Emergence and co-infections of West Nile virus and Toscana virus in Eastern Thrace, Turkey

H. Erdem[1], K. Ergunay[2], A. Yilmaz[3], H. Naz[4], F. Akata[5], A. S. Inan[6], A. Ulcay[1], F. Gunay[7], A. Ozkul[8], B. Alten[7], V. Turhan[1], O. Oncul[1] and L. Gorenek[1]

1) *Department of Infectious Diseases and Clinical Microbiology, Gulhane Military Medical Academy, Haydarpasa Training and Research Hospital, Istanbul,* 2) *Virology Unit, Department of Medical Microbiology, Faculty of Medicine, Hacettepe University, Ankara,* 3) *Turkish Ministry of Health, Corlu State Hospital,* 4) *Turkish Ministry of Health, Cerkezkoy State Hospital, Tekirdag,* 5) *Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Trakya University, Edirne,* 6) *Department of Infectious Diseases and Clinical Microbiology, Turkish Ministry of Health, Haydarpasa Training and Research Hospital, Istanbul,* 7) *Division of Ecology, Department of Biology, Faculty of Sciences, Hacettepe University and* 8) *Department of Virology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey*

Abstract

The objective of this study was to identify the impact of West Nile virus (WNV) and Toscana virus (TOSV) in febrile diseases of unknown aetiology in Eastern Thrace, Turkey; this study was conducted during August–October 2012, and included 18 clinical cases and 296 blood donors for local serosurveillance. Antibodies were determined via commercial assays and further tested for specificity via neutralization assays (NA). Viral RNAs were sought via specific and/or generic primers. WNV infections were diagnosed in seven patients (38.8%), detected via RNA+IgM in four, RNA in one and IgM and low avidity IgG in two cases. The most common symptom was fever (>38°C), followed by headache, malaise/fatigue, myalgia/arthralgia, muscle stiffness/lower back pain, anorexia, nausea/vomiting, diarrhoea, supraorbital/retrobulbar pain and abdominal pain. Neurological symptoms were noted in one individual. WNV strains in RNA-detectable patients were characterized as lineage 1. TOSV RNA or IgM were identified in two individuals with confirmed WNV infections and in one patient without evidence of WNV exposure. The clinical and laboratory findings in individuals with WNV/TOSV co-infection were comparable to those in WNV-induced disease. The TOSV strain in the patient with detectable viral RNA was characterized as genotype A. In local blood donors, seroreactivity for specific WNV and TOSV immunoglobulins was observed in 1.7% (5/296) and 14.4% (26/180), respectively. These findings indicate the emergence of WNV and TOSV-associated diseases in Eastern Thrace. WNV/TOSV co-infections were documented for the first time.

Keywords: Thrace, Toscana virus, TOSV, Turkey, West Nile virus, WNV
Original Submission: 25 February 2013; **Revised Submission:** 7 May 2013; **Accepted:** 19 June 2013
Editor: T. A. Zupanc
Article published online: 25 June 2013
Clin Microbiol Infect 2014; **20:** 319–325
10.1111/1469-0691.12310

Corresponding author: K. Ergunay, Virology Unit, Department of Medical Microbiology, Faculty of Medicine, Hacettepe University, Morphology Building 3rd floor, 06100 Sihhiye, Ankara, Turkey
E-mail: ekoray@hacettepe.edu.tr

Introduction

West Nile virus (WNV) and Toscana virus (TOSV) are causative agents of major vector-borne viral infections that induce central nervous system manifestations as well as febrile diseases in affected individuals [1,2]. WNV is a mosquito-borne Flavivirus (family *Flavivirdae*) maintained in an enzootic cycle between birds as amplifying hosts and mosquito vectors [1]. WNV infections in humans usually remain subclinical; a febrile illness called West Nile fever develops in 20% of exposed persons and neuroinvasive disease in <1% [3]. Since 1994, the virus has caused outbreaks of severe disease in humans and horses in Europe and the Mediterranean Basin [4]. TOSV, included in the *Phlebotomus* fever group in the *Phlebovirus* genus of the *Bunyaviridae* family along with Sandfly fever and Sicilian and Naples viruses, is transmitted to humans by the bite of phlebotomine sandflies [5]. While all three viruses may induce

a self-limited febrile disease known as phlebotomus, papatacci or sandfly fever, TOSV, having a distinct neurotropism, is considered to be one of the leading causes of human aseptic meningitis in endemic countries around the Mediterranean basin [5,6]. In this study, the activity and impact of WNV and TOSV were investigated in Eastern Thrace, Turkey, where no previous data were available.

