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Evaluation of a West Nile virus surveillance and early warning system in Greece, based on domestic pigeons



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ABSTRACT

In the summer of 2010 an epidemic of West Nile virus (WNV) occurred in Central Macedonia, Greece, with 197 human neuroinvasive disease (WNND) cases. In the following years the virus spread to new areas, with a total of 76 WNND cases in 2011, and 109 WNND cases in 2012 (14 and 12 WNND cases, respectively, in Central Macedonia). We established a surveillance system based on serological testing of domestic pigeons, using cELISA confirmed by serum neutralization test. In Central Macedonia, pigeon seroprevalence was 54% (95% CI: 49–59%) and 31% (95% CI: 24–37%) at the end of the 2010 and 2011 epidemic seasons, respectively. One serum was positive for neutralizing antibodies directed against Usutu virus. Pigeon WNV seroprevalence and incidence rates of human WNND after the 2010 epidemic were positively correlated ($\rho=0.94$, at the regional unit level), while in 2011 the correlation ($\rho=0.56$) was not statistically significant, possibly due to small number of human WNND cases recorded. To evaluate the efficacy of the system at alerting upon WNV enzootic circulation before the onset of human cases, we tested 270 pigeons in 2011 and 240 pigeons in 2012. In Central Macedonia, the first seroconversions in pigeons were recorded 44 and 47 days, respectively, before the first human WNND cases. Pigeon surveillance was used successfully for identification of areas with WNV enzootic transmission and for early warning. Timely diffusion of information to health authorities facilitated the implementation of preparedness plans to protect public health.

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1. Introduction

West Nile virus (WNV) is an RNA virus within the Japanese encephalitis virus group (genus *Flavivirus*, family

Flaviviridae) [1]. WNV is transmitted by *Culex* mosquitoes in a cycle involving birds as amplifying hosts. Infected mosquitoes carry WNV in their salivary glands and infect susceptible bird species during blood-meal feeding [2]. A mosquito requires approximately 10–14 days after its initial blood meal to become infectious and to transmit WNV to other animals. This time interval is known as the extrinsic incubation period [3]. Spillover infections may occur

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in mammals (including horses and humans), which are regarded as incidental or dead-end hosts, since they do not produce significant and long-lasting viremia, and do not contribute to the virus transmission cycle [2].

Antibodies against WNV have been present in domestic animals, as well as in ~1% of the humans in Greece since the 1960s [4–8]. Hemagglutination-inhibiting (HAI) antibodies against WNV were identified in sera obtained from sheep, goats, bovine, horses, mules, as well as in one rabbit, one hare and one pig. Furthermore, HAI antibodies against WNV were detected in sera obtained from pigeons, chickens, turkeys, a common snipe (*Capella gallinago*) and an Eurasian collared dove (*Streptopelia decaocto*) [5]. However, human WNV cases were not reported before 2010.

In 2010, a WNV epidemic occurred in Greece, with 197 human cases that suffered from neurological signs (WNND) and 35 fatalities [9]. Two other epidemics occurred the following years, with 76 human WNND cases, as well as 8 fatalities in 2011 and 109 reported WNND cases in humans and 16 fatalities in 2012 [10,11]. A lineage 2 WNV strain named “Nea Santa-Greece-2010” was detected in pools of *Culex pipiens* mosquitoes [12–14], a donated human blood sample [15], a Belgian traveler returning from Greece [16], captive sentinel chickens [14,17,18] and an Eurasian magpie (*Pica pica*) [19], between 2010 and 2012.

Worldwide surveillance efforts to detect WNV before infection of incidental hosts (humans, horses) were based on collection and testing of adult mosquitoes and/or birds [20,21]. Four categories of birds have been used for WNV surveillance; dead wild birds, trapped wild birds, captive sentinel birds, or domestic sentinel birds [22]. In the USA the value of using WNV-infected dead birds as an indicator of increased WNV disease risk has been demonstrated in several studies [23–25]. However, in Europe, and especially in Greece, dead bird surveillance could not be applied, since abnormal bird mortalities were not observed [19,26].

Sentinel birds can be used to detect the presence of WNV in a geographical location. An ideal sentinel bird is a species that is susceptible to infection, is resistant to disease, develops a detectable immune response rapidly, is maintained easily, presents negligible health risks to handlers, does not contribute to local pathogen transmission cycles and seroconverts to the target pathogen prior to the onset of disease outbreaks in the community [22]. Chickens and pigeons develop low-level WNV viremia [27] and are maintained easily in captivity, making them ideal sentinel species [28]. Sentinel chicken – based WNV surveillance systems have provided evidence of WNV transmission several weeks before the occurrence of human cases [29]. Moreover, pigeons have already been identified as potential sentinel birds [22]. Consequently, active surveillance of domestic birds has been used successfully in America and Europe for their early warning capacity [30–35].

The present study was initiated in Greece at the end of the 2010 WNV epidemic. It comprised two sub-studies with the following objectives: (a) to determine the geographical spread of WNV, immediately after the 2010 and 2011 epidemic seasons, using WNV antibodies in juvenile domestic pigeons (*Columba livia domestica*) as indicators of WNV circulation, (b) to assess the correlation between the WNV point seroprevalence in pigeons and the incidence

rates of human WNND cases (first study), and (c) to evaluate the early warning capacity of a pigeon surveillance system, e.g. its capacity to provide data on WNV circulation prior to the onset of human cases in Central Macedonia, during the 2011 and 2012 epidemic seasons (second study).

2. Materials and methods

2.1. First study: WNV point seroprevalence in juvenile pigeons after the 2010 and 2011 epidemics, and correlation with incidence rates of human WNND

2.1.1. Sampling in domestic pigeons

Two samplings were performed in juvenile domestic pigeons, in order to determine the spread of WNV in selected areas in Greece after the end of the 2010 and 2011 epidemics, respectively (Fig. 1). The first sampling took place between October 2010 and February 2011 and 655 pigeons (131 pigeon pens, 5 pigeons per pen) were sampled. During this period, 430 sera were obtained from pigeons in Central Macedonia (the area worst affected during the 2010 epidemics), specifically from the regional units (prefectures; geographical areas equivalent to Nomenclature of Territorial Units for Statistics – NUTS level 3 areas) of Imathia, Pella, Kilkis and Thessaloniki. In addition, 110 sera were obtained from Eastern Macedonia (Kavala) and Thrace (Xanthi), 65 from Thessaly (Larissa and Magnissia) and 50 from Attica. The second sampling took place in October 2011, where 210 pigeons were sampled (42 pigeon pens, 5 pigeons per pen). This sampling was limited to Central Macedonia.

