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Investigation of Tick- and Mosquito-Borne Flaviviruses in Blacksea Region

Harun ALBAYRAK[1]✉, Emre OZAN[2], Abdullah CAVUNT[2], Hamza KADİ[2], Mitat KURT[3], Cenk BOLUKBASİ[4], Zafer PEKMEZCİ[4], Selma KAYA[3]

1. Department of Virology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun.
2. Virology Laboratory, Veterinary Control Institute, Samsun.
3. Parasitology Laboratory, Veterinary Control Institute, Samsun.
4. Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun.

Abstract: Species within the *Flavivirus* genus pose public health problems around the world. Increasing cases of Dengue and Japanese encephalistis virus in Asia, frequent outbreaks of Yellow fever virus in Africa and South America, and the ongoing spread of West Nile virus throughout the America, show the geographical burden of flavivirus diseases. In this study, a total of 2340 adult ticks (675 hard tick pools) collected from the variety of mammalian species (cattle, sheep, goat and buffalo) and 3226 mosquitoes (142 mosquito pools) including *Culex* spp., *Anopheles* spp., *Aedes* spp., and *Culicoides* spp., trapped in Blacksea region of northern Turkey were surveyed for the presence of RNA from mosquito-borne flaviviruses (MBFV) and tick-borne flaviviruses (TBFV) by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. No flavivirus genomic RNA was detected in these samples. This is the first study about both the TBFV and MBFV infections in Turkey.

Key words: Mosquito, RT-PCR, Tick, Turkey, Vector-borne flavivirus.

Karadeniz Bölgesinde Kene ve Sineklerle Taşınan Flavivirusların Araştırılması

Özet: Flavivirus cinsi içinde yer alan türler dünya üzerinde halk sağlığı problemleri oluşturmaktadır. Asya'da artan Japon ensefalitis ve dang virus humması vakaları, Güney Amerika ve Afrika'da sık görülen sarı humma salgınları ve Amerika'da baştan başa yayılan batı nil virusu, flavivirusların coğrafi yoğunluğunu göstermektedir. Bu çalışmada, Karadeniz bölgesinde çeşitli memeli hayvan türlerinden (sığır, koyun, keçi ve manda) toplanan 2340 olgun kene (675 kene havuzu) ve *Culex* spp., *Anopheles* spp., *Aedes* spp., ve *Culicoides* spp.'den oluşan 3226 sinek (142 sinek havuzu) sinek ve kenelerle taşınan flavivirusların varlığı yönünden reverz-transkriptaz zincir reaksiyonu metodu ile test edildi. Örneklerin hiçbirisinde flavivirus genomik RNA'sı tespit edilemedi. Bu çalışma, hem kene hem de sineklerle taşınan flavivirusların varlığı yönünden Türkiye'de yapılan ilk çalışmadır.

Anahtar kelimeler: Kene, RT-PZR, Sinek, Türkiye, Vektör-taşıyıcılı flavivirus.

✉ Harun ALBAYRAK
Department of Virology, Faculty of Veterinary Medicine, Ondokuzmayis University, Samsun.
e-mail: harunalbayrak55@msn.com

INTRODUCTION

Over 70 viruses classified as members of *Flaviviridae* family *Flavivirus* genus are included in arboviruses. *Flaviviruses* have an 11 kb single-stranded, positive-sense RNA genome, encoding a single polyprotein, which is co- and post-translationally cleaved by host and virus encoded proteases (Burke and Monath, 2001). The *flaviviruses* form a monophyletic lineage that is currently divided into three main groups: tick-borne flaviviruses group (TBFV), mosquito-borne flaviviruses (MBFV) and no known vector (NKV) flaviviruses group. The tick-borne flaviviruses currently include twelve recognized species that are divided into two groups, the mammalian tick-borne (M-TBFV) and seabird tick-borne virus group (S-TBFV). Nevertheless, these viruses share a common ancestor within the genus Flavivirus (Burke and Monath, 2001; Thiel et al., 2005). The mammalian tick-borne flavivirus group includes six human and animal pathogens, previously known as the "tick-borne encephalitis (TBE) serocomplex," namely Louping ill (LIV), Tick-borne encephalitis (TBEV), Omsk hemorrhagic fever (OHFV), Langat (LGTV), Kyasanur Forest disease (KFDV) and Powassan virus (POWV) (Charrel et al., 2001). Dengue (DENV), West Nile (WNV), tickborne encephalitis (TBEV), and yellow fever virus (YFV) are among the most prevalent and clinically important *flaviviruses* throughout the world (Burke and Monath, 2001; Gaunt et al., 2001). The genus Flavivirus comprises more than 50 recognized species, including a large number (approximately 50 %) of human pathogens responsible for biphasic fever, encephalitis or hemorrhagic fever (Calisher et al., 1989).