Materials and Methods

Study area, setting and clinical cases

The study was carried out in the northwestern part of the Turkish Republic, also called the Eastern Thrace region, which includes territories of five provinces (Edirne, Tekirdag, Kirklareli, Canakkale and Istanbul) with a population of 1 569 388 (www.kalkinma.gov.tr/DocObjects/Download/10211/TR21_Trakya_Bolge_Plani.pdf). The region is bordered on the west-northwest by Greece and Bulgaria and separated from Anatolia or Asia minor by the Sea of Marmara (Fig. 1).

During early August 2012 two cases of febrile disease of unidentified aetiology were referred to the infectious diseases clinic of the Haydarpasa Training and Research Hospital of Gulhane Military Medical Academy. The cases were military recruits, located in the Corlu district of Tekirdag province (Fig. 1). As the preliminary evaluations indicated WNV infections, this study was undertaken to identify further cases and asymptomatic exposure in the region. The study was approved by the Turkish Ministry of Health Haydarpasa Research and Training Hospital ethical board (HNEAH-KAEK 2012/197).

Adult patients with the clinical diagnosis of febrile disease of presumed viral aetiology observed during late July to early October 2012 at state hospitals in the Corlu and Cerkezkoy districts of Tekirdag province, infectious diseases clinics of Trakya University Hospital in Edirne province and Haydarpasa State and Military Research and Training Hospitals in Istanbul province were included in the study (Fig. 1). After informed consent, physical examination and laboratory tests, which included haemoglobulin, leucocyte and platelet counts, sedimentation rate, C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), total protein, albumin and creatinine, were performed. The participants filled out a questionnaire to identify risk factors for vector-borne infections. Sera and/or cerebrospinal fluid (CSF) were obtained from patients within 1–10 days after the onset of symptoms, and subjected to nucleic acid purification using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) and reverse transcription using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Tokyo, Japan). For the detection of WNV RNA, nested and real-time reverse transcription PCRs, targeting distinct regions of the viral genome, were employed [7,8]. TOSV and other

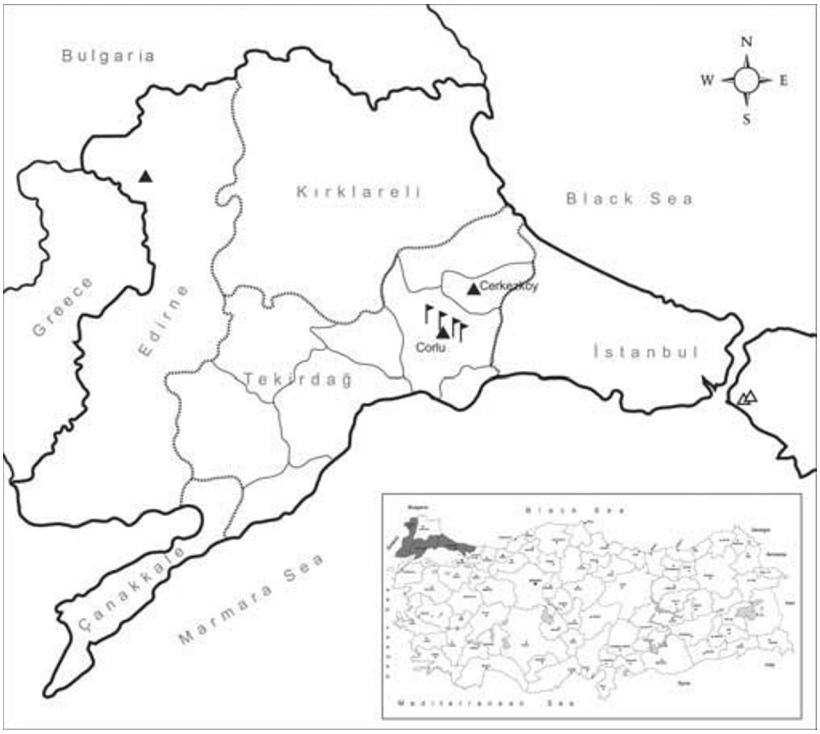

FIG. 1. Map of the Eastern Thrace region, showing the study area. Provincial boundaries and district borders in Tekirdağ province are demonstrated. See text for details (△, institution for patient evaluation; ▲, institution where confirmed WNV cases were identified; ⚐, mosquito sampling location).

phleboviruses were sought via a generic nested PCR utilizing degenerated primers [9].

