNV RNA in serum or plasma may give false-negative results due to the short duration of viraemia. Urine samples may be more appropriate when looking for the presence of WNV, because of longer shedding and higher viral load. Whole WNV genome reconstruction was also easily achieved from the urine sample.

The clinical presentation of the case here described was relatively mild. However, since this is the first case of WNV lineage 2 infection detected so far in the country, it is not possible to draw any conclusions on the virulence and neurotropism of the viral strain. Investigation of any future cases, as well as molecular analysis of the complete genome, could give further information about the presence of genetic determinants of virulence.
It should be noted, however, that the incidence of meningoencephalitis or fever of unknown origin did not increase this summer in the Ancona province.

Autochthonous WNV human infection has been reported in several European countries this summer, including those of the Mediterranean. As of 20 October 2011, 89 confirmed human cases of West Nile fever have been reported in the European Union (66 in Greece, 13 in Italy and 10 in Romania) and 149 in neighbouring countries (121 in the Russian Federation, 21 in Israel, 3 in Turkey, 2 in Albania and 2 in the Former Yugoslav Republic of Macedonia) [18,19]. Notably, cases of WNV infection in Greece in 2011 occurred in areas that had not been affected in 2010 [19].

The WNV lineage 1 sequences from human infections in 2008 to 2009 in Italy were grouped into a distinct cluster within the western Mediterranean cluster [12], suggesting autochthonous spread of a single virus strain, without de novo introduction. The finding of the case in Ancona described in this report might suggest that viral strains circulating in other European countries during this summer might be spreading to Italy. It is possible that the lineage 2 virus reached Ancona via infected mosquitoes carried by ships or via birds from the eastern part of Europe. An epidemiological investigation is under way in the Ancona area to identify risk factors for infection and the possible local spread of lineage 2 WNV among insect vectors and birds.

Whatever the origin of the virus, the finding of a case of WNV lineage 2 infection in the country deserves further attention, as it suggests that viral circulation routes may be expanding, and, possibly, that there is an increased opportunity for this lineage 2 virus to adapt to new environments and ecological niches. It will be important to determine whether the present molecular diagnostic assays, designed mainly to detect lineage 1 WNV, perform equally well for lineage 2 WNV. This may have important implications for effective screening of blood and organ donors.

As a result of this case of WNV infection, the same precautionary measures in force in the WNV-affected regions in north-east Italy were immediately adopted in the Marche, for the sake of safety of organ donation in the region. In particular, these measures concern the need to use a nucleic acid amplification test (NAAT) to check for the presence of WNV RNA in blood taken from organ donors living in the Marche region or who stayed at least one night in the region in the 28 days before notification of the case described in this report.

In conclusion, stronger vector control programmes, integrated human and animal WNV surveillance and implementation of diagnostic procedures that include testing of urine samples for WNV detection could provide a useful contribution to controlling WNV spread and human disease.

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Autochthonous *Plasmodium vivax* malaria in Greece, 2011

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Between May and September 2011, twenty cases of *Plasmodium vivax* infection were reported in Greek citizens without reported travel history. The vast majority of those cases were confined to a delimited agricultural area of Evrotas, Lakonia. Conditions favouring locally acquired transmission of malaria, including the presence of competent vectors and migrants from endemic countries exist in Greece, underscoring the need for the development of an integrated preparedness and response plan for malaria prevention.

In 2011, 20 malaria cases without reported travel history to endemic countries have been notified as of 27 September from Evrotas, Lakonia and other geographical areas in Greece. We conducted an investigation to describe the geographical and temporal distribution of those cases, determine the affected area(s) and identify the potential source of infection, in order to better understand the transmission dynamics and implement targeted control measures.

Malaria was officially eliminated from Greece in 1974, following an intense national malaria eradication programme that was implemented between 1946 and 1960 [1]. Between 1975 and 2005 approximately 50 cases of malaria were reported annually, the vast majority of whom were imported cases from countries endemic for malaria. However, sporadic cases of mosquito-transmitted malaria that could have been acquired locally were reported in 1991, 1999 and 2000 [2]. Between 2005 and 2009, 171 cases of malaria were reported in Greece with a mean number of 34 cases per year [3]. Of those, 98% were in people that likely acquired the infection in endemic countries and 78% of all cases were in migrants from those countries.

Between early August and October 2009, a cluster of eight malaria cases was notified to the Hellenic Centre for Disease Control and Prevention (HCDCP) from the Evrotas area of Lakonia district, which is located in the Peloponnese in southern Greece. The first two cases were in migrant workers from Pakistan and Afghanistan who reportedly arrived in Greece during the summer of 2009 and who were working in the agricultural holdings in this particular area. Four of the remaining six cases belonged to the local Roma community and the other two were Greek citizens residing in the area. None of those six cases reported travel history to a malaria endemic country. In 2010, another malaria case was notified from the same area in Evrotas, who also belonged to the local Roma community. Additionally, two Roma children with malaria were notified in Viotia with disease onset on 25 and 30 August 2010 and an unclear travel history.

Surveillance of malaria in Greece

As part of the mandatory notification system, physicians in Greece are asked to notify HCDCP of all cases of laboratory-confirmed malaria infection. Enhanced surveillance is implemented in areas where domestic transmission is suspected (i.e. where no clear recent travel history to a malaria-endemic country can be established), by tracing the reported cases, visiting their homes and if possible conducting face-to-face interviews. When this is not possible, telephone interviews are conducted. Translators are used where appropriate. The case investigation form for enhanced surveillance gathers information on: detailed travel history, potential modes of transmission, clinical manifestations and treatment, previous malaria clinical episodes, possible onward transmission and household characteristics. In addition, active surveillance is implemented by maintaining weekly communication with local laboratories to enquire about recent diagnosed cases of malaria. Residents in the neighbourhoods surrounding the homes of suspected locally-acquired cases are asked to report febrile illnesses to the local public health office and to seek healthcare promptly.