A two-stage stratified cluster sampling methodology was adapted in order to select a random sample of pigeons. The study area was divided into two strata: urban and rural areas. In the first stage, pens (clusters) were randomly selected from the study area using probability proportional to the human population size of the urban or rural areas. In the second stage, 5 pigeons were included in the study per selected pen. The pigeons were ringed and the coordinates of each pen were recorded using Global Positioning Systems (GPS) technology. All samples were obtained from pigeons over 45 days old, to ensure that the detected antibodies did not result from passive transfer of maternal IgY [36]. Given that there were no data on WNV circulation in pigeons prior to the 2010 epidemic, all pigeons that were sampled during October 2010–February 2011 period were younger than 12 months old, in order to exclude infections before 2010. In 2012, all sampled pigeons were either younger than 5 months old, or seronegative during the previous sampling period.

Blood samples were drawn with sterile syringes from the brachial vein. The volume of blood extracted did not exceed 500 µl. Blood was collected in 1.5 ml microcentrifuge tubes, allowed to clot and the tubes were centrifuged (21,000 × g, 5 min, 4 °C). Sera were transferred to clear microcentrifuge tubes and stored at –80 °C until they were assayed.

2.1.2. Serological testing

All sera ($n=865$) were tested for the presence of antibodies against WNV envelope protein (E), using

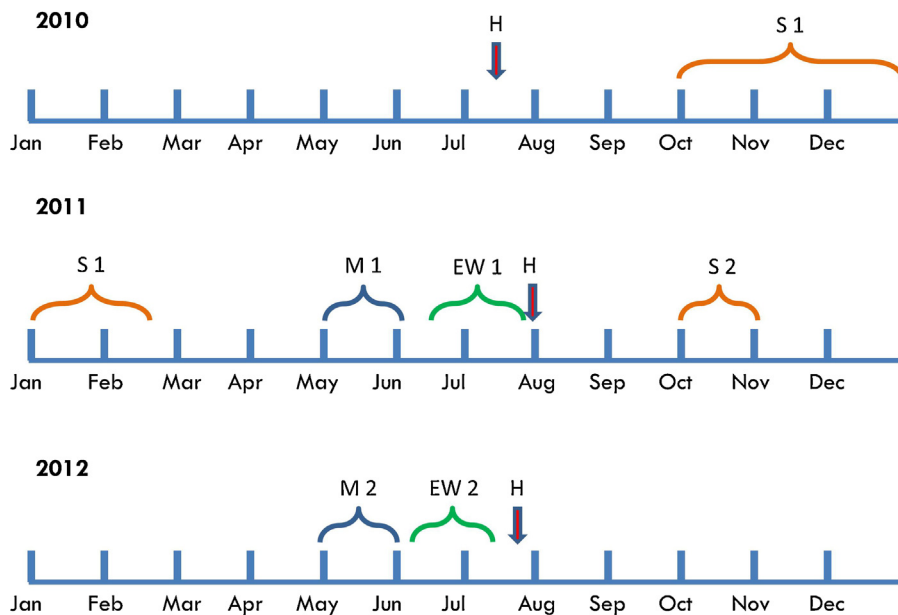


Fig. 1. Timeline of the samplings performed in domestic pigeons in Greece, during the 2010–2012 period. S 1, S 2: serum samplings in juvenile domestic pigeons for the determination of WNV spread after the 2010 and 2011 epidemics, respectively. EW 1, EW 2: serum samplings in juvenile domestic pigeons for early warning. H: onset of human WNNND cases. Collection of mosquitoes is also indicated (M 1, M 2).

a commercially available competitive enzyme-linked immunosorbent assay (cELISA) kit (ID Screen® West Nile Competition, ID.vet Innovative Diagnostics, Montpellier, France), according to the manufacturer's instructions with modifications. Sera were incubated overnight (16–20 h) at 4 °C to increase the assay sensitivity. Following the incubation, the plate was washed 5 times and 100 µl of HRP-conjugated anti-E antibody diluted 1:9 in dilution buffer were added to the wells. The plate was incubated for 1 h at room temperature and washed 5 times. After addition of TMB substrate, the plate was incubated at 21 °C for 20 min. OD values were recorded at 450 nm using a microplate spectrophotometer. Interpretation of the results was performed according to the manufacturer's criteria.

A representative number (25%) of the cELISA-positive sera were further assayed by microtiter virus neutralization test (micro-VNT) for the detection of neutralizing antibodies against WNV. Micro-VNT was performed in 96-well cell culture plates, following an existing protocol with modifications [37]. After heat inactivation at 56 °C for 30 min, sera were two-fold serially diluted (1:5–1:1280) in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen-Gibco, Groningen, The Netherlands) and mixed with an equal volume (50 µl) of DMEM containing 100 50% tissue culture infectious doses (TCID₅₀) of WNV, Is98 strain (kindly provided by Dr. Philippe Desprès, Institut Pasteur, France). The titer of the virus was determined in Vero cells (ATCC catalog no. CCL-81, ATCC, Manassas, VA), using the Reed and Muench formula. Each serum was tested in duplicate. Cell and virus (100 TCID₅₀ of WNV) controls, as well as reference sera (positive and negative), were added onto each plate. Moreover, 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ dilutions of the virus were prepared for back titration. After

incubation of the plates at 37 °C for 1.5 h, 2 × 10⁴ Vero cells in 100 µl of DMEM with 2% penicillin (100 IU/ml) and streptomycin (100 µg/ml), 2% sodium pyruvate and 10% fetal bovine serum (FBS; Invitrogen-Gibco, Groningen, The Netherlands) were added to every well. Plates were incubated at 37 °C for 3 days and wells were examined under an inverted light microscope for evidence of viral cytopathic effect (CPE). In order for the results to be valid, all the following criteria had to be met: (a) CPE was absent in the cell control wells, (b) CPE was present in the virus control wells, (c) back titration of the virus indicated that the virus titer per well was between 75 and 125 TCID₅₀ per well, (d) CPE was observed in every well with the negative reference serum, and (e) the positive reference serum protected cells from infection, and the average neutralizing antibody titer of this serum was 1:80. A test serum was considered negative if CPE was observed at each serum dilution and considered positive if cells were protected at ≥1:10 serum dilution. The serum neutralizing antibody titer was calculated as the highest serum dilution that conferred complete protection to the cell layer.

