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Laboratory and surveillance studies following a suspected Dengue case in Greece, 2012

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ABSTRACT

Objectives: To describe the laboratory assays used to investigate a suspected dengue case in summer 2012 in western Greece and to report the public health response.
Design of methods: Samples from the patient were tested for detection of Dengue virus (DENV) antibodies, NS1 antigen and RNA. Public health professionals interviewed residents of the village, and blood samples taken from 132 persons were tested for antibodies for DENV and West Nile virus (WNV). Samples from 10 persons who reported symptoms in the prior 15 days (including 3 persons who had fever at the time of sampling) were tested for DENV, WNV and additional arboviruses. Entomological missions were organized in the area of potential exposure to investigate the presence of competent DENV vectors.
Results: Based on a combination of serological and molecular methods, DENV infection was excluded, and the problems in the serology, especially in the DENV NS1 antigen, were attributed to interfering factors. A 6.1% WNV seroprevalence was detected in the region, and phlebovirus IgM and IgG antibodies were detected in two of three persons who had fever at the time of sampling. *Aedes albopictus* adult mosquitoes were present in the region.
Conclusions: A multi-disciplinary field and laboratory investigation showed no evidence of DENV infection. There is a need for industries to improve the immunometric assays to avoid interference with rheumatoid or other factors, and increased awareness is needed for the evaluation of the diagnostic assays. The high WNV seroprevalence in the investigated region highlights the need for strengthening awareness on vector borne diseases. The presence of *Ae. albopictus* suggests that the possibility of introduction of DENV exists, and preparedness plans are needed.

1. Introduction

Dengue virus (DENV) is a flavivirus (family *Flaviviridae*) transmitted to humans by bites from infected *Aedes* mosquitoes, mainly *Aedes aegypti*, whereas *Aedes albopictus* is considered a competent although secondary DENV vector. An estimated 50 to 100 million DENV infections occur annually in 125 countries, and DENV infection is considered a major public health threat by the World Health Organization (WHO), as Dengue fever global incidence has increased 30-fold in the last 50 years.[1] The main symptoms of Dengue fever include fever, myalgia, arthralgia, and rash, whereas severe cases lead to Dengue hemorrhagic fever or even Dengue shock syndrome. The disease is endemic in tropical

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and subtropical regions, and several imported cases are being reported annually in travelers returning from these regions. The last large epidemic in Europe was observed during 1927-28 in Greece (>80% of the population in the Athens area was affected), with more than 1,000 reported deaths.[2] The possibility of re-introduction of DENV to Europe exists, in view of the increasing global travel rates and the wide distribution of its secondary vector, *Ae. albopictus*, in more than 16 European countries,[3] also present in the wider region of West Greece since 2003.[3] Autochthonous cases of Dengue fever have been reported in 2010 in South France[4,5] and Croatia,[6] while a large outbreak was detected in 2012 in Madeira, Portugal,[7] suggesting that DENV is an emerging public health threat for Europe.

Another flavivirus, West Nile virus (WNV), transmitted to humans mainly by bites of infected *Culex* spp. mosquitoes, is widely distributed in the world, causing sporadic cases and outbreaks. Since 2010, when the virus emerged in Greece,[8] up to 2013, 609 human cases (432 of them accompanied by neurological symptoms) have been observed.[9] Cross-reactivity is often seen among flaviviruses, especially among those belonging to the same serocomplex.[10] In the present study we report the laboratory and field investigation of a suspected autochthonous Dengue case observed in Greece in 2012.

2. Case report

In late August 2012, an 84-year old male resident of a village in the municipality of Agrinio, western Greece, presented to a local private clinic with fever, fatigue, neck pain and weakness of his lower limbs. Laboratory tests showed elevated bilirubin, thrombocytopenia, and leukocytosis. The patient was transferred to a tertiary care center. His condition deteriorated rapidly, and he died on the 10th day of hospitalization due to *Staphylococcus aureus* septicemia despite adequate antimicrobial treatment and supportive care. During his hospitalization he developed epistaxis (not thrombocytopenic at the time) that was attributed to a medical history of Rendu-Weber-Osler syndrome. The patient had not received blood transfusions or other blood products during the incubation period, and he did not report any travel beyond the prefecture of his residence. His past medical history included atrial fibrillation, for which he was treated with an oral anti-coagulant.