Laboratory investigation

Light microscopic examination of Giemsa stained thick and thin blood smears is used to identify malaria

parasites in local laboratories. Blood smears are routinely analysed when general blood count tests identify anaemia, thrombocytopenia or other abnormal findings. All blood specimens positive for malaria and a number of negative ones are forwarded to the National Malaria Reference Laboratory (MRL) at the National School of Public Health in Athens to be validated with both microscopy and polymerase chain reaction (PCR). Rapid diagnostic antigen tests are not routinely used. Twenty per cent of positive samples are being genotyped at present.

Entomological investigation

Following the 2010 large outbreak of West Nile Virus infection in Greece [4], a study on vector distribution and mapping of risk areas was carried out. The adult mosquito population is monitored using CO_2 or CO_2-light traps at permanent sampling stations that are collected every 14 days. Several additional traps were used at locations of suspected malaria transmission in order to detect *Anopheles* mosquitoes. Collected specimens were counted and morphologically identified.

Situation in 2011

Epidemiological and clinical findings

Up to 27 September 2011, the HCDCP has received reports of 20 cases of *P. vivax* infection in Greek citizens who did not report travel to an endemic country. The majority of those cases (n=14) reside in the agricultural area of Evrotas, Lakonia district (Table, Figure 1). The remaining six Greek cases were reported from four other prefectures, namely Eastern Attiki (n=2), Evia (n=2), Viotia (n=1), and Larissa (n=1). From the area of Evrotas were further reported 16 cases of *P. vivax* infection in migrant workers from endemic countries (mainly from Pakistan) for whom no clear malaria importation status can be determined. In addition, two Romanian workers who had been working and living in the area of Evrotas developed symptoms in July 2011 and were diagnosed with *P. vivax* infection upon their return to Romania [5]. These two cases are not included in further analysis because not all the epidemiological information is available. All 36 cases have been confirmed as *P. vivax* infections, by both microscopy and PCR at the MRL. None of the cases had a history of recent blood transfusion or tissue/organ transplantation.

The first case from Evrotas reported disease onset on 23 May 2011 (Figure 2). An increasing number of cases residing in Evrotas area was observed during September (weeks 35-37). At the time of publication of this report, the outbreak is still ongoing.

The age distribution of the 36 reported cases ranged from 1.5-79 years (median: 36 years). The median age of migrant cases (24 years; range 15-55 years) was significantly lower ($p<0.001$) than of Greek cases (47 years; range 1.5-79 years), possibly reflecting the different age distributions of the two population groups. Seven of the Greek cases were female. As the majority of the migrant worker community is male, women with *P. vivax* infection were not reported among migrants.

Fever was reported as the main symptom by all cases, followed by splenomegaly (n=14) and anaemia (n=14). Three cases had central nervous symptom manifestations. All cases were hospitalised; one was admitted to an intensive care unit and has recovered fully. To date, there has been one fatality in an elderly male case from Evrotas area who had several underlying medical conditions, including cardiac insufficiency, arrhythmias and chronic obstructive pulmonary disease, and developed acute respiratory distress syndrome. This is the first death associated with *P. vivax* infection in the last three years in Greece. All other cases have fully recovered.

Almost all cases but three who were prescribed mefloquine and primaquine, received the current treatment regimen for uncomplicated *P. vivax* infection according to the national guidelines [6], which is three-day chloroquine treatment followed by 14-day primaquine treatment. Some cases in Lakonia received the alternative weekly primaquine outpatient regimen (higher dose than the daily regimen) for eight weeks, to achieve a higher compliance rate. Only one case among the reported 36 cases had glucose-6-phosphate dehydrogenase deficiency and did not receive primaquine.

When comparing the date of onset of symptoms to the date of hospitalisation (which is a proxy for receipt of anti-malaria treatment in Greece), the time period for all cases ranged between 0 and 27 days (median: 4 days). The median delay between symptom onset and treatment was shorter in the group of migrant workers (3 days; range 0-19 days) compared to Greek patients (4 days; range 2-27 days). However, this difference was not statistically significant (p= 0.12).

Entomological findings

Fifteen *Anopheles* species occur in Greece, of which five are considered as potential malaria vectors, namely *An. claviger*, *An. hyrcanus*, *An. maculipennis*, *An. sacharovi* and *An. superpictus* [7-9]. In the Evrotas area, *Anopheles* larvae were found in rivers, reed beds, the

Table

Reported *Plasmodium vivax* infections by district of residence, Greece, May–September 2011 (n=36)

District (region)	Number of cases
Lakonia (Peloponnese)	30[a]
Eastern Attiki (Attiki)	2
Evoia (Sterea Ellada)	2
Viotia (Sterea Ellada)	1
Larissa (Thessalia)	1
Total	**36[a]**

[a] This figure includes 16 cases in migrant workers from endemic countries residing in Evrotas area, Lakonia. The remaining cases are in Greek citizens without reported travel history to a malaria-endemic country.

Vivari lake and draining channels, but at very low densities. From 1 June to 15 September, 23 adult *Anopheles* specimens were collected from two sampling stations in the area, most of them *An. sacharovi* (n=21). Two specimens which were determined as *An. plumbeus* need further confirmation as they were not intact.

In the wetland area of Schinias national park in, Eastern Attiki, 19 mosquito species were identified, with *An. claviger* being the dominant *Anopheles* species in the area [9]. Other *Anopheles* species that were collected in that area included: *An. algeriensis*, *An. maculipennis s.s.*, *An. pseudopictus* and *An. sacharovi*. In the remaining affected areas in Greece, *Anopheles* species were identified, but their reported densities were often low. The most commonly identified species there were *An. sacharovi* and *An. claviger*.

Discussion

As of 27 September, 20 malaria cases were reported in Greece, affecting Greek citizens who did not have any reported history of travel to a country endemic for malaria. The vast majority of those cases were confined to a delimited geographical area in Evrotas, Lakonia, where a small number of malaria cases had already occurred in the previous two years. All other areas that reported cases were previously unaffected. In addition, 16 cases in migrant farm workers with unclear malaria importation status were notified in Evrotas. As none of these workers were documented, it is difficult to ascertain when they first arrived in Greece, where they travelled and worked and how long they had been residing in the area. Therefore, based on their self-reported travel, medical history and possibility of relapses, it cannot be determined conclusively whether they were non-imported cases.