Sera from clinical cases were screened for WNV IgM and IgG class immunoglobulins via commercial enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs) (anti-WNV ELISA and IFA, Euroimmun, Luebeck, Germany).

In IgG-positive samples, immunoglobulin avidity was determined via a commercial assay (WNV avidity ELISA, Euroimmun). For pathogenic phleboviruses including TOSV, a commercial IFA was employed (Sandfly Fever Virus Mosaic 1 IgG and IgM IFA, Euroimmun).

Positive samples in the commercial WNV and TOSV assays were subsequently analysed via neutralization assays (NAs) to confirm antibody specificity, using WNV strain NY99-4132, TOSV strain ISS.Phl.3 and Vero cells (ATCC CCL81) as previously described [10,11].

Blood donor surveillance

Sera from volunteer blood donors, obtained during September–October 2012 in Corlu and Cerkezkoy districts were screened for WNV and TOSV immunoglobulins via a commercial ELISA and IFA (Sandfly Fever virus type Toscana IgG and IgM IFA, Euroimmun). Reactive samples were included in NA and WNV IgG avidity assays.

Sequencing and phylogenetic analysis

Nested PCR amplicons were cleaned up using a High Pure PCR Product Purification Kit (Roche Diagnostics) and subsequently sequenced via forward and reverse primers using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were handled using CLC Main Workbench v5.5 (CLCBio, Aarhus, Denmark). Phylogenetic analyses were performed using the Jukes-Cantor substitution rate model with 500 bootstrap replicates. Maximum likelihood trees were generated based on a UPGMA tree.

Results

Evaluation of clinical cases

A total of 18 cases were evaluated during the study period. Evidence of acute WNV infections was identified in seven patients (38.8%). Diagnosis was based on viral RNA detection in one (1/7), viral RNA and NA-confirmed IgM detection in four (4/7) and NA-confirmed IgM and low avidity IgG detection in two cases (2/7). The average time for hospital admission and sampling after the onset of symptoms in cases with detecteable RNA was 1 day, whereas it was 2 and 10 days for serologically confirmed cases. Maximum composite likelihood analyses demonstrated 82.8–98.8% and 81.97–99.60% similarity among patient-derived WNV sequences and sequences from equine/human cases from Central Anatolia, respectively. All sequences belonged to the WNV lineage 1 clade 1a (Fig. 2a).

The most common symptom observed in laboratory-confirmed WNV infections was fever (38–39.5°C), noted in all patients (7/7), followed by headache (5/7), malaise/fatigue (5/7), myalgia/arthralgia (5/7), muscle stiffness/lower back pain (5/7), decreased appetite/anorexia (2/7), nausea/vomiting (2/7), diarrhoea (2/7), supraorbital/retrobulbar pain (2/7) and abdominal pain (1/7). No form of skin rash, photophobia or lymphadenopathy was present in any of the cases. Gastroentestinal symptoms were prominent in two individuals (Table 1). Laboratory evaluations revealed leucopenia in four, thrombocytopenia in four, elevated CPK levels in three, elevated AST levels in three and increased sedimentation rate in two cases. Leucocytosis and mildly elevated CRP and ALT levels were also noted in three separate individuals (Table 1). All patients with WNV infections recalled mosquito bites within 10 days prior to disease onset and no other risk factor associated with virus transmission could be identified.

One WNV-infected individual, a 65-year-old male farmer residing in Edirne province, presented with febrile disease and neurological manifestations, characterized by mental status changes, confusion, neck stiffness without Kernig or Brudzinski's sign, paresis and myoclonic seizures of the right arm. CSF examination displayed increased protein (74.2 mg/dL) and pleocytosis comprising 70% mononuclear cells. Diffuse slow wave activity was observed on electroencephalogram (EEG) and magnetic resonance imaging (MRI) demonstrated bilateral thalamic lacunar infarcts. In addition to the supportive therapy and anti-epileptics, a single 25 mg dose of prednisolone was administered. Although the patient recovered from the febrile condition, no neurological improvement was observed despite anti-epileptics and physical therapy after 35 days. All remaining patients responded favourably to the supportive therapy, with fever and other symptoms subsiding within an average of 3–4 days, and were discharged without residual sequelae.