Samples that were positive by cELISA and negative by WNV micro-VNT, as well as samples with weak antibody titers against WNV, were tested to determine the neutralizing antibody endpoint titers against Usutu virus (USUV). Micro-VNT was carried out as described above, with the USUV strain SAAR-1776 (kindly provided by Dr. Philippe Desprès, Institut Pasteur, France).

2.1.3. Human data

For WNV surveillance in humans, healthcare providers were asked to report laboratory confirmed WNNND cases (encephalitis, aseptic meningitis, or acute flaccid paralysis), or West Nile fever (WNF), to the Hellenic Center for Disease

Control and Prevention (HCDCP). However, the surveillance system was more likely to ascertain WNND cases due to more pronounced clinical signs. Thus, only WNND cases were included in the analysis of incidence of human cases.

2.1.4. Statistical analysis

Seroprevalence rates of pigeons were calculated per regional unit (NUTS level 3 areas) and municipality (equivalent to Local Administrative Unit – LAU level 1 areas) using the total number of tested pigeons as denominator. Incidence rates of human WNND were calculated using the 2011 census data of the Hellenic Statistical Authority (HSA) [38] as denominators. Regional units and municipalities were used as the unit of analysis for both 2010 and 2011 transmission seasons. For proportions, 95% confidence intervals (CIs) were calculated. Municipalities with less than three pigeon pens tested were excluded from this analysis to avoid inadequate numbers for reliable conclusions.

Spearman's rank correlation coefficients (ρ) were calculated to assess if the incidence rates of human WNND and the WNV point seroprevalence in juvenile pigeons were correlated or not, in 2010 and 2011. The variables were considered highly correlated, if ρ was close to 1. Additionally, Poisson regression was applied to assess the association between the incidence rates of human WNND and WNV point seroprevalence in juvenile pigeons. Incidence rate ratios (IRRs) were also calculated for both years. The analysis was carried out using STATA version 10 software (Stata Corporation LP, TX, USA).

2.2. Second study: evaluation of the early warning capacity of domestic pigeons and detection of the circulating WNV strain

2.2.1. Sampling in domestic pigeons

Two additional samplings were conducted in Central Macedonia in 2011 and 2012 to evaluate the early warning capacity of using domestic pigeons in a WNV surveillance system. Exact sample collection time from pigeons varied and was based on local temperatures and mosquito population data, in order to increase the probability of detection of WNV enzootic transmission early in the season, and before human outbreaks. More specifically, data on mosquito populations were collected in May 2011 and 2012, from 28 and 23 sampling stations, respectively, in Central Macedonia. The collection of adult mosquitoes was carried out with CO₂ traps every two weeks. The traps were placed within buffer zones of 5 km around the pigeon pens (considering the mean flying distance of *Culex*, *Anopheles* and *Aedes*, the three main mosquito genera present in Greece), and their positions were mapped with ArcGIS version 10.1 software (ESRI Inc., Redlands, CA, USA). Each mosquito collection session lasted for a period of 18–24 h. Counting and genus determination of adult mosquitoes were performed using a stereomicroscope and dichotomous determination keys [39]. Following the California Mosquito-Borne Virus Surveillance & Response plan [40], the criteria for the initiation of sample collection from pigeons were the following; (a) the average daily temperature range in the area, during the previous two weeks, had

to be between 18.6°C and 21.2°C, and (b) more than 200 adult *Culex* spp. mosquitoes per night and CO₂ trap had to be collected, within a buffer zone of 5 km around the participating pigeon pens.

As a result, the first pigeon sampling took place from June 15 to July 29, 2011, where 270 pigeons were sampled, while the second one was performed between June 6 and July 9, 2012, with 240 sampled pigeons (54 and 48 pigeon pens, respectively, 5 pigeons per pen) (Fig. 1). The sampling was performed as described above (Section 2.1.1), and all sampled pigeons were either younger than 5 months old, or seronegative during the previous sampling period.

2.2.2. Serological testing

All the collected sera ($n=510$) were assayed by cELISA as described previously (Section 2.1.2). Moreover, 100% of the cELISA-positive sera were tested by micro-VNT against WNV and USUV as described above (Section 2.1.2).

2.2.3. Molecular detection of WNV

RNA was extracted from all 510 sera obtained from the pigeons, using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For the molecular detection of WNV, RNA extracts were examined using a one tube real-time RT-PCR protocol, as described by Chaskopoulou et al. [14].

2.2.4. Human data

The data on human WNND cases collected from HCDCP (Section 2.1.3) was also used for the early warning study.

3. Results

3.1. First study: WNV point seroprevalence in juvenile pigeons after the 2010 and 2011 epidemics and correlation with incidence rates of human WNND

3.1.1. Geographical spread of WNV based on point seroprevalence in juvenile pigeons

After the 2010 epidemic season, ELISA testing on sera sampled in October 2010–February 2011 showed the presence of flavivirus-specific antibodies in 247 out of 655 analyzed pigeons (38%; 95% CI: 34–42%). WNV point seroprevalence in juvenile pigeons after the 2010 epidemics differed by regional units as follows: for Northern Greece/Central Macedonia: 232 out of 430 pigeons (54%; 95% CI: 49–59%) were found seropositive. The pigeon seroprevalence in the urban complex of Thessaloniki was significantly lower than the neighboring rural areas (Table 1). In Eastern Macedonia-Thrace, which is located east of Thessaloniki, WNV circulation in juvenile pigeons was low: 12 out of 110 pigeons (11%; 95% CI: 6–18%), whereas southwards, in Central Greece/Thessaly, the WNV point seroprevalence in juvenile pigeons was even lower: 3 out of 65 pigeons (5%; 95% CI: 1–13%). Finally, in Attica, the WNV point seroprevalence in juvenile pigeons was null, as WNV-specific antibodies were not detected in any of the 50 sera tested (0%; 95% CI: 0–7%) (Table 1 and Fig. 2).