Figure 1

Place of residence of reported malaria cases, Greece, May–September 2011 (n=36)

The affected area in Lakonia district is a plain agricultural area of about 20 km² in the delta of the Evrotas river. It was one of the historical hot spots of malaria transmission before elimination of the disease in Greece [10]. The area is characterised by freshwater springs, a complex network of 130 km of irrigation and drainage channels, the Evrotas river delta, the brackish Vivari lake, which seasonally dries out, and coastal wetlands. All other affected sites are located in agricultural areas, often closely associated with river deltas or wetland areas, providing favourable conditions for the presence and reproduction of potential malaria mosquito vectors. None of those areas are tourist destinations.

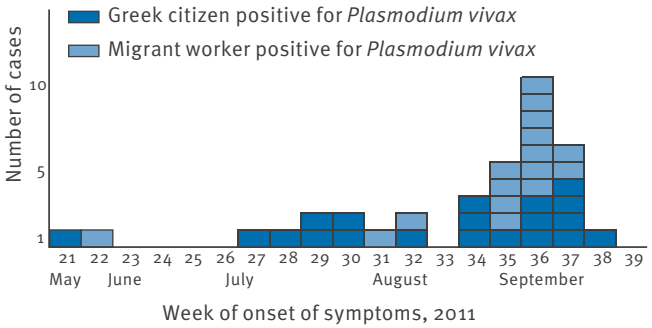
The affected area of Evrotas has a population of 4,485 and a large community of migrant farm workers (estimated between 2,000-4,000 depending on the period of the year), most of whom not registered [11]. Approximately 80% of all migrant workers in the area come from Pakistan, around 15% from Romania and the remaining from Morocco. The other affected areas have high numbers of migrant agricultural workers from malaria-endemic countries, predominantly from the Indian subcontinent.

Following the reports of malaria in Greece, the following control measures were introduced: Guidelines for the recognition, management and diagnosis of malaria were provided to healthcare professionals to improve their awareness of the disease. Interviewed patients were informed that persons in their close environment presenting with fever should get diagnosed as soon as possible. Support was provided to strengthen the laboratory capacity of local health centres in the affected areas to diagnose malaria. Surveillance of mosquitoes was enhanced in the affected areas. Guidance for blood and blood product safety according to European Union directives was implemented, including deferral from blood donation for a period of six months of persons residing or working in the affected areas within a radius of 10 km. Communication and health promotion activities were strengthened encouraging personal protection against mosquito bites in the general population. Intensified vector control activities were implemented using larviciding in breeding sites, ultra-low volume spraying in the affected villages and outdoor residual spraying in a zone of 50 meters around the houses of the cases, including backyards, neighbouring stockyards and other installations favourable for the resting of *Anopheles* adults. Furthermore, all households in the area have been visited fortnightly since 30 September to detect people with fever and to ensure early detection and prompt treatment of all malaria cases. During those visits, multidisciplinary health teams screened blood smears from all persons with fever of 37.5 °C or higher, current or reported during the previous 15 days.

Since the malaria eradication in 1974 in Greece, sporadic cases of probable local mosquito-borne transmission have occurred. Because of its climate, proximity of human and mosquito populations, and the increased number of migrants from malaria-endemic countries, Greece and possibly other Mediterranean countries might be vulnerable to the re-establishment of endemic malaria [12,13]. However, provided that current healthcare, mosquito control and public health infrastructures remain intact in Greece, the re-establishment of endemic areas for malaria remains unlikely. Nevertheless, conditions may exist for small clusters of locally acquired mosquito-borne transmission to occur sporadically. The development of an integrated preparedness and response plan for malaria that covers all aspects from surveillance, clinical management, laboratory diagnosis, entomological surveillance, vector control and communication is necessary to prevent transmission and control the disease on the long term. Such a plan should not only address the most affected area of Evrotas, Lakonia, but also other parts of Greece where ecological parameters are favourable for malaria transmission.

Figure 2

Reported cases of malaria by week of symptom onset and region of residence, Greece, May–September 2011 (n=36)



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Plasmodium vivax malaria in a Romanian traveller returning from Greece, August 2011

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In August 2011, a *Plasmodium vivax* malaria infection was diagnosed in a Romanian traveller returning from Greece. This case together with several reports over the past decade of autochthonous cases in Greece highlight that malaria should be considered as differential diagnosis in symptomatic travellers returning from this country. Travellers may serve as sentinels of emerging vector-borne diseases.

Malaria is considered to be eradicated in several European countries since 1975, although *Anopheles* spp. mosquito vectors remain prevalent in parts of southern and central Europe [1]. A few cases of autochthonous transmission of malaria to local residents have been reported over the last 10 years in areas where the disease has been declared eradicated (Bulgaria, France, Germany, Greece, Italy, and Spain), including the so-called airport malaria, but there has not been sustained local transmission in any specific location [2].

In this report, we describe a case of malaria in a Romanian traveller returning from Greece.

Case report

On 1 August 2011, a 25-year-old Romanian man developed an acute febrile illness with chills, myalgia, fever, and left abdominal flank pain. He had returned from Greece on 30 July. His past medical history included a splenectomy 14 years earlier. As the symptoms persisted, on 3 August, the patient visited the family doctor who suspected a respiratory infection and prescribed a symptomatic and antibiotic treatment (amoxicillin-clavulanate). On 9 August, as there was no improvement in his symptoms, as the fever persisted and he felt an increased pain in the left flank, and after an episode of near syncope, he was admitted to the local hospital in his area of residence. Abdominal ultrasound showed a hematoma in the splenic bed (5.5 by 6.6 cm). The hematoma was surgically drained. On 11 August laboratory results revealed thrombocytopenia (57,000/mm^3; norm: 150,000-400,000), anaemia (Hb: 8.7 g/dl; norm 11.5-15), and leukocytosis (16,600/mm^3; norm: 4,000-10,000) with normal white blood cell differential count. Blood cultures taken upon each hospital admission remained negative. Thin blood smear revealed *Plasmodium* spp. trophozoites and schizonts. On the same day, the patient was transferred to 'Victor Babes' Hospital for Infectious and Tropical Diseases in Bucharest. On arrival, physical examination revealed a reduced general condition, a temperature of 37.8°C, abdomen with diffuse sensitivity to palpation, and moderate hepatomegaly. Thin and thick films revealed *Plasmodium vivax* parasitaemia of 0.05% with mature trophozoites, young schizonts, and gametocytes of *P. Vivax*. Whole blood DNA quantification using a LightScanner 32 (Idaho Technology, USA) demonstrated *P. Vivax* (1,500 copies/µl) and was negative for the other *Plasmodium* species. The patient responded quickly to the seven-day treatment of oral quinine combined with doxycycline. The clinical response was good (fever ceased after 48 hours of treatment) and the parasite clearance appropriate (negative thin and thick smear after 72 hours of antiparasitic treatment). After this treatment, the patient was given primaquine for 14 days to prevent relapses.