Mosquitoes and ticks are important for public health because they can be infected by a number of pathogenic microorganisms that are transmissible to humans. Among them, the most important ones are emerging infectious diseases that are recently recognized or previously known diseases appearing in a new population or are rapidly increasing in incidence or geographic area (Kurt et al., 2002). Although, the mosquito and tick species are known to transmit vector-borne diseases have been observed (Dik et al., 2006; Albayrak et al., 2010) and some vector-borne flaviviruses such as West Nile (WNV) and Tick-borne encephalitis virus (TBEV) antibodies have been detected in humans and animals in Turkey (Ozkul et al., 2006; Uyar et al., 2007; Ergunay et al., 2007a, 2007b), except three human West Nile cases in 2010, there has been no report of acute infections humans and animals in Turkey.

The aims of this study were to survey tick samples collected from different mammalian species and mosquito pools for the presence of RNA from mosquito- (MBFV) and tick-borne flaviviruses (TBFV) in the northern Turkey.

MATERIALS and METHODS

Mosquito and Tick Processing

A total of 2340 adult ticks (675 tick pools) were collected from cattle, sheep, goat and buffalo and 3226 mosquitoes (142 mosquito pools) were trapped in the Blacksea region of Turkey (Samsun, Sinop, Ordu, Giresun, Trabzon, Rize, Tokat, Amasya, Sivas) (Figure 1). Mosquitoes were collected primarily by using the CDC miniature light traps. Traps were set in mid- to late afternoon. Mosquitoes were collected the following morning, taken to the laboratory, and frozen. Sorting and identification were performed on a chilled table, after which the specimens were stored at −70 °C until testing for the presence of viral RNA. Mosquitoes were pooled by species, location and date of collection and shipped on dry ice to our laboratory. The numbers and distribution of tick and mosquito species according to the collection points on farms are illustrated in Tables 1–2. Although ticks were collected between May and July of 2008, mosquitoes were trapped between June and

October of 2011 and 2012. They were pooled according to the size and pools ranged from one to 50 mosquitoes and ticks. They were placed in 2 ml PBS diluent with MagNA Lyser Green Beads (Roche, Mannheim, Germany). Pools were homogenised at 3.000 *g* for 3 min by MagNa Lyser (Roche, Mannheim, Germany). Homogenates were centrifuged in eppendorf tubes at 12.000 *g* for 3 min to remove the suspended solids, without removing the beads. The supernatants were stored at −70 °C until being used.

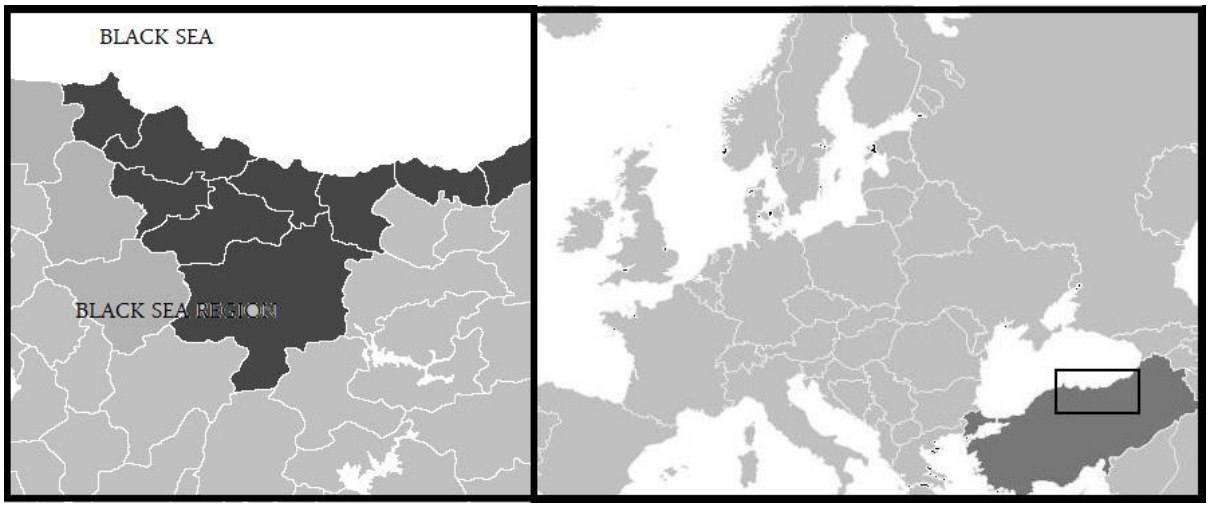

Figure 1. The sampling area.
Şekil 1. Örnekleme alanı.