3. Laboratory testing

Since an outbreak of WNV infection was ongoing at the time in Greece in August 2012, blood and cerebrospinal fluid (CSF) samples drawn on the 8th day of illness were sent to the National Reference Laboratory for Arboviruses to be tested for probable WNV infection. Samples were tested for presence of WNV IgM and IgG antibodies using ELISA (WNV IgM capture DxSelect and WNV IgG DxSelect, Focus Diagnostics, Cypress, CA). No WNV IgM and IgG antibodies were detected in the CSF; a borderline WNV IgM antibody index (1.6 with cut-off 1.5) was seen in the patient's serum sample, which became negative after subtraction of the background).

Due to the presence of epistaxis and thrombocytopenia in the patient, samples were additionally tested for DENV IgM and IgG antibodies using ELISA (DENV IgM capture DxSelect and DENV IgG DxSelect, Focus Diagnostics, Cypress, CA). No DENV IgM and IgG antibodies were detected in the CSF; results also were negative for DENV IgG antibodies in the serum and CSF. A high positive DENV IgM index (out of range) was seen in the patient's serum, which resulted in a negative after subtracting the background.

Blood and CSF samples were also tested for DENV NS1 antigen (Platelia Dengue NS1 Ag-ELISA (Biorad Laboratories, Marnes-La-Coquette, France); CSF was negative, but a positive result was obtained in the serum sample (3.98, cut-off 1). An NS1 kit was kindly provided by Focus Diagnostics, and serum and CSF samples results were negative. The large difference between indices before and after subtraction of the background in the DENV IgM ELISA prompted us to repeat the Biorad NS1 antigen assay after treatment of the patient's serum with rheumatoid factor (Rf)-absorbent (although it is not written in the instructions for use) to investigate any interference with IgM- Rfs, and the result was negative.

Two RT-nested PCRs, one using generic flavivirus primers[11] and a second using DENV-specific primers,[12] as well as a DENV-specific real time RT-PCR (reagents kindly provided by Ana Vazquez and Antonio Tenorio, Spain), were applied on serum and CSF samples and had negative results. Based on all of the above results, the case was declared DENV-negative.

Serum samples from another WNV-suspected case tested in the Department of Microbiology in the University of Athens also gave borderline WNV IgM and a high DENV IgM antibody index; in that case the DENV infection was easily excluded, since the DENV IgM was negative after the background subtraction and the NS1 antigen was negative. Five serum samples from additional WNV-suspected cases with borderline WNV IgM antibodies were negative after subtraction of the background, and when tested for DENV IgM and IgG antibodies and NS1 antigen were all negative. Table 1

4. Public health response

Immediately after the first suspicion of DENV infection, and before finalization of the laboratory testing, an active case-finding mission was organized by the Hellenic Centre for Disease Control and Prevention (HCDCP) in the village in western Greece where the patient lived. Public health (PH) professionals from the HCDCP and the University of Thessaly, in cooperation with regional PH staff, visited the area and offered a physical exam for all residents of the village; the participants were interviewed through a structured questionnaire aiming to identify persons with possible DENV infection. A total of 132 persons [57 (43.18%) male] were examined, focusing on an area up to 200-m radius from the case's residence, also including the patient's relatives. Their median age was 57 years (range 7-85 years). Ten persons reported fever, headache, arthralgia, myalgia, nausea, vomiting or upper respiratory infection in the last 15 days (3 at the time of examination).

Table 1
Initial laboratory testing results of the suspected Dengue case, Greece, August 2012. CSF: cerebrospinal fluid; neg: negative.

Sample	WNV IgM before/after subtraction cut off: 1.5	WNV IgG	DENV IgM cut off: 1.0	DENV IgG	NS1 antigen cut off: 1.0	PCR for flaviviruses and DENV-specific
	Focus Diagnostics				BioRad	
Serum	1.6/neg	neg	Out of range/neg	neg	3.98	neg
CSF	neg	neg	neg	neg	neg	neg

Table 2
IgM and IgG antibodies against four phleboviruses: Toscana virus (TOSV), sandfly fever Naples virus (SFNV), sandfly fever Sicilian virus (SFNV) and Cyprus virus (CYPV) in two febrile persons detected during the epidemiological investigation. Serum dilution 1:100. T: body temperature; neg: negative; F: female.