Patient history revealed that he had worked intermittently in agriculture in Greece, for about six years (in 2005 in Argos region and then every October to February from 2006 to 2010 in Lakonia region, in Skala and Elos localities of the Evrotas river basin). More recently, from November 2010 to 30 July 2011 he worked in the same regions (Skala and Elos) in agriculture. He had no history of travel to any malaria-endemic areas and his only other travel abroad was a two–month trip to Italy (Sicily) from September to October 2010. He had never travelled by plane, he does not live in the proximity of any international airport and there have been no reports of imported malaria cases within his residence

area in Romania. Malaria tests in the patient's relatives with whom he had travelled and worked in Greece were negative.

Epidemiological situation in Greece

Several Anopheline vector species are known to breed in Greece, including some of the historically most important vectors in Europe including *A. atroparvus*, *A. sacharovi* and *A. superpictus* that are competent for *P. Vivax* [3]. As early as 1994 and 1995 four autochthonous malaria cases (*P. falciparum*, *P. malariae* and two *P. Vivax*) were diagnosed in Evros, northern Greece, in native residents from rural areas who stated that they had never left Greece or visited an international airport [4]. In the summer of 1998 two additional malaria cases (*P. Vivax*) were diagnosed in the same region in two patients who had been living for four years in Feres (in the Evros peripheral area, East Macedonia and Thrace, Greece) after they had immigrated from southern Albania [5]. Later, an autochthonous cluster of *P. Vivax* malaria occurred in the Evrotas river basin, Lakonia, southern Greece, from August to October 2009, with eight patients hospitalised in Sparta General Hospital, including two immigrants from Pakistan and Afghanistan and six patients, natives of Lakonia and living in different areas from the first two [6]. More recently, between June and 18 August 2011, six cases of locally-acquired *P. Vivax* malaria have been notified to the Hellenic Centre for Disease Control and Prevention through the mandatory notification system in Greece: four cases from the same agricultural wetland area of the Evrotas river basin, Lakonia, in the Peloponnese, southern Greece and two cases who reside near the city of Chalkida, Evoia, in the eastern part of Greece [7]. All cases were local residents with no history of travel to a malaria endemic area.

Epidemiological situation in Romania

The *P. Vivax* malaria case in Romania was reported to the National Institute of Public Health within the National Malaria Surveillance Programme. In Romania, there is a national programme for surveillance and rapid communication of malaria cases. In 1948, a total of 333,198 cases of malaria were reported, but starting with 1968 Romania was declared a malaria-free country. No local transmission of malaria has been reported in Romania since then. Nevertheless, malaria remains a possible re-emerging disease especially in the southern and south-eastern part of the country, where vector-competent *Anopheles* species are prevalent [8]. In the past 10 years, 107 cases of malaria were diagnosed in the 'Victor Babes' Hospital of Infectious and Tropical Diseases in Bucharest and all of them were in travellers who had returned from malaria endemic areas, mostly from Africa [9,10].

Discussion and conclusions

The occurrence of presumably vector-competent *Anopheles* species, together with increasingly favourable climatic conditions and the frequent availability of reservoirs of infection such as imported cases, produce an ongoing probability of autochthonous malaria appearing time and again.

This case report presented epidemiological evidence and patient history point to an infection in the Skala and Elos areas Greece. Prior to 2011, the patient had not been exposed in Greece during summer, or during periods when autochthonous cases were reported in Greece. However, it is unclear whether the reservoir of infection for this case was from migrant workers in Greece coming from South Asia or from the local population.

To the best of our knowledge, this is the second case of malaria reported recently in an international traveller who acquired the infection in an European Union (EU) country. In 2000, a German couple was diagnosed almost simultaneously with *P. Vivax* malaria after a one-week holiday in Kassandra, Chalkidiki, a Greek tourist resort [11]. No local cases had been reported at that time but it seems that sporadic local transmission must have been occurring.

Since Greece is a frequent destination for people visiting or working, any re-emergence of malaria will be of concern. Furthermore, the occurrence of malaria cases in areas considered to be malaria-free may lead to delay in diagnosis raising the possibility of exposure to vectors and even the risk of incidence of local malaria cases.

To date, there has been no recommendation for travellers to the affected areas in Greece to take any antimalarial chemoprophylactic drug. However, advice has been issued on avoidance measures regarding insect bites particularly during the evening and at night [12,13].

In the authors' opinion, the Greek interventions with the current cases should be followed closely so that Romania and other formerly malaria endemic countries in the EU remain malaria-free. Both local health authorities and practicing clinicians need to be aware that they should also include malaria in the differential diagnosis in travellers and temporary residents returning from Greece and maybe also other southern European countries. However, according to a risk assessment related to the six autochthonous cases of *P. Vivax* malaria in Greece, published by the European Centre for Disease Prevention and Control, the risk for further extension of malaria transmission into the EU is considered low at present [7].

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Malaria among patients and aid workers consulting a primary healthcare centre in Leogane, Haiti, November 2010 to February 2011 – a prospective observational study

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Plasmodium falciparum malaria is endemic in Haiti, but epidemiological data are scarce. A total of 61 cases of malaria were diagnosed between November 2010 and February 2011 among 130 Haitian patients with undifferentiated fever. Three additional cases were diagnosed in expatriates not taking the recommended chemoprophylaxis. No cases were diagnosed among aid workers using chemoprophylaxis. In conclusion, malaria is a significant health problem in Leogane, Haiti. Aid workers and visitors should use chemoprophylaxis according to existing guidelines.