WNV-specific antibodies, as determined by serum neutralization test were present in 61 out of 62 (~25% of the 247) cELISA-positive sera tested, and WNV neutralizing

Table 1

Number and seroprevalence of WNV-seropositive pigeons recorded after the 2010 and 2011 WNV epidemic seasons, and incidence rates (per 100,000 population) of human WNND cases by area.

Area/regional unit		2010 pigeons number seroposi- tive/number tested (WNV point seroprevalence)	2010 humans ^a number of WNND cases (incidence rate per 100,000)	2011 pigeons number seroposi- tive/number tested (WNV point seroprevalence)	2011 humans ^a number of WNND cases (incidence rate per 100,000)
Central Macedonia	Imathia	88/130 (68%; 95% CI: 59–76%)	39 (27.7)	32/90 (36%; 95% CI: 26–46%)	3 (2.1)
	Pella	87/130 (67%; 95% CI: 58–75%)	41 (29.4)	22/75 (29%; 95% CI: 19–40%)	5 (3.6)
	Kilkis	11/20 (55%; 95% CI: 32–77%)	12 (14.9)	0/20 (0%; 95% CI: 0–17%)	0 (0.0)
	Thessaloniki R	32/70 (46%; 95% CI: 34–58%)	33 (9.3)	10/20 (50%; 95% CI: 27–73%)	2 (0.6)
	Thessaloniki U	14/80 (18%; 95% CI: 10–28%)	27 (3.6)	0/5 (0%; 95% CI: 0–52%)	4 (0.5)
Eastern Macedonia-Thrace	Kavala	11/40 (28%; 95% CI: 15–44%)	1 (0.8)	N/A	0 (0.0)
	Xanthi	1/70 (1%; 95% CI: 0–8%)	0 (0.0)	N/A	0 (0.0)
Thessaly	Larissa	3/50 (6%; 95% CI: 1–17%)	8 (2.8)	N/A	12 (4.2)
	Magnissia	0/15 (0%; 95% CI: 0–28%)	0 (0.0)	N/A	0 (0.0)
Attica		0/50 (0%; 95% CI: 0–7%)	0 (0.0)	N/A	21 (0.5)

R, rural areas; U, urban complex; CI, confidence interval; N/A, not applicable.

^a Human WNND cases in areas where pigeon surveillance was not applied were not included in the table.

antibody titers ranged between 1:10 and 1:320. In brief, in 15 out of the 61 pigeon sera (25%; 95% CI: 15–37%) a weak neutralizing response was observed (titers of 1:10 or 1:20), while 29 out of the 61 pigeon sera (48%; 95% CI: 35–61%) had a high WNV neutralizing activity (titers 1:80 and above). Only one of the 62 cELISA-positive sera (2%; 95% CI: 0–9%) was found negative by WNV micro-VNT (titer < 1:10) but positive by USUV micro-VNT, with an antibody titer of 1:40. The pigeon which was found positive for USUV neutralizing antibodies was sampled from a pigeon pen in Veria city (Latitude: 40°30'45", Longitude: 22°12'36"), on November 17, 2010.

After the 2011 epidemic season in Central Macedonia, WNV point seroprevalence in juvenile pigeons was found to be lower than the corresponding seroprevalence value from 2010. Particularly, 64 out of 210 tested pigeons (31%; 95% CI: 24–37%) were found seropositive by cELISA (Table 1). WNV neutralizing antibodies were detected in 100% of the 16 (25% of 64 sera) cELISA-positive sera tested, with antibody titers ranging between 1:20 and 1:80.

3.1.2. Correlation of WNV point seroprevalence in pigeons with incidence rates of human WNND

Overall, 197 and 76 human WNND cases were reported to HCDCP in 2010 and 2011, respectively. Of these cases, 161 (81.7%) and 14 (18.4%) were reported from regional units where domestic pigeons were being monitored, respectively [9,10]. Specifically, during 2010, WNND cases were reported in 60 humans in Thessaloniki (27 in urban and 33 in rural areas), 41 in Pella, 39 in Imathia and 12 in Kilkis. Furthermore, 8 WNND human cases were recorded in Larissa and 1 case was recorded in Kavala (Table 1) [9].

During 2011, 6 cases were recorded in Thessaloniki (4 in urban and 2 in rural areas), 5 cases were recorded in Pella, and 3 cases in Imathia (Table 1) [10].

In 2010 the observed Spearman's rank correlation coefficient (ρ) between the incidence rates of human WNND by municipality and the WNV point seroprevalence in juvenile pigeons was as high as 0.54 (P -value = 0.01). When the incidence rates of human WNND and WNV point seroprevalence in juvenile pigeons were considered at the regional unit level (larger scale of analysis), the correlation between these two variables was even higher (ρ = 0.94, P -value < 0.001) (Fig. 3). In 2011, the Spearman's rank correlation coefficients between the incidence rates of human WNND and the WNV point seroprevalence in juvenile pigeons were 0.32 (P -value = 0.47) and 0.56 (P -value = 0.32) at the municipality and regional unit levels, respectively (Fig. 3). Poisson regression model suggested that the incidence of WNND human cases in municipalities was almost 16 (IRR 15.5; 95% CI: 9.5–25.2) and 21 (IRR 21.2; 95% CI: 0.7–634) times higher, for every percentage increase in the WNV point seroprevalence in juvenile pigeons in 2010 and 2011, respectively.

