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Investigation of Tick-Borne Encephalitis Virus (TBEV) in Ixodid Ticks Collected from Central Black Sea Region in Turkey

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Abstract –Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* causes thousands of Tick-borne encephalitis (TBE) cases annually throughout Europe. TBE is an infectious disease involving the central nervous system, which may result in death. TBEV is transmitted to humans by ixodid tick species such as *Ixodes ricinus*, *Ixodes persulcatus* and *Haemaphysalis concinna*. To date, there is no evidence for the presence of TBEV in Turkish ticks. In the present study, presence of TBEV in Ixodid ticks collected from humans in Amasya, Tokat and Ordu provinces was investigated using Revers Transcriptase Polimerase Chain Reaction (RT-PCR). A total 918 tick specimens were collected from humans applying health centers with tick bites in Amasya (n=630), Tokat (n=60) and Ordu (n=228), provinces located in Central Black Sea Region. According to RT-PCR results, no TBEV detected in *Ixodes ricinus* or other ixodid tick species tested in this study. This result indicates that the tick species tested from the region have no potential for transmission of TBEV.

Keywords -
Ticks, TBEV, Human infestation,, Tokat, Ordu, Amasya

1. Introduction

Many members of the Flaviviridae family cause significant public health problems in different regions of the world. Tick-borne encephalitis virus (TBEV) causes a serious encephalitic illness with a mortality ranging from 1% to 30% [1]. Three subtypes of the TBEV are known: the European, Siberian, and Far Eastern [2]. The virus is usually transmitted by tick bites. The main vector for the European subtype, *Ixodes ricinus* (Linnaeus) [3], is prevalent in Europe and Middle East. Whereas, the main vector of Siberian and Far Eastern subtypes, *Ixodes persulcatus* Schulze ranges from Eastern Europe to China and Japan [4].

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Tick-borne encephalitis (TBE) cases have been reported in many European countries [5]. TBEV were determined in ixodid ticks collected from humans, cows and rodents in Germany [6], Sweden [7], and Czech Republic [8], Italy [9], and Poland [10]. As reported by Takeda *et al.* [11], the distribution of the TBEV in ticks and vertebrate hosts covers almost the entire southern part of the non-tropical Eurasian forest belt, from Alsace-Lorraine in the west to Vladivostok and the northern and eastern regions of China in the East. The seroprevalence studies on TBEV demonstrated that TBE is prevalent in especially in Thrace and Black Sea regions of Turkey [12]. However, determination of TBEV seropositivity in humans from central and southeastern Turkey [13], indicates that TBEV infections is not limited to a specific geographic location. Although there have been vaccine programs in European countries, there are no awareness programs for TBE and other tick-borne disease in Turkey, because of low mortality and asymptomatic progress of the diseases. Since, the adverse effects of the disease may appear after years and cause permanent health problems and decrease in life quality, TBEV should not be ignored.

Even though *I. ricinus* is very common in many provinces with mild or moderate climate and coastal provinces and harbor Crimean-Congo Hemorrhagic Fever (CCHF) [14], studies on TBEV prevalence in ticks are very limited in Turkey. Abundance of *I. ricinus* and detection of TBE cases in humans especially in the Black Sea and Thrace regions suggest the possibility of transmission of TBEV by ticks in these locations.

The aim of this study was investigated presence of TBEV in several tick species collected from humans in Amasya, Tokat and Ordu provinces in Turkey by using molecular methods.

2. Material and Methods

2.1 Ticks

Total of 918 tick samples (Table 1) were collected from humans applying health centers with tick bites in provinces of Central Black Sea Region; Amasya (40° 39′ N, 35° 51′ E), Tokat (40° 19′ N, 36° 43′ E) and Ordu provinces (Central, 41° 00′ N, 37° 53′ E and Fatsa Districts, 41° 03′ N, 37° 49′ E) (Figure 1). Ticks were detached from human skin by health personnel under aseptic conditions. Tick samples were transported to laboratory in 15 ml plastic tubes containing 70% ethanol. Then tick samples were transferred to 2 ml sterile Eppendorf tubes containing RNA/DNA stabilization solution (Roche, Germany). Tick samples were stored at -80 ^{o}C after they were identified to species using identification keys by Pomerantzev [15], Filippova [16], Apanaskevich and Horak [17]. Before testing for TBEV presence, ticks were pooled by genus/species and location. For Tokat and Amasya provinces, 63 (630 ticks) *Hyalomma spp*, 4 (40 ticks) *I. ricinus* and 2 (20 ticks) *Haemaphysalis concinna* Koch pools each of 10 ticks were formed. For the Central and Fatsa districts of Ordu province, 41 (205 ticks) adult and 4 nymph (20 ticks) pools each of 5 ticks and 1 pool of larvae (3 ticks) were included in *I. ricinus* pools.