Introduction

Plasmodium falciparum malaria is endemic in Haiti [1-5]. Epidemiological data from Haiti are scarce, but before 2010 the prevalence of malaria in most areas of Haiti was estimated to be low [2-5]. The effects of the 2010 earthquake and the severe flooding that followed the 2010 hurricane on the incidence of malaria are unknown. We report the incidence of malaria among febrile patients in two primary-care clinics in the West (Ouest) Province of Haiti. In addition we report all cases of malaria in expatriate aid workers seen in our clinic.

Methods

The study was conducted in two newly established primary healthcare clinics in the West Province of Haiti. The main clinic is situated in the town of Leogane, 30 km west of Port-au-Prince. Leogane has an estimated population of 200,000. The other clinic is situated in Magandou, a rural village in the same region. Since November 2010 the Leogane clinic has been operating daily, and the Magandou clinic is open once a week. Both clinics are staffed by nurses and doctors from Haiti, Israel, and Canada. Medical services are provided free of charge. All cases of undifferentiated fever were tested for malaria. Diagnoses of malaria were reached with the help of a rapid diagnostic test for detection of histidine-rich protein II (Paracheck Rapid Test, Orchid Biomedical Systems). The tests were performed in both clinics by the same experienced doctors using the same diagnostic kits. The clinical and epidemiological features of all cases of malaria were collected prospectively.

Results

Over a period of 14 weeks, between November 2010 and February 2011, a total of 61 cases of falciparum malaria were diagnosed among Haitian patients in the Leogane clinic. This period roughly correlates with the peak malaria transmission season in Haiti [3]. These 61 cases accounted for 46.9% of the 130 patients with undifferentiated fever, and 1.9% of all 3,166 patient visits. The average age of the patients with malaria was 22.5 years (range 3 to 67 years) with 25 of 61 cases occurring in patients younger than 16 years. Thirty-two cases occurred in females. All malaria cases were acquired in Leogane, as none of the patients had travelled outside the Leogane area during the three weeks preceding the onset of symptoms.

All patients with malaria reported a febrile disease; although upon presentation only 43 of 61 had a fever higher than 37.5°C. Two patients had severe malaria and were transferred to a referral hospital. Nearly all patients (60 of 63) were treated with chloroquine. Three patients were treated with artemether/lumefantrine; two because of difficulty in accurately dividing the chloroquine pills for young children, and one because of an allergic reaction to chloroquine.

No cases of malaria were found among a total of 258 patients examined in the village of Magandou. Eleven of these patients had presented with an undifferentiated fever.

Three expatriates diagnosed with malaria were aid workers living in Leogane. None of the three were using anti-malaria chemoprophylaxis. Since the total number of aid workers residing in the area of Leogane is unknown, the risk of acquiring malaria in this population can not be calculated. In our organisation two out of the ten aid workers who stayed in Haiti for a total of 57 person-weeks and did not use chemoprophylaxis contracted malaria. No cases of malaria occurred in 52 additional aid workers who stayed in Haiti for a total of 346 person-weeks and used chemoprophylaxis with chloroquine.

Discussion

Studies before the earthquake reported a low risk of acquiring malaria in most areas of the country [2-5]; data from Leogane itself were not available. According to our data, collected after the earthquake and hurricane of 2010, the incidence of malaria among patients with undifferentiated fever in Leogane, Haiti was around 47%. Although the sensitivity of the Paracheck Rapid Test has been reported to be sub-optimal [6], its specificity is very high. Therefore we think that the number of malaria cases has not been overestimated. A recent report from a post-earthquake national surveillance system indicated that suspected malaria and fever of unknown cause accounted for 10.3% and 10%, respectively, of total visits to 51 pre-specified clinics [1]. Although laboratory diagnoses of malaria were not performed, these results seem to indicate that the incidence of malaria in certain parts of post-earthquake Haiti may be appreciable.

In a study published in 1995 only 4% of peripheral blood smears taken from febrile patients in several different provinces of Haiti were positive for *P. falciparum* [4]. It is not known whether the incidence of malaria among febrile patients was underreported in the past, or whether the natural disasters that recently affected the country have caused an increase in malaria incidence. It is also unclear whether the incidence of malaria in other areas of the country is similar to the one in Leogane. Leogane is situated near the epicenter of the 2010 earthquake. Approximately 80% of Leogane was destroyed, and tens of thousands of its inhabitants were made homeless. Since *Anopheles albimanus*, the mosquito vector of malaria in Haiti, usually bites outdoors, people living in temporary shelters are probably at an increased risk of contracting malaria in post-earthquake Haiti. In addition hurricane Tomas caused severe floods in Leogane in November 2010, and may therefore have expanded the breeding sites for the vector.

In contrast, no cases were found in Magandou, located in the hilly areas 25 km south-west of Leogane. The elevation of Magandou (941 meters above sea level) does not fully explain this finding. The reasons for such a significant regional variation in the incidence of malaria within a relatively small area are unclear. Possible explanations include a more mountainous terrain, and less damage caused by both the 2010 earthquake and hurricane Tomas.

Not surprisingly cases of malaria also occurred among aid workers residing in the Leogane area. Cases of malaria among emergency responders after the 2010 earthquake were reported in other areas in Haiti, too, but since data regarding incidence are unavailable, a comparison of the risk of infection in different areas is impossible [7]. It is important to note that no cases of malaria were detected among aid workers receiving chloroquine chemoprophylaxis. Apparently, the risk of acquiring malaria in expatriates using chemoprophylaxis is appropriately low.

In conclusion, malaria is a significant health problem in Leogane, Haiti. It is unknown whether this holds true for other areas of Haiti. It is also unclear whether the high malaria incidence among febrile patients was underreported in the past, or whether it is related to the deteriorated infrastructure of the area following the earthquake and the hurricane that occurred in 2010. Aid workers and visitors should use chemoprophylaxis according to existing guidelines. We have not detected any cases of chloroquine chemoprophylaxis failure, thus supporting the current malaria prevention guidelines [8]. Further entomologic surveys and vector control efforts are warranted if malaria incidence is to be reduced in Leogane, Haiti.