3.2. Second study: evaluation of the early warning capacity of domestic pigeons and detection of the circulating WNV strain

During the 2011 early warning sampling period (15 June–29 July), WNV seroconversions were detected in 20 out of the 270 pigeons sampled from Central Macedonia (7%; 95% CI: 5–11%). Specifically, 11 of these pigeons were detected in Pella, where the first 3 seroconverted pigeons

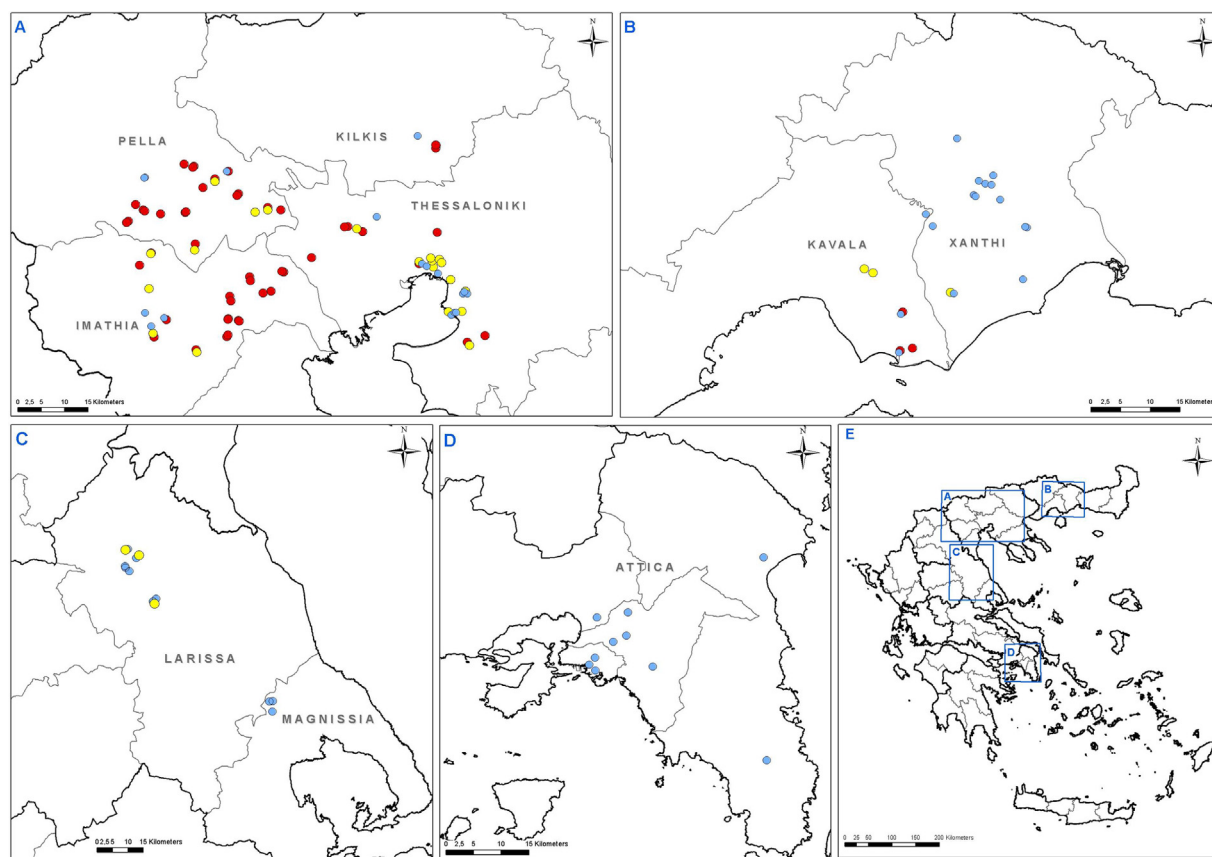


Fig. 2. Locations of the pigeon pens sampled after the 2010 epidemic period in Greece. (A) Central Macedonia (Thessaloniki, Kilkis, Imathia and Pella), (B) Eastern Macedonia (Kavala) and Thrace (Xanthi), (C) Thessaly (Magnissia and Larissa) and (D) Attica. Blue dots represent 0/5 seropositive pigeon per pigeon pen, yellow dots represent 1–2/5 seropositive pigeons per pen, while red dots represent 3–5/5 seropositive pigeons per pen. Serological results indicate that during the 2010 epidemic, WNV circulation was low in Eastern Macedonia-Thrace and Thessaly, whereas it was absent from Southern Greece (Attica).

Table 2

Number and percent seroconversions of the pigeons tested prior to the 2011 and 2012 WNV epidemic seasons, and incidence rates (per 100,000 population) of human WNND cases by area.

Regional unit	2011 pigeons number seroconverted/number tested (% seroconversions)	2011 humans number of WNND cases (incidence rate per 100,000)	2012 pigeons number seroconverted/number tested (% seroconversions)	2012 humans number of WNND cases (incidence rate per 100,000)
Imathia	4/95 (4%; 95% CI: 1–10%)	3 (2.1)	4/90 (4%; 95% CI: 1–11%)	3 (2.1)
Pella	11/100 (11%; 95% CI: 6–19%)	5 (3.6)	2/80 (3%; 95% CI: 0–9%)	2 (1.4)
Kilkis	0/20 (0%; 95% CI: 0–17%)	0 (0.0)	2/20 (10%; 95% CI: 1–32%)	0 (0.0)
Thessaloniki R	5/35 (14%; 95% CI: 4–30%)	2 (0.6)	4/30 (13%; 95% CI: 4–31%)	6 (1.7)
Thessaloniki U	0/20 (0%; 95% CI: 0–17%)	4 (0.5)	0/20 (0%; 95% CI: 0–17%)	1 (0.1)
Total	20/270 (7%; 95% CI: 5–11%)	14 (1.0)	12/240 (5%; 95% CI: 3–9%)	12 (0.8)

R, rural areas, U, urban complex, CI, confidence interval.

were detected on June 15–16, four in Imathia, where the first seroconverted pigeon was sampled on June 27, and 5 in Thessaloniki, with the first one being detected on July 6 (Table 2 and Fig. 4). The first seroconverted pigeons were sampled and detected right at the beginning of the sampling period, about 1.5 months prior to the diagnosis of the first human WNND case in Central Macedonia. Specifically, the first human case in the area was diagnosed on July 29,

in a resident of the urban complex of Thessaloniki. The second human WNND case was diagnosed on August 2, in a resident of Agkathia village (Imathia). Twelve more WNND cases in humans followed (Table 2).