No	Age	Sex	T (°C)	IgM antibodies				IgG antibodies			
				TOSV	SFNV	SFSV	CYPV	TOSV	SFNV	SFSV	CYPV
1	24	F	37.5	-	-	++	+++	-	-	+	++
2	12	F	37.2	-	-	+	+	-	-	+	-

Blood samples were obtained from all of them and sent in optimal conditions to the National Reference Laboratory for arboviruses.

Serum samples were tested for potential detection of WNV and DENV IgM and IgG antibodies using the same ELISA methods as for the patient's samples. All samples were negative for WNV and DENV IgM antibodies, while WNV IgG antibodies were detected in 8 (8/131, 6.1%) persons, confirmed by plaque reduction neutralization test ($PRNT_{90}$). The ages of these 8 persons ranged from 74 to 84 years (median 79 years).

Sera from the ten persons who reported symptoms in the prior 15 days were also tested for IgM and IgG antibodies against Chikungunya virus (CHIKV) and phleboviruses by indirect immunofluorescence assays (Euroimmun, Lübeck, Germany). Regarding phleboviruses, samples were tested for antibodies against Toscana virus (TOSV), sandfly fever Naples virus (SFNV), sandfly fever Sicilian virus (SFSV) and Cyprus virus (CYPV) (Sandfly fever virus Mosaic 1, Euroimmun, Lübeck, Germany). CHIKV IgM and IgG antibodies were not detected. However, phlebovirus IgM and IgG antibodies were detected in two of the three persons who had fever at the time of sampling (Table 2), suggesting an acute phlebovirus infection. An RT-nested PCR using phlebovirus generic primers[13] applied on the patients' blood samples was negative.

PH staff raised awareness of local clinicians and PH professionals on the clinical presentation of DENV infection. Information and communication material was also provided to health professionals and the public on personal protection measures against mosquitoes, although this activity was on-going due to the recent outbreaks of WNV infections (since 2010[8,14,15]) and the report of autochthonous malaria cases.[16]

5. Entomological investigation

Two entomological missions were organized in the area of potential exposure to investigate the presence of competent DENV vectors. A team of experts from Benaki Phytopathological Institute and the HCDCP carried out mosquito field surveys in the patient's village residential area, and in nearby villages per his reported movement history. Entomological surveillance detected a variety of potential mosquito breeding sites, which yielded only *Culex* spp. larvae and pupae. In addition, three octenol baited adult traps (2 Biogents Mosquitaire Plus & 1 BG Sentinel) were placed in the area surrounding the patient's residence and a nearby village, where the patient reportedly spent some of his summer vacation. A total of 3 *Ae. albopictus* adult mosquitoes were captured during the mission, which tested DENV-negative at the Department of Parasitology, Entomology and Tropical Diseases at the National School of Public Health. Furthermore, a number of oviposition traps were placed in the immediate surroundings. Eggs were collected from all three locations, which after hatching in the laboratory, were identified as *Ae. albopictus*.

6. Discussion

One of the priorities of PH preparedness for emerging pathogens is the capacity for prompt and accurate diagnosis. The present study illustrates the need for a thorough interdisciplinary investigation of a first reported case of an emerging pathogen, as well as the value of a Reference Centre, which, being a member of the European Network of Imported Viral Diseases (ENIVD), participated in several external quality control studies, including those on diagnostics on DENV[17,18] and WNV.[19]

The main problem in flavivirus diagnostics is the extensive cross-reactivity among members of the family, and, especially, among members of the same serocomplex. The present report shows an additional problem: the interference of various factors, such as the Rf or the heterophilic antibodies, in the performance of the assays for the detection of WNV and DENV antibodies and the DENV NS1 antigen. The extremely high index (out of range) of DENV IgM antibodies observed without the subtraction method was exceptional. Previous studies on WNV cases have shown no interfering factors in high IgM positive samples (index value of >3.5) tested by Focus Diagnostics capture ELISA (MAC-ELISA)[20] (which applies also for DENV ELISA). The negative result after application of the subtraction method (which is suggested by the manufacturers) facilitated the conclusion that the patient did not have IgM (and IgG) antibodies against WNV and DENV.