In one individual with WNV RNA and specific IgM, generic phlebovirus PCR also yielded positive results (Table 1, patient No.6). After repeat assays and sequencing, the amplicons were characterized as TOSV, grouped with genotype A strains (Fig. 2b). The sequence was identical and 99.19% similar to TOSV sequences detected in Central Anatolia during 2009 (not included in the tree; GenBank accession,HM151316) and 2012, respectively. Moreover, a neutralizing TOSV IgM reactivity was observed in another individual with serologically confirmed WNV (Table 1, patient no.7). Symptoms and clinical presentation of these cases were indistinguishable from West

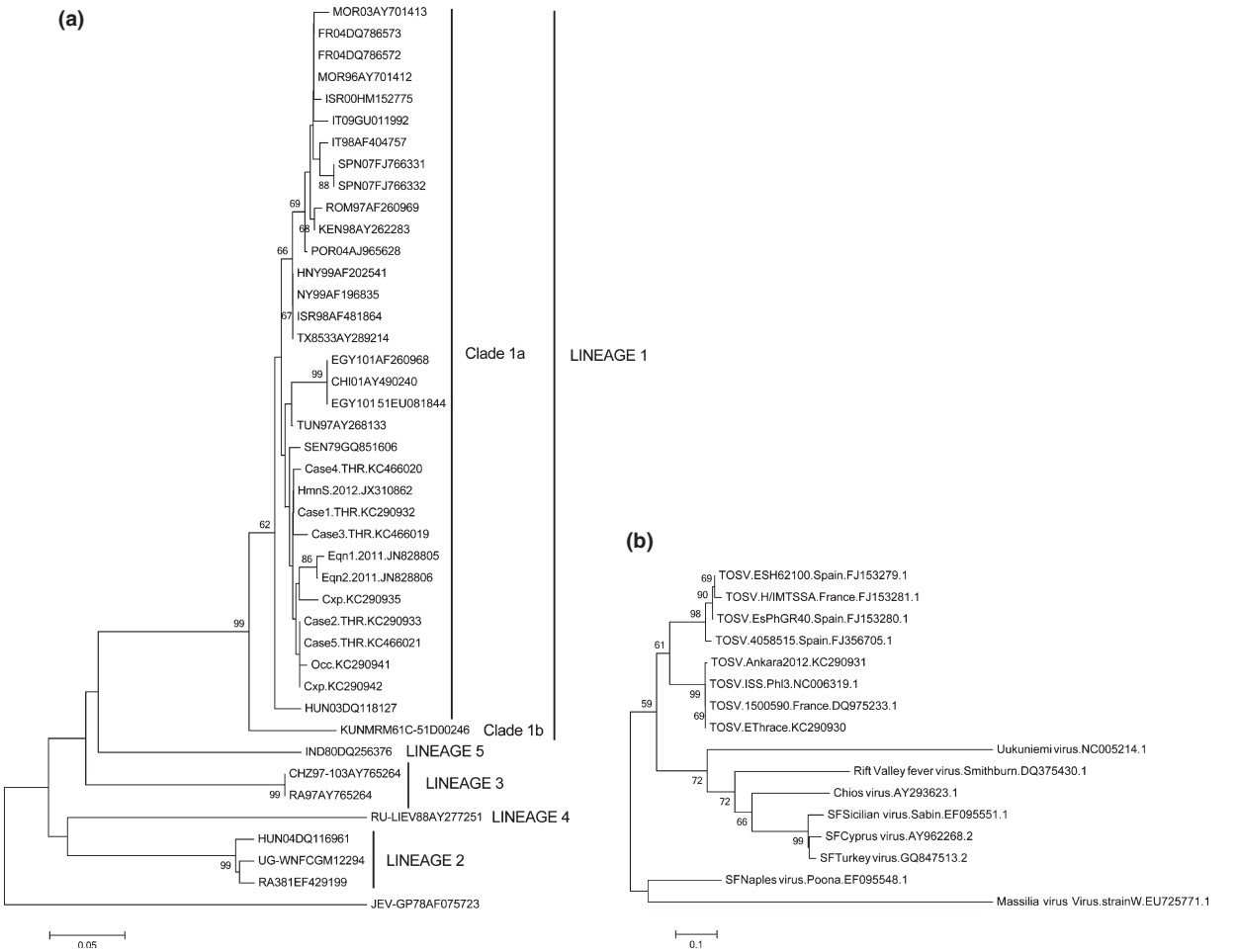

FIG. 2. Neighbour-joining tree of West Nile virus (WNV) partial E protein (a) and Toscana virus (TOSV) partial L segment (b) sequences. Virus strains represented are indicated by strain name and GenBank accession number. Bootstrap values are given in per cent. (a) Case1.THR.KC290932, Case2.THR.KC290933, Case3.THR.KC466019, Case4.THR.KC466020 and Case5.THR.KC466021: sequences characterized in this study. Eqn1.2011.JN828805, Eqn2.2011.JN828806 and HmnS.2012.JX310862: equine and human sequences from Central Anatolia. Cxp.KC290935, Cxp.KC290942 and Occ.KC290941: sequences detected in mosquitoes in Eastern Thrace [21]). (b) TOSV.EThrace.KC290930: sequence characterized in this study. TOSV.Ankara.KC290931n: human sequence detected in 2012 from Central Anatolia.

Nile fever patients (Table 1). Moreover, a 35-year-old housewife from Cerkezkoy province presenting with febrile disease with negative WNV assays also demonstrated a neutralizing TOSV IgM without viral RNA in serum. Lacking a history of insect bites, this patient had leucopenia, thrombocytopenia, and elevated AST (128 IU/L), ALT (108 IU/L) and LDH (1056 U/L) (Table 1 patient No.8).

Virus exposure in blood donors

WNV and TOSV exposures were sought in 296 and 180 asymptomatic blood donors, respectively. WNV seroreactivity was observed in 22 individuals (7.4%), whereas specificity could be confirmed via NA in five (1.7%). High avidity IgGs were detected in all NA-confirmed donors, also accompanied by IgM in one individual. A higher rate of TOSV exposure was observed, detected in 42 (23.3%) and confirmed in 26 (14.4%) donors. The majority of the individuals with TOSV exposure had IgM antibodies (22/26), suggesting recent exposure. In two (0.6%) blood donors, dual exposure to these viruses was identified by the detection of WNV IgG with TOSV IgG and/or IgM. The distribution of serosurveillance results is given in Table 2.

