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Original Article
Özgün Araştırma

Serological survey and molecular investigation of Tick-borne encephalitis virus in Northern Turkey

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Abstract: Tick-borne encephalitis (TBE) is an endemic viral zoonotic disease in many regions of Eurasia. In this study, whole blood, serum samples and the hard ticks collected from a variety of ruminant species (cattle, goat and sheep) in the middle Black Sea region of Turkey were investigated for the presence of RNA and IgG antibodies against Tick-borne encephalitis virus (TBEV). No TBEV genomic RNA was found in 2625 tick and 708 blood samples. However, serological examination for anti-TBEV antibodies revealed that TBEV IgG antibody was found as follow: cattle 61 of 198 (30.8%), goat 7 of 115 (6.1%) and sheep 15 of 147 (10.2%), and confirmed by an enzyme-linked immunosorbent assay (ELISA). Positivity rates for the provinces were as follows: Samsun 12.7%, Sivas 35.2% and Tokat 13.2%. This information supports previous findings of TBEV in ticks in Turkey and may be of relevance for public health considerations (in respect to vaccination recommendations for those exposed).

Keywords: Domestic animals, Real time RT-PCR, Seroprevalence, Tick, Tick-borne encephalitis virus

Türkiye'nin kuzeyinde Tick-borne ensefalitis virüsünün serolojik ve moleküler araştırması

Özet: Kene kaynaklı ensefalit (TBE), Avrasya'nın birçok bölgesinde endemik viral zoonotik bir hastalıktır. Bu çalışmada, Türkiye'nin Orta Karadeniz bölgesinde çeşitli ruminant türlerinden (sığır, keçi ve koyun) toplanan tam kan, serum örnekleri ve sert kenelerde Tick-borne ensefalitis virusuna karşı RNA ve IgG antikorlarının varlığı araştırıldı. 2625 kene ve 708 kan örneğinde TBEV genomik RNA bulunamadı. Bununla birlikte, anti-TBEV antikorları için yapılan serolojik inceleme, TBEV IgG antikorunun: 198 sığırdan 61'i (%30.8), 115 keçiden 7'si (%6.1) ve 147 koyundan 15'i (%10.2) ELISA yöntemiyle pozitif bulunmuştur. İller için pozitiflik oranları şu şekildedir: Samsun %12,7, Sivas %35,2 ve Tokat %13,2. Bu çalışma, Türkiye'deki kenelerde TBEV'nin önceki bulgularını desteklemektedir ve halk sağlığı açısından (maruz kalanlara yönelik aşı önerileri açısından) önem arzetmektedir.

Anahtar kelimeler: Evcil hayvanlar, Kene, Real time RT-PCR, Seroprevelans, Tick-borne ensefalitis virüsü

Introductıon

Tick-borne encephalitis virus (TBEV) is one of the vector-borne viruses that belong to the *Flavivirus* genus in the *Flaviviridae* family (Suss, 2011). It is transmitted to humans either by bites of Ixodid ticks, by ingestion of non-pasteurized milk or milk-products (milk from goats and sheep rather than from cows), or by contact with blood or tissues from TBE patients or viremic livestock (Gritsun et al., 2003a; Lindquist and Vapalahti, 2008; Holzmann et al., 2009; Kerlik et al., 2018; Ruzek et al., 2019).

TBEV is found in a wide area covering northern Asia and Europe. It has been classified into five main subtypes, the European subtype (transmitted by *Ixodes ricinus*), the Far Eastern, the Siberian subtype (both transmitted by *I. persulcatus*), the Baikalian and the Himalayan subtypes (Ecker et al., 1999; Gritsun et al., 2003b; Suss 2011; Kovalev and Mukhacheva, 2017; Dai et al., 2018). This disease in humans ranges from asymptomatic to severe infection in the central nervous system (Kaiser, 2012). The fatality rate of the European and Siberian are approximately 1%–3%, while the fatality rate of the Far Eastern subtype might be reached at 20%–40% (Gritsun et al., 2003a; Dorrbecker et al., 2010). In Turkey, only the European subtype is found. However, no outbreaks have been reported until now (Karan et al., 2014; Whitby et al., 1993).