In June 2012, WNV seroconversions were detected in 12 out of the 240 pigeons sampled (5%; 95% CI: 3–9%) from Central Macedonia. Two of these pigeons were sampled from Kilkis (June 7), 4 more pigeons were detected in

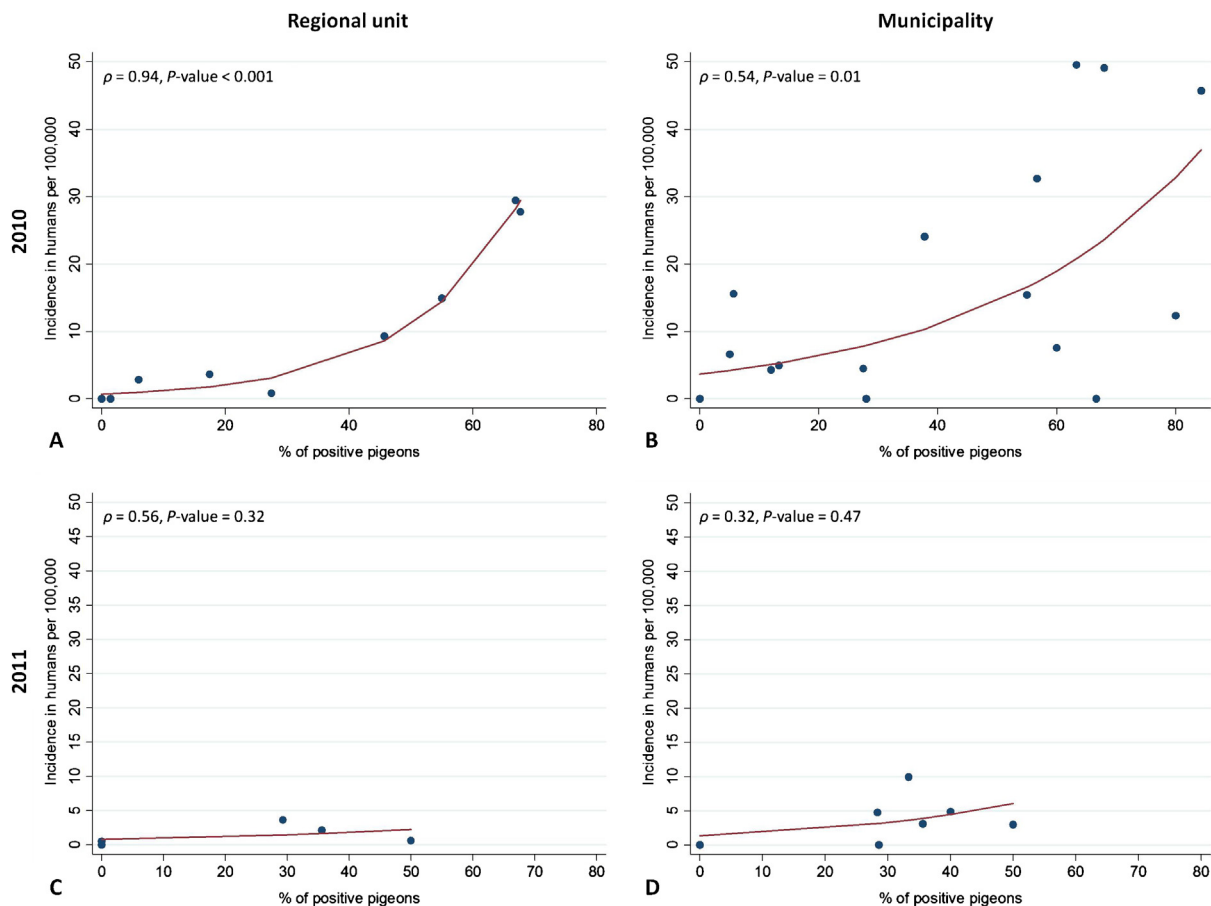


Fig. 3. Correlation matrix and associations (Poisson regression model) between the WNV point seroprevalence in juvenile pigeons and the incidence rates of reported human WNND cases by regional unit (left) and municipality (right) of residence, in 2010 (A and B, respectively) and in 2011 (C and D, respectively). The prediction of curves was performed from Poisson regression. ρ : Spearman's rank correlation coefficient.

Imathia (June 13), while seroconversions were detected in 2 more pigeons from Pella (June 20), as well as in 4 pigeons sampled in the rural areas of Western Thessaloniki (June 22) (Table 2 and Fig. 4). The first human WNND case in Central Macedonia was diagnosed on July 24 in a resident of Diavata town (rural area-Western Thessaloniki), about 1.5 month after the sampling and detection of the first seroconverted pigeons in the study area. The second human WNND case was diagnosed on July 27, in Palaio Zervochori village (Imathia). Ten more WNND cases in humans followed (Table 2).

The presence of WNV specific antibodies subsequent to WNV infections were confirmed by micro-VNT in all 32 cELISA-positive pigeon sera obtained during the 2011 and 2012 early warning periods. None of the 510 tested serum samples collected during the two early warning periods was found positive by real-time RT-PCR.

4. Discussion

This is the first time that domestic pigeons were evaluated extensively for their efficacy in providing data on the circulation of a lineage 2 WNV strain, during

two consecutive years in a highly affected area. Through serological assays performed in different areas of Greece during October 2010–February 2011, a high WNV point seroprevalence in pigeons, implying intense virus circulation, was evidenced in Central Macedonia, and particularly in the Regional Units of Imathia, Pella, Kilkis, and the rural areas of Western Thessaloniki, indicating the epicenter of the 2010 epidemic. Contrary, seroprevalence in pigeons from the urban complex of Thessaloniki was found significantly lower than the values obtained from the neighboring rural areas (Table 1). This lower rate of virus spread is a possible indication that the city urban complex comprises an environment with a different host-exposure and virus transmission patterns.