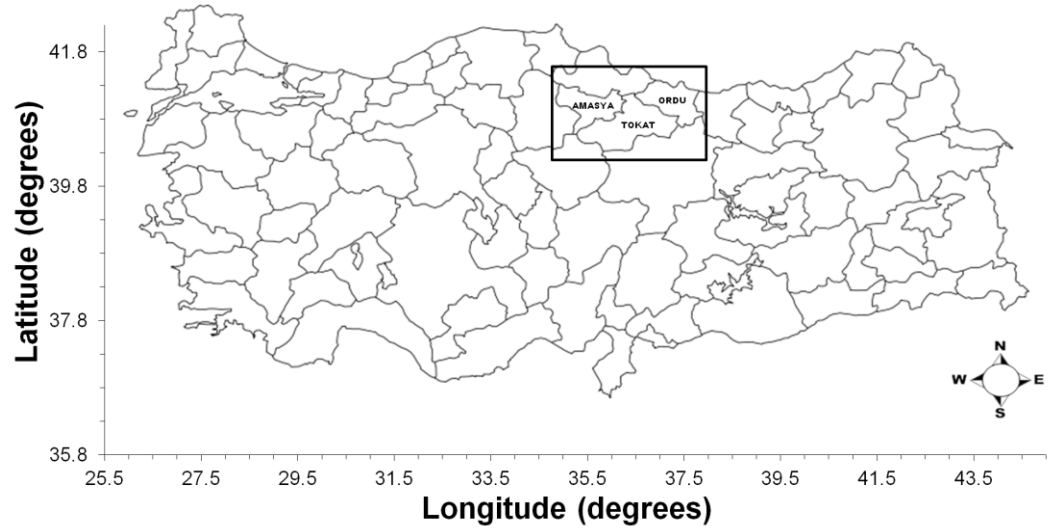

Figure 1. Map of Amasya, Tokat and Ordu Provinces in Central Black Sea Region in Turkey

Table 1. Numbers and location of tick species used in this study.

Tick species	**Location [number of ticks (stage)]**		
	Tokat	**Amasya**	**Ordu Central and Fatsa**
Hyalomma spp.		630 (Adult)	-
Haemaphysalis concinna	20 (Adult)		-
Ixodes ricinus	40 (Adult)		205 (Adult) / 20(Nymph) / 3(Larvae)
Total	**60**	**630**	**228**

2.2 Viral RNA Isolation

Ticks in each tick pool was individually cut half in the middle and were crushed in a micro centrifuge tube containing 500 µl RNA/DNA stabilization solution using a sterile plastic pestle. The homogenates were centrifuged at 2000 x g for 5 min and 250 µl supernatant used for viral RNA isolation using High Pure RNA isolation kit (Roche, Germany).

2.3 RT-PCR

Presence of TBEV in tick pools were determined by one step RT-PCR assay using Transcriptor One Step RT-PCR Kit (Roche, Germany) with several TBEV specific primers (Table 2). Reaction conditions were 55°C 30 min, 94°C for 10 min, 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 10 sec and 72°C for 5 min for final elongation. In all reactions, total volume was 25 µl. The amplicons were visualized on a 2% agarose gels stained with ethidium bromide. Any PCR products from tick pools obtained by RT-PCR were purified and sequenced using an ABI 3130XL Genetic Anaylzer (Applied Biosystems, Fostercity, CA) with a BigDye Cycle Sequencing kit (Applied Biosystems, Fostercity, CA).

Table 2. The primers used to test the presence of TBEV [25].

Primers	*Sequence (5'-3')*	*TBEV Region (nt)*
5'NCR-OF	5'-AGA TTT TCT TGC ACG TGC AT-3'	1-20 (+ sense)
5'NCR-OR	5'-CTC TTT CGA CAC TCG TCG AGG-3'	195-175 (-sense)
NS4B-NS5-OF	5'-ATG AGT GGT GTC GTC AGG GGG-3'	7582-7601 (+ sense)
NS4B-NS5-OR	5'-TGC ATG CTG AAC ACG TCC ATT CC-3'	8072-8050 (-sense)
NS4B-NS5-IF	5'-GGG GTT TTT GCC TCT TGG GC-3'	7611-7630 (+ sense)
NS4B-NS5-IR	5'-CCC AGG CTT GTT ACC ATC TTT GG-3'	8024-8002 (-sense)

3. Results and Discussion

3.1 Presence of TBEV in Tick Species

According to RT-PCR results, no TBEV was detected neither in *I. ricinus* nor in *H. concinna* and *Hyalomma sp.* in tick pools tested in this study. This result indicated that at least, the most common TBEV vector, *I. ricinus* ticks of the region and other ticks tested were not harbored TBEV and have no potential for transmission of TBEV.

In Turkey, 39 hard and 8 argasid tick species have been reported [18, 19]. Presensences of various tick-borne pathogens in Turkish ticks has been reported. The Crimean-Congo Hemorrhagic Fever Virus (CCHFV) was demonstrated in various *Hyalomma* [20, 21] *Rhipicephalus* [21], *Haemaphysalis* [20, 21] and *Ixodes* species [14]. In addition to a rich diversity of tick species, numerous fatal cases of tick-borne CCHF have been reported in Turkey in 2010 [22]. Besides, presence of *Borrelia* species in ticks was reported by [23]. Although, presence of several tick-borne pathogens in ticks and seroprevalence of TBE in humans have been reported, there is no enough evidence for the presence of TBEV in ticks in Turkey. Therefore, in the present study, presence of TBEV was investigated in several tick species including *I. ricinus*, a major vector of TBEV.

According to results of this study no TBEV detected in any tick pools of *I. ricinus*, *Hyalomma spp* and *H. concinna* collected on humans from Amasya, Tokat and Ordu provinces. In fact, absence of TBEV in *Hyalomma spp.*, and *H. concinna* pools were not surprising because of not being a common vector of TBEV. Eventhough, some evidences of human cases of TBE reported [24], absence of TBEV in *I. ricinus* pools from Black Sea region was unusual. These results may be associated with number of tick samples and location. Therefore, a tick survey should be conducted for the investigation of TBEV using numerous of samples from several provinces of Black Sea Region in Turkey. In addition, using real time PCR may be preferred because of its higher sensitivity.

4. Conclusion

As a result, according to RT-PCR tests no TBEV was detected in especially the common vector of TBEV, *I. ricinus* and other tick species tested, indicating that ticks of these provinces may have no potential for transmitting TBEV to humans. However, a further and detailed study should be conducted to reveal TBEV potential of ixodid ticks by using more Ixodes tick samples from all provinces of Black Sea and Thrace region.

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