The life cycle of the TBEV in nature includes transovarial and transstadial transmission among ticks and a tick–vertebrate host cycle involving wild and domestic animals. TBEV causes infections in humans, horses and dogs as well (Weissenbock et al., 1998; Klaus et al., 2013; Paulsen et al., 2019, 2020). Ticks are very important to the ecology of

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TBEV. The biological role of the ticks is remarkable, not only as virus vectors but also as reservoirs of the virus. However, there is little information about tick species diversity or tick vector capacity in the endemic regions of Turkey. *Ixodes ricinus* ticks are mainly distributed in Northern Turkey. It is likely that the high rainfall and the intensive forest in this area may contribute to this observation (41°25′21″N; 36°57′38″E). Data available on TBEV infection in Turkey, which shares similar climatic and ecological conditions with the endemic regions, is limited (Aydin and Bakirci, 2007; Paulsen et al., 2019, 2020; Suss, 2011). Although studies have found that vector tick (*Ixodes ricinus*) is distributed in Northern Turkey, the number of human cases of TBE in Turkey has been low since the first case occurred in 1968 (Serter,1968). There are no clinical reports in terms of TBEV in humans in Turkey so far (Ergunay et al., 2011; Yilmaz et al., 2019).To date, there is no evidence of the presence of TBEV in ticks in Turkey (Karan et al., 2014).

With this study, we aimed to determine the prevalence of TBEV among ticks and animals in Northern Turkey, as they are the main organisms involved in the virus life cycle, and also this study was performed to investigate the seroprevalence of anti-TBEV antibodies in domestic ruminants of three provinces of Turkey.

Material and Methods

Tick processing whole blood and serum samples

A total of 2625 ticks and 708 whole blood samples were collected between March and July of 2017 from 93 cattle (99 pools, 183 ticks in total), 106 goats (124 pools, 408 ticks in total) and 509 sheep (556 pools, 2034 ticks in total), grazing in the middle Black Sea region of Turkey. The ticks were collected directly from the animals. After identification using standard keys, ticks were stored at −80°C until testing for the presence of viral RNA (Aktaş and Vatansever., 2014). They were pooled according to size and pools ranged from one to 10 ticks. They were placed in 2 ml of PBS diluent with MagNA Lyser Green Beads (Roche, Mannheim, Germany). Pools were homogenized at 3000 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 used for the RNA extraction. At the same time, 460 serum samples were collected from cattle, goats and sheep in the same region (Table 3).

RNA extraction, TaqMan based real-time RT-PCR and enzyme-linked immunosorbent assay (ELISA)

Viral RNA extraction was performed from 200 µl of tick pool supernatant and whole blood samples by using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at −20°C. The TaqMan primers and probe were described previously (Schwaiger and Cassinotti, 2003) (Table 1). The real-time RT-PCR (rRT-PCR) was performed with the iTaq™ Universal Probes One-Step Kit (Biorad, California, USA) by using 5 µl of RNA combined with 10 pmol of each primer and 10 pmol of the FAM- and TAMRA-labelled probe in a 25 µl total reaction volume according to the manufacturer's protocol with the following cycling times and temperatures: 1 cycle of 50°C for 10 min and 95°C for 3 min and 40 cycles of 95°C for 5 s, 60°C for 15 s. The rate of TaqMan assay positivity was calculated by using the 99.9% confidence level settings in the CFX Connect Optic Module Maestro Software (Biorad, Singapore, Singapore). Positive control plasmid of TBEV was kindly provided by Prof. Dr. Manfred Weidmann (Institute for Virology of the University of Gottingen, Germany). The presence of specific IgG antibodies against TBEV was investigated by a commercial ELISA kit (Test Line, Brno, Czech Republic). The sensitivity and specificity of the IgG ELISA for TBEV are 95.7% (data obtained from Test Line).