The pattern of the WNV epidemics in humans and animals in 2011 and 2012 suggests that after its exceptional amplification in 2010, WNV overwintered in Greece and subsequently spread to Central and Southern Greece. Our data indicate that during 2010 the virus was already present in North-Eastern (Xanthi) and Central Greece (Thessaly), but not in Attica. WNND and WNF cases in humans were reported in Attica one year later, in 2011 (Table 1) [10]. We assume that WNV was introduced in Attica during the spring of 2011. This further supports a

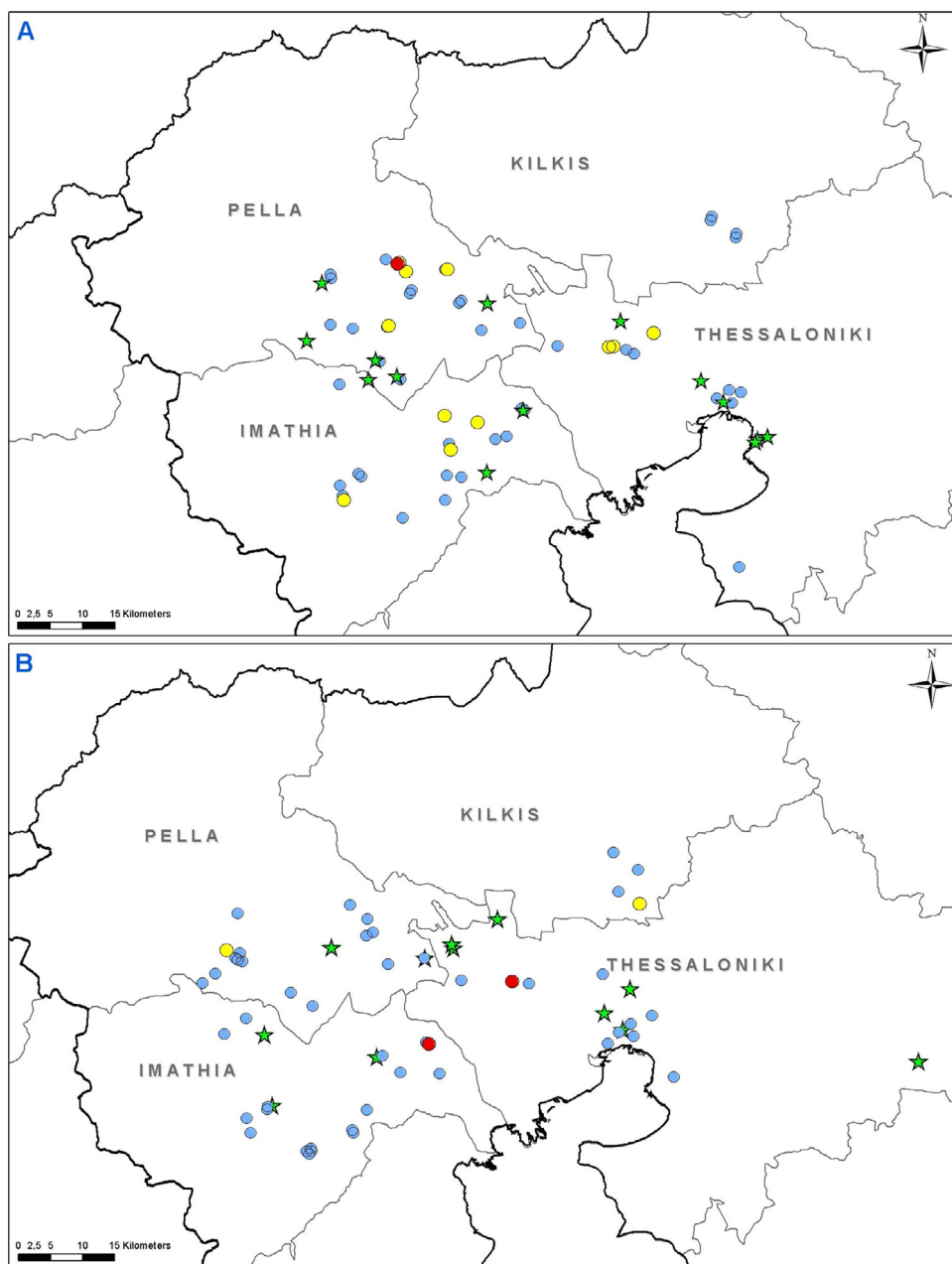


Fig. 4. Locations of the pigeon pens sampled during the 2011 (A) and 2012 (B) early warning sampling periods in Central Macedonia. Blue dots represent 0/5 seropositive pigeon per pigeon pen, yellow dots represent 1–2/5 seropositive pigeons per pen, while red dots represent 3–5/5 seropositive pigeons per pen. Stars depict the human WNND cases recorded during the 2011 and 2012 (14 and 12 cases, respectively) WNV epidemic seasons, in residential areas adjacent to the pigeon pens. During the 2011 early warning period, 20 seroconverted pigeons were identified before the onset of WNND cases in humans. During the 2012 early warning period, 12 seroconverted pigeons were identified before the onset of WNND cases in humans. For both periods, the first seroconversions in pigeon pens were detected at least 1.5 months prior to human cases in Central Macedonia.

study conducted in wild birds (Eurasian magpies), where sera obtained in February 2011 were found negative for WNV-specific antibodies [19].

In Central Macedonia, WNV point seroprevalence in domestic pigeons and WNND cases in humans for 2011 was reduced compared to the 2010 data, indicating a lower level of virus circulation in this initial focus (Table 1). Our serological results suggested that high numbers of

pigeons were found seropositive after the 2010 epidemic season, in areas west of Thessaloniki (seroprevalence > 67%, Table 1). Based on these results, we can hypothesize that wild birds, which act as amplification hosts of WNV, were also highly affected during the 2010 epidemic and the resulting herd immunity among them might be a contributing factor for the less intense WNV circulation observed in 2011 (Table 1).