The remaining problem was the positive result of the DENV NS1 antigen when the Biorad kit was used (the manufacturer reports no interference with heterophilic antibodies). It is of interest that when the test was repeated after treatment of the patient's serum with Rf-absorbent (for the removal of IgM-Rf rheumatoid factors), the result was negative, suggesting that further studies are needed on this topic to investigate the role of Rfs in the NS1 antigen performance. The problem was solved by the negative result in NS1 antigen when the Focus kit was used, and by the negative result of the three PCRs (two nested RT-PCRs and one real-time RT-PCR).The epidemiological and entomological investigation showed no evidence of DENV infection in the region, neither in humans nor in mosquitoes. However, the high WNV seroprevalence detected in the population sample of the region (6.1%), together with the fact that only two human cases have been reported from the municipality of Agrinio (one in 2010 and one in 2012), suggests that some WNV cases probably went undetected, indicating the continuing need for increased awareness in the region. The detection of two cases with phlebovirus infection was not unexpected, since the phlebovirus seroprevalence in Greece is high (up to 60%), especially in the islands and the coastal areas of the mainland[21,22]; western Greece, where the cases were detected, is among the regions with high seroprevalence. Patients with mild symptoms, such as the two cases in the present study, do not seek medical advice, and remain undetected. However, phlebovirus infections are not usually included in the differential diagnosis of summer febrile cases, even when they present neurological symptoms, and the number of reported cases is currently very limited.[23–26]

The capture of *Ae. albopictus*, although DENV-negative, suggests that the possibility of re-introduction of DENV to Greece exists (similar to other southern European countries), and preparedness plans must be in place. The recent autochthonous dengue case reported in southern France on Aug 20, 2014, emphasises the present risk of dengue transmission in Europe in areas where competent mosquito vectors are established.[27]

In conclusion, the present case shows that the laboratory diagnosis of a flavivirus infection presents certain challenges and that critical evaluation of the results by experts in the field is essential; this applies for all cases, but especially when the emergence of a pathogen in a new area is suspected. There is a need for industries to improve the immunometric assays to avoid interference with RF or heterophilic antibodies, while increased awareness is needed by laboratories about the vulnerability of diagnostic assays, especially when only one sample is available. The present investigation was the trigger for a comprehensive

approach to detection of arboviral diseases in the area, which can serve as a basis for future studies; it was also a good opportunity to further explore collaborations already formed for the management of WNV and malaria in Greece regarding the prompt mobilization of a multi-disciplinary team to a particular area for field and laboratory investigations.