RNA Extraction, Reverse-transcriptase PCR Assay

Viral RNA was extracted from 350 µl of supernatant by using the MagNA Pure LC RNA Isolation Kit III (Roche, Mannheim, Germany) and stored at −80 ^{o}C. PCR were performed with Titan One-tube RT-PCR system (Roche, Mannheim, Germany) acoording to manufacturer's instructions. Two sets of universal primers were used for detection of mosquito- and tick-borne flaviviruses. These primers correspond to sequences in the 3'-UTR and in the NS5 gene which are highly conserved among the mosquito- and tick-borne flaviviruses (Table 3), (Pierre et al., 1994; Maher-Sturgess et al., 2008). Briefly, RT-PCR was performed in a 50 µl volume containing 5 µl of viral RNA, 1 µl of each primers, 10 µl of 5x RT buffer, 1 µl of 10 mM dNTPs, 1 µl of enzyme mixture, 2.5 µl of 100 mM DTT, 0.25 µl of RNase inhibitor (10 U/ µl), and 28.25 µl of HPLC water. PCR products were analysed in 1 % agarose gels after the electrophoresis at 100 V for 30 min. The DNA bands were observed under the ultraviolet light. Positive controls RNA of WNV and TBEV were kindly provided by Dr. Nicholas Johnson (Veterinary Laboratories Agency, Weybridge, UK) and Dr. Manfred Weidmann (Institute for Virology of the University of Göttingen, Germany), respectively.

RESULTS

A total of 3226 mosquitoes (142 pools) and 2340 adult ticks (675 pools) collected from cattle, sheep, goat and buffalo in northern Turkey were surveyed for the presence of RNA from mosquito- (MBFV) and tick-borne flaviviruses (TBFV) by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. No flavivirus genomic RNA was detected in any sample. This is the first study about both the TBFV and MBFV infections in Turkey.

Table 1. Numbers of tick and mosquito species sampled.
Tablo 1. Örneklenen kene ve sinek türlerinin sayıları.

Ticks	Total number of ticks tested	No. of tick pools	Mosquitoes	Total number of mosquitoes tested	No. of mosquito pools
Hyalomma anatolicum excavatum	71	32	*Aedes* spp.	1427	43
Hyalomma anatolicum anatolicum	10	2	*Anopheles* spp.	934	41
Hyalomma detritum	87	52	*Culex* spp.	711	43
Hyalomma marginatum marginatum	425	173	*Culicoides* spp.	154	15
Rhipicephalus bursa	755	174			
Rhipicephalus turanicus	682	126			
Ixodes ricinus	207	58			
Haemaphysalis punctata	1	1			
Haemaphysalis sulcata	18	14			
Dermacentor marginatus	81	40			
Boophilus annulatus	3	3			
Total	2340	675	Total	3226	142

Table 2. Numbers of ticks, mosquitoes and pools sampled according to province.
Tablo 2. İllere göre örneklenen kene, sinek ve havuzların sayıları.

No	Provinces	Total number of ticks tested	No. of tick pools	Total number of mosquitoes tested	No. of mosquito pools
1.	Sinop	398	128	744	27
2.	Samsun	532	123	1803	43
3	Ordu	112	23	113	8
4.	Giresun	302	79	30	9
5.	Amasya	193	54	89	16
6.	Tokat	314	107	375	25
7.	Sivas	489	161	50	3
8.	Trabzon	-	-	14	7
9.	Rize	-	-	8	4
Total		2340	675	3226	142

Table 3. Oligonucleotide primers used in the RT-PCR assay.
Tablo 3. RT-PZR testinde kullanılan oligonükleotid primerleri.