At the end of the 2010 epidemic, WNV point seroprevalence in juvenile domestic pigeons was positively associated with the incidence rates of WNND in humans in the same geographic areas. By applying the Poisson regression model, a strong correlation between WNV point seroprevalence in domestic pigeons and the incidence of WNND in humans was observed, when data analysis was conducted at the regional unit level. When the Poisson regression model was applied at a smaller size scale (municipality level), the correlation appeared to be lower, a fact that might be attributed to the lower number of data available per municipality (figures more prone to random fluctuations). Such a strong correlation between the obtained values in domestic pigeons and humans suggests that domestic pigeon surveillance could be a valuable system for the monitoring of WNV in rural areas. After the 2011 outbreak, a tendency for positive correlation was observed between incidence of WNND in human and WNV seroprevalence in pigeons, but this correlation was not statistically significant, which was possibly related to small numbers of human WNND cases reported in 2011.

Early enzootic transmission of WNV in the epicenter of the 2010 epidemic was successfully determined during 2 consecutive years (2011 and 2012), at least 1.5 months before the onset of human cases. This enabled the timely dissemination of information to public health authorities, in order to increase preparedness and implementation of adapted vector control measures and minimize the impact of the epidemic for humans and horses. The sensitivity of this WNV surveillance system based on pigeons is comparable to other surveillance systems applied for WNV surveillance in other countries [30–35], as well as in Greece [14,17,18]. In our case, and in order to increase the possibilities to detect seroconverted pigeons early in the epidemic seasons, we monitored mosquito populations and environmental temperatures, before the initiation of the pigeon samplings. In this regard, it should be noted that some of the detected seroconversions might have occurred long before the onset of the samplings. Overall, our data indicate that that pigeons used as sentinels before the onset of human WNND cases can constitute one of the important prediction factors of the incidence rates of WNND cases in humans in the following months. It is interesting that, despite the small number of areas studied, the comparison of the percentages of seroconverted pigeons early in the season and incidence rates of human WNND cases in Central Macedonia between the two epidemics (2011 and 2012), showed similar results (7% and 1.0, compared to 5% and 0.8, respectively) (Table 2). However, it should be noted that prediction of human case incidence based on seroconverted pigeons early in the season, is not an easy task to achieve, since it depends not only on the initial foci of WNV enzootic circulation, but also on the immunity of the susceptible animals and humans, as well as on the mosquito populations during the epidemic. In order to perform such predictions and determine relationships between the seroconverted pigeons at the beginning of the epidemic season and the incidence rates of human WNND cases at the end of the season, all factors that contribute to changes in the mosquito populations during the whole epidemic period (e.g. climatic conditions, migration of mosquitoes

and human interventions – larviciding and adulticiding operations), should be taken into account. More research is necessary to assess the effects of initial WNV enzootic circulation foci in relation to bird populations, herd immunity, mosquito populations, and climatic conditions for the final prediction of the WNND human cases onset.

eELISA-positive results were confirmed by WNV micro-VNT in all of the tested sera, with only one exception, where the detected antibodies were specifically directed against USUV and not against WNV. Such a case highlights the importance of systematically confirming ELISA results by performing serum neutralization tests, in order to avoid misdiagnosis due to frequent immunological cross-reactivities within flaviviruses. Nevertheless, in our case, since the majority of the eELISA-positive results were due to WNV infections, data obtained from ELISA testing could reliably be used for further analysis (e.g. statistical tests, temporal and spatial epidemiological analysis). This is the first report of serological evidence of USUV infection in Greece. USUV is also a member of the genus *Flavivirus* and the Japanese encephalitis serocomplex [41]. In Europe, USUV emerged in Austria in 2001 [42] and outbreaks have also been reported in Hungary [43], in Switzerland, in Italy [44], in Spain [45], and in Germany [46]. The detection of neutralizing antibodies against USUV in our WNV surveillance system is similar to findings in Northern Italy, where antibodies against USUV were detected in sentinel animals tested within the framework of the WNV surveillance system, providing evidence of co-circulation of WNV and USUV in the same area [47]. The geographical spread of USUV in Greece is still unknown and further studies are needed to assess its importance as a human pathogen, since it can cause severe neurological syndromes in immunocompromised patients [48].

In our study, molecular detection and characterization of the circulating WNV strain was not possible in any of the 510 pigeon sera tested. This was anticipated, since the duration of viremia in pigeons is short (3–4 days) [49] and in our surveys, pigeons were sampled only once. If molecular characterization of the circulating WNV strains is necessary, the surveillance system should include multiple collections of blood samples at different time points from the same domestic birds. Since it would be difficult to get permission from pigeon owners for repetitive blood sampling, surveillance systems like the one we describe are not ideal for fast virus isolation and molecular characterization. Consequently, weekly sampling from captive chickens and retrospective RT-PCR analysis in samples collected from seroconverted birds proved to be a more efficient surveillance approach [14,17,18]. However, it should be emphasized that, the success of early warning surveillance systems with captive birds depends heavily on the identification of WNV initial enzootic transmission foci. These foci cannot be identified easily, since many factors, such as microclimate, mosquito overwintering, and vector-bird populations are involved. This is even more difficult to achieve in complex environmental areas. In our case, the high number of areas surveyed enabled us to determine foci of WNV early enzootic circulation in specific areas of Central Macedonia, west of Axios River. In this regard, it seems that domestic pigeon surveillance can help in the

selection of these candidate areas of early WNV circulation, in order to achieve higher efficiency of captive bird surveillance systems during following epidemic seasons.

Based on our findings, we can conclude that domestic pigeons proved to be a flexible and convenient arbovirus surveillance system. Importantly, the cost for such a system was minimized, with no costs for setting up and maintaining the flocks. Juvenile domestic pigeon surveillance is much more approachable, compared to surveillance of wild birds, especially in areas where WNV circulation is established. In such areas, past WNV infections in wild birds might occur, resulting in difficulties in the interpretation of serological testing results and need for increased surveillance costs. Moreover, our pigeon surveillance approach enabled sampling from many different areas in Greece. Development of integrated surveillance systems (e.g. domestic pigeon surveillance, captive chicken surveillance, mosquito surveillance, collection of temperature data) should be encouraged. Based on combined data analysis of all these systems, information could be provided for more efficient protection of the population of horses, as well as of the public health and for better targeted control interventions in areas at-risk for increased WNV transmission.

Conflict of interest

The authors have declared that no competing financial interests exist.

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