Table 1. The primers and probe used in the rRT-PCR assay against TBEV (Schwaiger & Cassinotti, 2003).

Primers and Probe	Sequence (5′-3′)	Genome position
F-TBE 1	5′-GGG CGG TTC TTG TTC TCC-3′	11054-11071
F-TBE 2	5′- ACA CAT CAC CTC CTT GTC AGA CT-3′	11099-11121
F-TBE probe	5′FAM-TGA GCC ACC ATC ACC CAG ACA CA-TAMRA3′	11073-11095

Results

Tick species and distribution

A total of 2625 adult ticks were collected from cattle (99 pools, 183 ticks in total), goat (124 pools, 408 ticks in total) and sheep (556 pools, 2034 ticks in total), in the middle Black Sea region of Turkey (Samsun, Sivas, Tokat) (Fig. 1). The numbers and distribution of tick species according to the collection points on farms are documented in Table 2. Eight tick species were identified and the most abundant were *Haemaphysalis sulcata* 32.4% (850/2625), *Rhipicephalus turanicus* 27.2% (715/2625), *Dermacentor marginatus* 16.6% (437/2625) and *Hyalomma marginatum* 13.3% (349/2625). *Rhipicephalus bursa* represented 6.1% (160/2625) of the total number of ticks. *Haemaphysalis punctata, Hyalomma detritum* and *Ixodes ricinus* were less common and represented 3.2% (85/2625), 0.6% (16/2625) and 0.5 (13/2625) of the tick population, respectively. *Ixodes ricinus* was mainly found in Samsun province, which lies in the coastal area of the surveyed region. *Dermacentor marginatus, Rhipicephalus bursa* and *R. turanicus* were found in all provinces in the surveyed region. *Hyalomma detritum* was found in only one location (Tokat). *Hyalomma marginatum*, *Haemaphysalis punctata* and *Haemaphysalis sulcata* were found in only two localities (Sivas and Tokat).

Figure 1. Areas where ticks and samples were collected for Tick-borne encephalitis virus

Table 2. Distribution of tick samples collected by host and provinces.

Provinces	Samsun				Sivas				Tokat				Total		
Ticks	**Cattle**	**Goat**	**Sheep**	**Total**	**Cattle**	**Goat**	**Sheep**	**Total**	**Cattle**	**Goat**	**Sheep**	**Total**	**Cattle**	**Goat**	**Sheep**
Rhipicephalus turanicus	39		101	140	12	150	407	569	1		5	6	52	150	513
Rhipicephalus bursa	4		18	22		47	66	113	1	19	5	25	5	66	89
Dermacentor marginatus			2	2		13	340	353		7	75	82	-	20	417
Ixodes ricinus	11		1	12						1		1	11	1	1
Hyalomma marginatum					23	5	37	65	80	47	157	284	103	52	194
Haemaphysalis punctata						5	8	13		6	66	72	-	11	74
Haemaphysalis sulcata						1		1		103	746	849	-	104	746
Hyalomma detritum									12	4		16	12	4	-
Total				176				1114				1335	183	408	2034
											Total tick samples:		183+408+2034:		2625

TBEV nucleic acid and antibody detection

A total of 779 tick pools (2625 adult ticks), 708 whole blood samples were tested by rRT-PCR for TBEV. No TBEV genomic RNA was detected in tick pools and whole blood samples. Serological examination of serum samples for anti-TBEV infection revealed that TBE IgG antibody was present in 61 of 198 (30.8%), 7 of 115 (6.1%) and 15 of 147 (10.2%) cattle, goat and sheep, respectively (Table 3). The provincial distribution of chosen serum samples in this study was as follows: Samsun 12.7% (21/165), Sivas 35.2% (37/105) and Tokat 13.2% (25/190).