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Autochthonous dengue fever in Croatia, August–September 2010

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After information about a dengue case in Germany acquired in Croatia, health professionals and the public in Croatia were alerted to assess the situation and to enhance mosquito control, resulting in the diagnosis of a second case of autochthonous dengue fever in the same area and the detection of 15 persons with evidence of recent dengue infection. Mosquito control measures were introduced. The circumstances of dengue virus introduction to Croatia remain unresolved.

Introduction

The epidemiology service of the Croatian National Institute of Public Health (CNIPH) has registered six imported cases of dengue virus (DENV) infection since 2007. All except one were Croatian citizens who had spent time in areas with local transmission of this disease (Southeast Asia, South America) and had a mild clinical presentation of dengue fever. The sixth case occurred in 2007 in a tourist visiting Croatia who developed haemorrhagic fever and had previously travelled in South-east Asia [1,2]. Although a seroepidemiology study conducted in 1980 in a limited area of north-eastern Croatia in healthy young inhabitants proved the presence of antibodies to DENV type 2 (3,9%) and type 1 (2,1%) [3], no cases of dengue fever were registered by the health services.

Aedes albopictus was for the first time recorded in Croatia in 2004 in the area surrounding Zagreb [4]. Within two years, *Ae. albopictus* was found on the entire territory of the Adriatic coast from northern Istria to Dubrovnik in the south. According to routine monitoring of mosquitoes and published articles, *Ae. albopictus* is now permanently established in the coastal but not yet in the continental areas of Croatia [5].

Soon after reports on the first autochthonous DENV infection diagnosed in France in September 2010 [6], the Epidemiology Service of the CNIPH was notified on 30 September by the Robert Koch Institute (RKI) in Germany of a German citizen, who fell ill with symptoms of dengue fever immediately after returning to Germany from a 15-day stay on the Pelješac peninsula in Croatia. Virological investigation revealed the presence of DENV-specific IgM, a rise in DENV-specific IgG and the presence of NENV NS1 antigen in the patient's blood [7]. As this was the first case of dengue fever probably acquired in Croatia, an epidemiological investigation was conducted and outbreak control measures implemented. We present here the first results of the epidemiological investigation.

Active case finding

According to information received by the RKI, the German citizen travelled from Germany via Austria and Slovenia along the Croatian coast in August 2010 and stayed on the Pelješac peninsula and the island of Korčula for 15 days. The disease onset was on the day after he returned to Germany. The information received from RKI on the first autochthonous case of dengue fever was sent to the World Health Organization (WHO) via the International Health Regulations (IHR) information network [8] by the national IHR focal point (the epidemiology service of the CNIPH) and disseminated to the Croatian and international public via the media in order to increase local and international awareness.

The epidemiological investigation started in Pelješac and Korčula, and will be gradually expanded to the entire Croatian littoral. The CNIPH released a circulatory letter informing all epidemiology services and hospital infectology clinics in the country to consider the possibility of dengue fever in clinically compatible cases including those with no history of travelling

abroad. The circulatory letter contained the entire range of clinical manifestations of dengue fever, and for the purposes of the epidemiological investigation, a question on diseases accompanied with fever occurring in the village where the German tourist had stayed. At the time of the first epidemiological investigation among local physicians in the beginning of October 2010, there were no such acute diseases in the area. In the following weeks, a number of clinically suspect cases were reported and serum samples were sent to the CNIPH, but tested negative for dengue virus (Anti-Dengue virus Elisa IgM/IgG, Euroimmun, Germany).

On 22 October 2010, a possible case of dengue fever was reported in a resident of the same village where the German patient had stayed. The Croatian patient, a woman in her fifties, who had not travelled outside her place of residence, developed symptoms compatible with dengue fever on 17 October, including temperature up to 39°C, skin rash, chill, headache and joint and muscle pain, and was admitted to the infectology ward at Dubrovnik hospital on day 6 after onset of disease.

Laboratory diagnosis

A serum sample was taken from the Croatian patient on admission to hospital and sent to the National Reference Laboratory for Arboviral Infections at the Virology department of the CNIPH in Zagreb. Virological analysis by ELISA (Anti-Dengue virus ELISA IgM/IgG Euroimmun, Germany) detected DENV-specific IgM (ratio 1.9). The sample was negative for DENV-specific IgG (10 relative units (RU)/ml), West Nile virus (WNV) IgM and IgG (Anti-West Nile virus ELISA IgM/IgG, Euroimmun, Germany), and chikungunya virus IgM and IgG. (Anti-Chikungunya virus ELISA, IgM/IgG, Euroimmun, Germany).The patient had not been vaccinated against yellow fever or tick-borne encephalitis (TBE). The village of residence as well as whole southern Croatia is not endemic for TBE [9]. Considering the current epidemiological situation in Croatia these laboratory results pointed to a diagnosis of dengue fever [10].

The patient's first serum was also sent to the regional reference laboratory in Ljubljana (Slovenia) where an RT-PCR was negative for dengue virus [11]. The second (paired) serum was taken on day 19 after onset of illness when the patient was already discharged and recovered, and the sample was analysed at CNIPH with the following results: DENV: IgM-positive (ratio 4.9) and IgG-positive (110 RU/ml); WNV: IgM-positive (ratio 1.2) and IgG-negative; and chikungunya virus: IgM- and IgG-negative. These results confirmed this case as the second autochthonous case of dengue fever in Croatia.

Analysis of serum samples collected in the area

We collected 14 blood samples from healthy inhabitants living near the case's place of residence. The samples were analysed by ELISA for the presence of DENV and WNV IgM/IgG antibodies. Nine of those were found positive for DENV infection (IgG) and seven had positive or borderline results for DENV-specific IgM (Table 1).