Primer	Genome position	Sequence (5'-3') Binding of YF ref (NC_002031)	RT-PCR product size (bp)
Flav 100F	8276–8296	AAY TCI ACI CAI GAR ATG TAY	
Flav 200R	9062–9078	CCI ARC CAC ATR WAC CA	802
EMF1	10055–10074	TGG ATG ACS ACK GAR GAY ATG	
VD8	10709–10728	GGG TCT CCT CTA ACC TCT AG	673

DISCUSSION

Mosquito- and tick-borne flaviviruses are emerging as the cause of some of the most serious and widespread arthropod-borne viral diseases in the world. Flavivirus outbreaks are influenced by intrinsic (e.g., viral strain, vector competence, host susceptibility) and extrinsic factors (e.g., temperature, rainfall, human land use) that affect the biologies of mosquitos and ticks in complex ways. The influence of extrinsic factors such as temperature, rainfall, seasonal and multi-year weather patterns, and human behavior affecting the mosquito and tick biologies and thereby flavivirus transmission, is explored. Reservoir–vector–climate

trio was very important at the epidemiology for all the vector-borne flaviviruses. Climatic conditions of Blacksea region appeared convenient for mosquitoes and ticks. The average annual values of heat, humidity, and rainfall of Blacksea region were 13.0 (4.2–22.1), 71 %, 842.6 mm^3 (Anonim, 2010). Mosquitoes and ticks play a main role for the epidemiology of some vector-borne diseases because they are the main amplifying host of the virus in nature. These viruses have been isolated from a number of mosquito and tick species in different areas (Kurt et al., 2002).

These primer sets (EMF1-VD8 and Flav 100 F-Flav 200 R) capable of amplifying 673 and 802 bp from the 3'UTR and NS5 genes from almost every recognised member of the genus Flavivirus. Since the amplified products represent 7-8 % of the genome, this is sufficient sequence to determine the species of the virus and thus potentially to identify the flaviviruses unrecognised. Indeed, traditional serological methods based on the neutralisation and ELISA, have proven effective for identifying the flaviviruses and classification. By using this technology however, some flaviviruses could not be classified due to; the difficulties in interpreting the antigenic cross-reactivity or the failure to identify relatively closely the antigenic relationships, depending on the epitopes encoded by the regions of genome not being reflected by the serological tests. Moreover, the serology is time-consuming, requires highly experienced personel and is less precise than the PCR. Using the molecular methods, it is now possible to analyse the archival material and confirm the identification of tentatively identified flaviviruses (Pierre et al., 1994; Maher-Sturgess et al., 2008).

Flaviviruses have a wide geographical range that includes the portions of Europe, Asia, Africa, Australia and America (Burke and Monath, 2001). Some vector-borne flaviviruses such as West Nile (WNV) and Tick-borne encephalitis virus (TBEV) antibodies have been detected in humans and animals in Turkey (Ozkul et al., 2006; Uyar et al., 2007; Ergunay et al., 2007a, 2007b) and antibodies and viruses have been detected among the mammals and vectors (mosquitoes and ticks) in the neighbouring countries of Balkan peninsula (Hubalek and Halouzka, 1999). In addition, the mosquito and tick species known to transmit the vector-borne diseases have been observed in Turkey (Dik et al., 2006; Albayrak et al., 2010). However, with the exception of three human West Nile cases in 2010, there has been no report on acute infection in humans and animals in Turkey. All the cases in human were detected in the Aegean region of our western border. This region is also the border between Turkey and Greece where West Nile-born human cases were observed in 2010, along with eighteen people died in Greece. The average annual values of heat, humidity and rainfall were 16.3 °C (6.4–26.8 °C), 63.2 %, 725.9 mm^3 in the Aegean region, while the annual heat changes were reported to be more dramatic in Blacksea region (Anonim, 2010). Undoubtedly, a higher vector activity ultimately causes to an increase in the vector-dependent diseases. For mosquitoes, the climatic conditions of Aegean region appeared more suitable than the Blacksea region. However, there seems no report available on the presence of TBEV and WNV in Blacksea region. To our knowledge, although Turkish sheep encephalitis virus (Gene Bank no. DQ235151.1, previously recognized as Turkish subtype of Louping ill virus) was reported by Grard et al. (2007), there seems no data available for the georgraphical source of this isolate.

Herein, the viral nucleic acid was not detected in ticks and mosquitoes in the northern Turkey. The existing data in Turkey is not enough to determine the region-based profile of the TBFV and MBFV infections. Besides, further studies are mandatory for understanding the vector dynamics, interactions between various sensitive species and risk factors of exposure.

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