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References

1. World Health Organization. Global Strategy for dengue prevention and control, 2012–2020; 2012.
2. Papaevangelou G, Halstead SB. Infections with two dengue viruses in Greece in the 20th century. Did dengue hemorrhagic fever occur in the 1928 epidemic? *J Trop Med Hyg* 1977;**80**:46–51.
3. Reiter P. Yellow fever and dengue: a threat to Europe? *Euro Surveill* 2010;**15**: 19509.
4. La Ruche G, Souares Y, Armengaud A, Peloux-Petiot F, Delaunay P, Despres P, et al. First two autochthonous dengue virus infections in metropolitan France, September 2010. *Euro Surveill* 2010;**15**:19676.
5. Marchand E, Prat C, Jeannin C, Lafont E, Bergmann T, Flusin O, et al. Autochthonous case of dengue in France, October 2013. *Euro Surveill* 2013;**18**:20661.
6. Schmidt-Chanasit J, Haditsch M, Schoneberg I, Gunther S, Stark K, Frank C. Dengue virus infection in a traveller returning from Croatia to Germany. *Euro Surveill* 2010;15.
7. Alves MJ, Fernandes PL, Amaro F, Osorio H, Luz T, Parreira P, et al. Clinical presentation and laboratory findings for the first autochthonous cases of dengue fever in Madeira island, Portugal, October 2012. *Euro Surveill* 2013;18.
8. Papa A, Danis K, Baka A, Bakas A, Dougas G, Lytras T, et al. Ongoing outbreak of West Nile virus infections in humans in Greece, July - August 2010. *Euro Surveill* 2010;**15**. pii: 19644.
9. Marka A, Diamantidis A, Papa A, Valiakos G, Chaintoutis SC, Doukas D, et al. West Nile virus state of the art report of MALWEST Project. *Int J Environ Res Public Health* 2013;**10**:6534–610.
10. Papa A, Karabaxoglou D, Kansouzidou A. Acute West Nile virus neuroinvasive infections: Cross-reactivity with dengue virus and tick-borne encephalitis virus. *J Med Virol* 2011;**83**:1861–5.
11. Sanchez-Seco MP, Rosario D, Domingo C, Hernandez L, Valdes K, Guzman MG, et al. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. *J Virol Methods* 2005;**126**:101–9.
12. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992;**30**:545–51.
13. Sanchez-Seco MP, Echevarria JM, Hernandez L, Estevez D, Navarro-Mari JM, Tenorio A. Detection and identification of Toscana and other phleboviruses by RT-nested-PCR assays with degenerated primers. *J Med Virol* 2003;**71**:140–9.
14. Danis K, Papa A, Papanikolaou E, Dougas G, Terzaki I, Baka A, et al. Ongoing outbreak of West Nile virus infection in humans, Greece, July to August 2011. *Euro Surveill* 2011;**16**. pii: 19951.
15. Pervanidou D, Detsis M, Danis K, Mellou K, Papanikolaou E, Terzaki I, et al. West Nile virus outbreak in humans, Greece, 2012: third consecutive year of local transmission. Euro Surveill;19.
16. Danis K, Baka A, Lenglet A, Van Bortel W, Terzaki I, Tseroni M, et al. Autochthonous Plasmodium vivax malaria in Greece, 2011. *Euro Surveill* 2011;16.
17. Domingo C, Niedrig M, Teichmann A, Kaiser M, Rumer L, Jarman RG, et al. 2nd International external quality control assessment for the molecular diagnosis of dengue infections. *PLoS Negl Trop Dis* 2010;4.
18. Donoso Mantke O, Lemmer K, Biel SS, Groen J, Schmitz H, Durand JP, et al. Quality control assessment for the serological diagnosis of dengue virus infections. *J Clin Virol* 2004;**29**:105–12.
19. Sanchini A, Donoso-Mantke O, Papa A, Sambri V, Teichmann A, Niedrig M. Second international diagnostic accuracy study for the serological detection of West Nile virus infection. PLoS Negl Trop Dis;7:e2184.
20. Sambol AR, Hinrichs SH, Hogrefe WR, Schweitzer BK. Performance of a commercial immunoglobulin M antibody capture assay using analyte-specific reagents to screen for interfering factors during a West Nile virus epidemic season in Nebraska. *Clin Vaccine Immunol* 2007;**14**:87–9.
21. Anagnostou V, Papa A. Seroprevalence of Toscana virus among residents of Aegean Sea islands, Greece. *Travel Med Infect Dis* 2013;**11**:98–102.
22. Papa A, Andriotis V, Tzilianos M. Prevalence of Toscana virus antibodies in residents of two Ionian islands, Greece. *Travel Med Infect Dis* 2010;**8**:302–4.
23. Anagnostou V, Pardalos G, Athanasiou-Metaxa M, Papa A. Novel phlebovirus in febrile child, Greece. *Emerg Infect Dis* 2011;**17**:940–1.
24. Anagnostou V, Sdouga M, Volakli H, Violaki A, Papa A. Phlebovirus meningoencephalis complicated by *Pseudomonas aeruginosa* pneumonia: a case report. *Vector Borne Zoonotic Dis* 2010;**11**:595–6.
25. Papa A, Mallias J, Tsergouli K, Markou F, Poulou A, Milidis T. Neuroinvasive phlebovirus infection in Greece: A case report. Intervirology: accepted 2014.
26. Papa A, Paraforou T, Papakonstantinou I, Pagdatoglou K, Kontana A, Koukoubani T. Severe encephalitis caused by Toscana virus, Greece. *Emerg Infect Dis* 2014;**20**:1417–9.
27. Schaffner F, Fontenille D, Mathis A. Autochthonous dengue emphasises the threat of arbovirosis in Europe. *Lancet Infect Dis* 2014;(14):70925–9. pii: S1473-3099.