Discussion

Following the identification of two cases of febrile disease associated with WNV in the Eastern Thrace region in Turkey, further WNV and phlebovirus infections were investigated in this region during August–October 2012 with an accompanying

TABLE 1. Clinical symptoms and laboratory findings in patients with West Nile virus (WNV) and/or Toscana virus (TOSV) infections

No.	Age/gender/place of residence/occupation	Signs and symptoms[a]	Laboratory findings	Diagnostic assay results[b]
1	25, male, Corlu, military recruit	Headache, malaise/fatigue, myalgia/arthralgia, muscle stiffness/lower back pain	Leucopenia (2800/mm3) Thrombocytopenia (129 000/mm3) Increased CPK (665 U/L) Increased AST (51 IU/L)	WNV RNA (+), WNV IgM (+)
2	24, male, Corlu, military recruit	Headache, malaise/fatigue, myalgia/arthralgia	Leucopenia (3300/mm3) Increased CPK (427 U/L)	WNV RNA (+),WNV IgM (+)
3	21, male, Corlu, military recruit	Muscle stiffness/lower back pain Supraorbital/retrobulbar pain	Thrombocytopenia (112 000/mm3)	WNV RNA (+)
4	21, male, Corlu, military recruit	Headache, malaise/fatigue, myalgia/arthralgia, muscle stiffness/lower back pain, Supraorbital/retrobulbar pain, abdominal pain, nausea/vomiting, anorexia, diarrhoea	Leucopenia (3100/mm3)	WNV RNA (+),WNV IgM (+)
5	65, male, Edirne, farmer	Headache, malaise/fatigue, myalgia/arthralgia, abdominal pain, nausea/vomiting, anorexia, diarrhoea, neurological symptoms[c]	Leucocytosis (15 800/mm3) Increased ESR (81 mm/hr) Increased CPK (246 U/L)	WNV IgM (+) WNV IgG (+) (low avidity)
6	21, male, Corlu, military recruit	Muscle stiffness/lower back pain	Thrombocytopenia (137 000/mm3) Increased CRP (5.2 mg/dL)	WNV RNA (+),WNV IgM (+) TOSV RNA (+),
7	19, male, Corlu, student	Headache, malaise/fatigue, myalgia/arthralgia, muscle stiffness/lower back pain, abdominal pain	Increased ESR (45 mm/h)	WNV IgM (+) WNV IgG (+) (low avidity) TOSV IgM (+)
8	35, female, Cerkezkoy, housewife	Headache, malaise/fatigue, myalgia/arthralgia	Leucopenia (2500/mm3) Thrombocytopenia (105 000/mm3) Increased AST (128 IU/L), ALT(108 IU/L) Increased LDH (1056 U/L)	TOSV IgM (+)