A further 112 sera collected from anonymous patients who had sought medical care from various reasons during October 2010 were available at the laboratory of the local health centre. These sera were tested at

Table 1

Distribution of antibodies to dengue virus in nine persons from a pool of 14 neighbours of the autochthonous case from Pelješac, Croatia, October 2010

Examinee number	DENV IgM (ratio) [a]	DENV IgG (RU/ml) [b]
1	+ (2.4)	+ (155)
2	+/- (1.04)	+ (126)
3	+ (2.2)	+ (98)
4	+ (1.2)	+ (140)
5	- (0.3)	+ (170)
6	- (0.5)	+ (155)
7	+ (2.3)	+ (138)
8	+ (2.4)	+ (94)
9	+ (2.6)	+ (170)

DENV: dengue virus.

[a] ‹0.8negative (-), 0.8-1.1 borderline (+/-), ≥1.1 positive (+). Results are expressed as ratio according to the manufacturer's specifications.

[b] ‹16 negative (-), 16-22 borderline (+/-), ≥22 positive (+). Results are expressed in RU/ml according to the manufacturer's specifications.

Table 2

Distribution of antibodies to dengue virus in six anonymous serum samples, Croatia, October 2010

Examinee number	1	2	3	4	5	6
DENV IgM (ratio)[a]	+/- (0.9)	+/- (0.8)	+/- (1.08)	+ (4.6)	+ (2.2)	-
DENV IgG (RU/ml)[b]	+ (72)	+ (46)	+ (40)	+ (46)	+/- (‹2)	+ (153)

DENV: dengue virus.

[a] ‹0.8 negative (-), 0.8-1.1 borderline (+/-), ≥ 1.1 positive (+). Results are expressed as ratio according to the manufacturer's specifications.

[b] ‹16 negative (-), 16-22 borderline (+/-), ≥ 22 positive (+). Results are expressed in RU/ml according to the manufacturer's specifications.

Table 3

Adult mosquitoes caught in Podobuče, Orebić and Korčula, Croatia, October 2010

Species	Number
Aedes albopictus	49
Ochlerotatus mariae	4
Ochlerotatus sp.	2
Culex pipiens	5
Culiseta annulata	1
Total	**61**

CNIPH for the presence of DENV and WNV antibodies. Ethics approval was not required and informed consent was not sought. The work was carried out under the Communicable Disease Protection and Control Act which provides statutory support for investigations conducted for the purpose of communicable disease control.

Of those 112 samples, six were positive for DENV-specific antibodies. In all six positive sera, DENV-specific IgG was found (one sample with a borderline value). DENV-specific IgM were found in five sera: clearly positive in two and borderline in three (Table 2). All 112 sera were negative for WNV IgM and IgG.

Entomological investigation

During the field investigation the presence of mosquitoes was noticed, despite mandatory disinsection implemented on the entire Croatian territory. Mosquitoes were caught in the place of probable transmission and also on the island of Korčula in October 2011 with the aim of identifying the mosquito species present and determining whether they were carrying DENV. Two days after the mosquito had been caught, adulticidal and larvicidal disinsection was conducted at the village where the German patient had stayed.

The 61 caught mosquitoes were identified by an entomologist (Table 3). The species *Ae. albopictus* dominated (49 of 61).

Virological investigation was conducted for 44 *Ae. albopictus* adults in eight pools containing between five and seven mosquitoes. All eight pools tested negative for DENV in the RT-PCR conducted at the WHO Regional Reference Laboratory for Arboviruses at the Institute for Microbiology and Immunology in Ljubljana, Slovenia [11].

Discussion

After France, Croatia is the second country in Europe in which autochthonous transmission of dengue infection has been shown, which had not been recorded in Europe since the epidemic in Greece in 1925 to 1928 [12-15]. According to data of the communicable disease epidemiology service of the CNIPH, registered imported cases of dengue are not frequent (six cases in three years). Although until recently dengue fever was not a notifiable disease in Croatia, it is unlikely that the epidemiology service network which collaborates with the laboratories that conduct the diagnosis would have missed the occurrence of a confirmed case of imported dengue fever.

The assumption that the German tourist acquired dengue fever in the region of the Pelješac peninsula was confirmed by the identification of a second case of dengue fever in a local citizen who had not travelled outside the area. Although the antigen was not confirmed by RT-PCR in the acute serum of the patient, taken six days after illness onset, the presence of specific IgM antibodies (IgG was negative) pointed to acute infection. This was confirmed in a sample taken on day 19 of the illness when IgM and IgG antibodies were found.

Nine of the 14 samples taken from the Croatian patient's neighbours, none of whom had travelled outside Croatia, were IgG-positive, and we assume that these were relatively recent infections because seven of them were also IgM-positive. Some of them reported having an influenza-like disease in August and early September. Moreover, DENV-specific antibodies were found in 5.4% of the anonymous serum samples collected in October 2010 by the laboratory that covers the area of Pelješac and Korčula. Five of the six DENV-positive sera in this panel showed borderline or positive values of IgM antibodies against DENV. Based on the available serological and epidemiological data we therefore assume that a cluster of acute DENV infection occurred in the area, most probably during August and September 2010.

Each cluster of infectious diseases is reported using the national communicable diseases early warning system. During summer 2010 there were no such reports from Pelješac. Only four of the DENV-positive villagers contacted health services for febrile illness in August and September and were not recognised as an outbreak. At that time of year, there is an increased circulation of enteroviruses which can manifest with similar symptoms, but we believe that the main reason why dengue fever was not suspected in these patients is the fact that this illness had not been registered in Croatia or Europe so far and is therefore not considered in persons who have no travel history to endemic areas. Although the health services had been alerted of the possibility of new diseases transmitted by *Ae. albopictus*, particularly following the chikungunya fever outbreak in Italy in 2007 [16], it was only after our circulatory letter in October that dengue virus infection in local inhabitants was suspected by general practitioners and subsequently confirmed in the second autochthonous Croatian case described in this paper. There may also have been other dengue virus infections with an inapparent or mild course. We believe that other tourists staying in the area may have returned to their home countries with dengue fever, but there have been no reports of exported cases other than the German case.