ALT, alanine aminotrasferase; AST, aspartate aminotransferase; CPK, creatinine phosphokinase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LDH, lactate dehydrogenase.
[a]High fever (>38°C) was present in all patients, whereas skin rash or lymphadenopathy was not observed.
[b]IgM and IgG seropositivities reported for the patients have been confirmed via virus neutralization assays.
[c]Include mental status changes, confusion, stiff neck and myoclonus and paresis affecting right arm. See text for details.

TABLE 2. West Nile virus (WNV) and Toscana virus (TOSV) seroepidemiology according to the sampling locations in the study region

	WNV				TOSV			
	ELISA		NA		IFA		NA	
Location	Negative	Positive	Confirmed	Total	Negative	Positive	Confirmed	Total
Corlu (41°17′23″N,28°0′0″E)								
IgM	135	15	0		70	16	11	
IgG	148	2	2		82	4	1	
IgM+IgG	150	0	0		83	3	2	
Total		17 (11.3%)	2 (1.3%)	150		23 (26.7%)	14 (16.3%)	86
Cerkezkoy (41°9′23″N,27°48′37″E)								
IgM	144	2	0		80	14	11	
IgG	144	2	2		91	3	0	
IgM+IgG	145	1	1		72	2	1	
Total		5 (3.4%)	3 (2.1%)	146		19 (20.2%)	12 (12.7%)	94

NA, neutralization assay; ELISA, enzyme-linked immunosorbent assays; IFA, immunofluorescence assays.

serosurveillance study. The results revealed seven laboratory-confirmed WNV cases and a local seroprevalence of 1.7% in asymptomatic blood donors. Moreover, TOSV infections have been detected in three individuals with febrile diseases with a 14.4% seroprevalence rate. In two febrile patients, laboratory-confirmed co-infections of WNV and TOSV have been identified via serology and/or viral RNA detection. These findings are the first reports of WNV and TOSV activity from this region. In addition, evidence of WNV and TOSV co-infections in the same individual has been described for the first time.

Turkey is located in the endemic zone for WNV and serosurveillance data demonstrated animal and human WNV exposure throughout Anatolia [12]. However, reports of symptomatic infections have been few, mostly involving serologically identified neuroinvasive diseases [12–14]. Here, we described six cases of West Nile fever and one individual with central nervous system involvement. Fever exceeding 38°C and accompanying symptoms documented in these patients are generally in accordance with the previously reported case series [2,3,15]. Symptoms involving the gastrointestinal system predominated only in two individuals (Table 1). Detection of WNV RNA was successful if the blood sampling could be performed within the first day of hospital admission. Neuroinvasive disease was documented in

a 65-year-old male, who demonstrated meningoencephalitic as well as focal neurological symptoms with increased protein and pleocytosis in CSF. Encephalitis occurs in about 60% of persons with WNV-associated neuroinvasive disease and is more frequent in persons over 55 years and in those with underlying medical conditions [16,17]. No risk factor other than advanced age could be identified for our patient, who has not completely recovered despite medical attention.

Five genetic lineages of WNV have been identified, and the majority of the strains responsible for the European and the Mediterranean outbreaks belong in Lineage 1 [4,18]. In Turkey, human cases as well as equine infections with lineage 1 WNV strains have been documented in 2011–2012 from Central Anatolia [19,20]. Sequences from West Nile fever cases identified in Eastern Thrace in this study were grouped with lineage 1 viruses and were closely related to the strains from Central Anatolia (Fig. 2a). These findings indicate that lineage 1 viruses are in circulation not only in Central Anatolia but in the European part of Turkey as well. All cases have indicated recent mosquito exposure prior to the initiation of symptoms. We performed a field study in locations associated with WNV emergence in early September (Fig. 1), but a limited number of *Culex pipiens s.l.* specimens, negative for WNV RNA and antigens, could be collected. However, WNV-infected *Cx pipiens s.l.* and *Ochlerotatus caspius* pools were identified during an arboviral field survey performed during early July in locations 80–100 km west-northwest of the current sites of disease emergence [21]. These findings strongly suggest mosquito bites as the route of WNV transmission in the Eastern Thrace region.

Outbreaks of febrile diseases due to phleboviruses, especially the recently identified phlebovirus variant Sandfly Fever Turkey virus (SFTV), have been previously demonstrated and this strain appears to be in circulation in Central Anatolia [22,23]. Acute TOSV infections have initially been identified in 2009 in Ankara (Central Anatolia) and in 2011 in Izmir provinces (Western Anatolia), in patients with symptoms of mild meningitis, usually without encephalitic symptoms [14,24]. Partial L and S segment sequences detected in patients suggested TOSV genotype A strains to be prevalent, with serosurveillance data indicating asymptomatic human exposure in these regions [24,25]. In this study, we have identified an individual with febrile disease due to TOSV via IgM detection and a confirmed TOSV seroprevalence of 14.4% in the study region. Furthermore, TOSV RNA or neutralizing IgM antibodies were identified in two individuals with confirmed WNV infections, indicating dual infections with these agents. The symptoms and laboratory findings noted in WNV/TOSV co-infected individuals were comparable to those in WNV-induced febrile disease (Table 1). Virus serosurveillance also provided indirect evidence for WNV/TOSV co-infections by the demonstration of WNV IgG and or TOSV IgM/IgG in blood donors. It remains to be determined whether simultaneous infections with WNV/TOSV are observed in other endemic regions and result in enhanced pathogenicity with more severe clinical outcomes in various patient groups.

Partial sequences characterized in the patient with detectable TOSV RNA demonstrate the circulation of genotype A strains in Eastern Thrace as well as in Central Anatolia and a more widespread zone of TOSV activity than previously anticipated [12].

Various sandfly species capable of transmitting TOSV as well as other pathogenic phleboviruses have been observed in Turkey and *Phlebotomus major s.l.* has been identified as the probable SFTV vector [12,26]. Currently, sandflies indigenous to Eastern Thrace are not thoroughly investigated and further studies are required to demonstrate whether *P. perfiliewi* and/or other species, established as TOSV vectors, are present in the region.

In conclusion, we have revealed cases of WNV and TOSV-associated diseases as well as asymptomatic virus exposure in the Eastern Thrace region. Moreover, the first co-infections of WNV and TOSV have been documented.

Acknowledgements

Preliminary findings of this study were presented as an oral presentation at the EKMUD Congress, which was held in Antalya, Turkey, between 20 and 24 March 2013.

Author Contributions

Hakan Erdem: project planning and execution, patient evaluation, sampling and follow-up. Koray Ergunay: project planning, serological and PCR assays, evaluation of results and manuscript preparation. Filiz Akata, Asuman Sengoz Inan, Asim Ulcay, Vedat Turhan, Oral Oncul and Levent Gorenek: patient identification, evaluation, sampling and follow-up. Hasan Naz and Asiye Yılmaz: patient and blood donor evaluation and sample collection. Filiz Gunay: mosquito field sampling and species identification. Bulent Alten: planning and execution of mosquito field sampling and species identification. Aykut Ozkul: DNA sequencing, phylogenetic analyses, neutralization and PCR assays.

Transparency Declaration

The authors declare no conflicts of interests.