It is likely that the dengue virus was imported into this community during the summer months of 2010. Regarding the manner of importation, we can only speculate. It could have been through infected travellers arriving from endemic areas in whom the infection was not recognised. Bearing in mind that *Ae. albopictus* species have spread along the Adriatic coast, also in the region of Pelješac and Korčula mainly through transport by sea [5], importation of infected mosquitoes in the same manner cannot be excluded. Since the role of transovarial transmission in mosquitoes is questionable [17,18], it is not likely that importation

happened through infected eggs or larvae (e.g. in used car tires).

Croatia will continue to alert health practitioners to the presence of this disease. Larvicidal and adulticidal mosquito control measures already applied in the affected area will be continued and expanded to the entire country to prevent further establishment of dengue fever or other diseases transmitted by the same vector, such as chikungunya fever [19,20]. Selection of blood donors in the country is in line with all internationally accepted criteria, in that any febrile illness in a potential donor presents a contraindication including the period of convalescence. This covers dengue virus infections well, as cases are only infective during the febrile phase and there is no chronic infection stage. A short prodromal period can pose a risk, but the regular delayed use of blood donations allows to investigate all donors who fall ill in the first two days after donation and to discard their blood if necessary. However, the possibility of transient asymptomatic dengue viraemia needs to be taken into account in practice if the disease were to become established.

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Notes

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Quarterly, print and online. In Dutch, summaries in English.
http://www.infectieziektebulletin.be

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Institut Scientifique de la Santé Publique, Brussels
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Bulgaria

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Cyprus

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Medical and Public Health Services, Ministry of Health, Nicosia
Biannual, print and online. In Greek.
http://www.moh.gov.cy

Czech Republic

Zpravy CEM (Bulletin of the Centre of
Epidemiology and Microbiology)
Centrum Epidemiologie a Mikrobiologie Státního
Zdravotního Ústavu, Prague.
Monthly, print and online. In Czech, titles in English.
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http://www.szu.cz/cema/epidat/epidat.htm

Denmark

EPI-NEWS
Department of Epidemiology, Statens Serum Institut, Copenhagen.
Weekly, print and online. In Danish and English.
http://www.ssi.dk

Finland

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Department of Infectious Disease Epidemiology, National Public Health Institute, Helsinki.
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Institut de veille sanitaire, Saint-Maurice Cedex.
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Germany

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Robert Koch-Institut, Berlin
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Hungary

Epinfo (az Országos Epidemiológiai Központ epidemiológiai információs hetilapja)
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http://www.oek.hu/oek.web?to=839&nid=41&pid=7&lang=hun

Iceland

EPI-ICE
Landlæknisembættið
Directorate Of Health, Seltjarnarnes
Monthly, online. In Icelandic and English.
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Ireland

EPI-INSIGHT
Health Protection Surveillance Centre, Dublin.
Monthly, print and online. In English.
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Italy

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Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome.
Monthly, online. In Italian.
http://www.iss.it/publ/noti/index.php?lang=1&tipo=4

Bolletino Epidemiologico Nazionale (BEN)
Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome.
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http://www.epicentro.iss.it/ben

Latvia

Epidemiologijas Bileteni
Sabiedribas veselibas agentura
Public Health Agency, Riga.
Online. In Latvian.
http://www.sva.lv/epidemiologija/bileteni

Lithuania

Epidemiologijos žinios
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Center for Communicable Disease Prevention and Control, Vilnius.
Online. In Lithuanian.
http://www.ulac.lt/index.php?pl=26

Netherlands

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Rijksinstituut voor Volksgezondheid en Milieu
National Institute of Public Health and the Environment, Bilthoven
Monthly, print and online. In Dutch.
http://www.rivm.nl/infectieziektenbulletin

Norway

MSIS-rapport
Folkehelseinstituttet, Oslo.
Weekly, print and online. In Norwegian.
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Poland

Meldunki o zachorowaniach na choroby zakazne i zatruciach w Polsce
Panstwowy Zaklad Higieny,
National Institute of Hygiene, Warsaw.
Fortnightly, online. In Polish and English.
http://www.pzh.gov.pl/epimeld/index_p.html#01

Portugal

Saúde em Números
Ministério da Saúde,
Direcção-Geral da Saúde, Lisbon.
Sporadic, print only. In Portuguese.
http://www.dgs.pt

Slovenia

CNB Novice
Inštitut za varovanje zdravja, Center za nalezljive bolezni, Institute of Public Health, Center for Infectious Diseases, Ljubljana.
Monthly, online. In Slovene.
http://www.ivz.si

Romania

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Centrul pentru Prevenirea si Controlul Bolilor Transmisibile, National Centre of Communicable Diseases Prevention and Control, Institute of Public Health, Bucharest.
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Spain

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Centro Nacional de Epidemiología, Instituto de Salud Carlos III, Madrid.
Fortnightly, print and online. In Spanish.
http://www.isciii.es/ htdocs/centros/epidemiologia/epi_boletines.jsp

Sweden

Smittskyddsinstitutets nyhetsbrev
Smittskyddsinstitutet, Stockholm.
Weekly, online. In Swedish.
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United Kingdom

England and Wales
Health Protection Report
Health Protection Agency, London.
Weekly, online only. In English.
http://www.hpa.org.uk/hpr

Northern Ireland
Communicable Diseases Monthly Report
Communicable Disease Surveillance Centre, Northern Ireland, Belfast.
Monthly, print and online. In English.
http://www.cdscni.org.uk/publications

Scotland
Health Protection Scotland Weekly Report
Health Protection Scotland, Glasgow.
Weekly, print and online. In English.
http://www.hps.scot.nhs.uk/ewr/index.aspx

Other journals

EpiNorth journal
Norwegian Institute of Public Health, Folkehelseinstituttet, Oslo, Norway
Published four times a year in English and Russian.
http://www.epinorth.org

European Union
"Europa" is the official portal of the European Union. It provides up-to-date coverage of main events and information on activities and institutions of the European Union.
http://europa.eu

European Commission - Public Health
The website of European Commission Directorate General for Health and Consumer Protection (DG SANCO).
http://ec.europa.eu/health/index_en.htm

Health-EU Portal
The Health-EU Portal (the official public health portal of the European Union) includes a wide range of information and data on health-related issues and activities at both European and international level.
http://ec.europa.eu/health-eu/index_en.htm

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