References

1. Burke D, Monath T. Flaviviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia: Lippincott William & Wilkins, 2001; 1043–1126.
2. Mertz GJ. Bunyaviridae: Bunyaviruses, Phleboviruses, Nairoviruses and Hantaviruses. In: Richman DD, Whitley RJ, Hayden FG, eds. *Clinical Virology*. New York: Churchill-Livingstone, 1997; 943–971.
3. Hayes EB, Gubler DJ. West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. *Annu Rev Med* 2006; 57: 181–194.
4. Calistri P, Giovannini A, Hubalek Z *et al.* Epidemiology of West Nile in Europe and in the Mediterranean basin. *Open Virol J* 2010; 4: 29–37.
5. Dionisio D, Esperti F, Vivarelli A, Valassina M. Epidemiological, clinical and laboratory aspects of sandfly fever. *Curr Opin Infect Dis* 2003; 16: 383–388.
6. Charel RN, Gallian P, Navarro-Mari JM *et al.* Emergence of Toscana virus in Europe. *Emerg Infect Dis* 2005; 11: 1657–1663.
7. Johnson DJ, Ostlund EN, Pedersen DD, Schmitt BJ. Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. *Emerg Infect Dis* 2001; 7: 739–741.
8. Ergunay K, Saygan MB, Aydogan S *et al.* West Nile virus seroprevalence in blood donors from Central Anatolia, Turkey. *Vector Borne Zoonotic Dis* 2010; 10: 771–775.
9. 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: 140–149.
10. Bunning ML, Bowen RA, Cropp CB *et al.* Experimental infection of horses with West Nile virus. *Emerg Infect Dis* 2002; 8: 380–386.
11. Ergunay K, Litzba N, Lo MM *et al.* Performance of various commercial assays for the detection of Toscana virus antibodies. *Vector Borne Zoonotic Dis* 2011; 11: 781–787.
12. Ergunay K, Whitehouse CA, Ozkul A. Current status of human arboviral diseases in Turkey. *Vector Borne Zoonotic Dis* 2011; 11: 731–741.
13. Kalaycioglu H, Korukluoglu G, Ozkul A *et al.* Emergence of West Nile virus infections in humans in Turkey, 2010 to 2011. *Euro Surveill* 2012; 17: pii: 20182.
14. Ergunay K, Sayiner A, Litzba N *et al.* Multicentre evaluation of central nervous system infections due to Flavi and *Phleboviruses* in Turkey. *J Infect* 2012; 65: 343–349.
15. Chowers MY, Lang R, Nassar F *et al.* Clinical characteristics of the West Nile fever outbreak, Israel, 2000. *Emerg Infect Dis* 2001; 7: 675–678.
16. Davis LE, DeBiasi R, Goade DE *et al.* West Nile virus neuroinvasive disease. *Ann Neurol* 2006; 60: 286–300.
17. Murray KO, Koers E, Baraniuk S *et al.* Risk factors for encephalitis from West Nile virus: a matched case-control study using hospitalized controls. *Zoonoses Public Health* 2009; 56: 370–375.
18. Bakonyi T, Ivanics E, Erdelyi K *et al.* Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerg Infect Dis* 2006; 12: 618–623.
19. Ocal M, Onder H, Arsava EM, Alp S, Ozkul A, Ergünay K. A case of central nervous system infection due to west nile virus lineage-1 in ankara province, Turkey. *Mikrobiyol Bul* 2013; 47: 164–172.
20. Ozkul A, Ergunay K, Koysuren A *et al.* Concurrent occurence of human and equine West Nile virus infections in Central Anatolia, Turkey: the first evidence for circulaton of lineage 1 viruses. *Int J Infect Dis* 2013; 17: e546–e551.
21. Ergunay K, Gunay F, Oter K *et al.* Arboviral surveillance of field-collected mosquitoes reveals circulation of West Nile Virus (WNV) Lineage 1 strains in Eastern Thrace, Turkey. *Vector Borne Zoonotic Dis* 2013; 13: doi:10.1089/vbz.2012.1288.
22. Carhan A, Uyar Y, Ozkaya E *et al.* Characterization of a new phlebovirus related to sandfly fever Sicilian virus isolated during a sandfly fever epidemic in Turkey. *J Clin Virol* 2010; 48: 264–269.
23. Kocak Tufan Z, Weidmann M, Bulut C *et al.* Clinical and laboratory findings of a sandfly fever Turkey Virus outbreak in Ankara. *J Infect* 2011; 63: 375–381.
24. Ergunay K, Saygan MB, Aydogan S *et al.* Sandfly fever virus activity in central/northern Anatolia, Turkey: first report of Toscana virus infections. *Clin Microbiol Infect* 2011; 17: 575–581.
25. Ergunay K, Aydogan S, Ilhami Ozcebe O *et al.* Toscana virus (TOSV) exposure is confirmed in blood donors from Central, North and South/Southeast Anatolia, Turkey. *Zoonoses Public Health* 2012; 59: 148–154.
26. Ergunay K, Erisoz Kasap O, Kocak Tufan Z, Turan HM, Ozkul A, Alten B. Molecular evidence indicates that *Phlebotomus* major sensu lato (*Diptera*: *Psychodidae*) is the vector species of the recently-identified sandfly fever Sicilian Virus variant: sandfly fever Turkey virus. *Vector Borne Zoonotic Dis* 2012; 12: 690–698.