Table 3. Seroprevalence of IgG antibodies against TBEV collected from domestic animals in Northern Turkey.

	Cattle (%)	Goat (%)	Sheep (%)	Total
Samsun	20/85 (23.5)	0/38 (0)	1/42 (2.4)	21/165 (12.7)
Sivas	24/50 (48)	-	13/55 (23.6)	37/105 (35.2)
Tokat	17/63 (27)	7/77(9.1)	1/50 (2)	25/190(13.2)
Total	61/198 (30.8)	7/115 (6.1)	15/147 (10.2)	83/460 (18.04)

Discussion and Conclusion

The presented study is aimed to compare the presence of TBEV in various domestic animals in Turkey. We identified TBEV antibodies in cattle, goats and sheep. The majority of the positive serum samples collected from cattle included in this study originated from inland Turkey. This is in the area where human Crimean-Congo Hemorrhagic Fever (CCHF) cases have been reported in Turkey, which is transmitted by ticks (Bakir et al., 2005). TBEV positive samples were furthermore detected in the counties of Samsun and Tokat, which are also endemic for tick-borne human cases. This result supports previous findings of TBE antibodies in human blood donors (Ergunay et al., 2007, 2011). The presence of TBE antibodies in domestic animals (sheep) has only been studied in Turkey in the early 1970s. This study from 1971 detected a seroprevalence of 1.3% in sheep sera in southern Turkey (Radda, 1971). In the present study, a total of 460 serum samples was analyzed. The seropositivity rate in cattle (30.8%) was found to be higher than the rate in sheep (10.2%) and goats (6.1%). The previous study from 1971 seems to be based on animals taken almost exclusively from non-endemic areas, which might help explain the low prevalence found in that study (Radda, 1971). Antibodies are detectable in ruminants even 28 months after infection (Balogh et al., 2012; Klaus et al., 2014). The antibody response seems to vary between species, and it is unclear if all animals exposed to the virus develop an immune response (Klaus et al., 2010, 2012; Paulsen et al., 2020). While the age of the animals and their entry into new areas may affect the results of the study, adult animals using the same pasture in more than one season were used in this study. TBEV specific antibodies were detected in all three provinces of our study in the North of Turkey.

Seroprevalence studies on TBE in domestic animals in Europe have demonstrated that animals may serve as useful sentinels for the detection of TBEV risk areas (Klaus et al., 2010, 2012; Rieille et al., 2017; Salat et al., 2017; Paulsen et al., 2020). A few human seroprevalences were reported in different regions in Turkey concerning IgG antibodies to TBEV, and overall seroprevalence was found to be between 1.1% and 10.5% (Ergunay et al., 2007, 2011; Uyar et al., 2007; Esen et al., 2008; Inci et al., 2016; Yılmaz et al., 2019;). While TBEV antibodies were detected in 1% in Central Anatolia and 0.3% in the Marmara region, no antibodies were detected in other regions (Blacksea, Aegean, Eastern Anatolia, and Southeastern Anatolia regions) (Uyar et al., 2007). In addition, Ergunay et al. (2007) detected 10.5% TBEV seropositivity in the Southeast Anatolia region. The only animal seroprevalence data on TBEV come from the Mediterranean region (Hatay province-southern border) of Turkey. A study from 1971 detected a seroprevalence of 1.3% in sheep sera in southern Turkey (Radda, 1971).

Contrary to our research, Juceviciene et al. (2005) found that the rate of seropositivity in sheep in Lithuania was higher than in cattle and goats. It is thought that the difference between these two studies may occur for two reasons. First, it is thought that ovine breeders in our country are more conscious in the fight against ticks, and the second is that small ruminant herds are grazed in high areas where the humidity and temperature are low in the summer months when ticks are active. It is very difficult

to compare studies that have differences in content (methods, species, sample size and time, geography etc).

Since there is no vaccination program against TBEV in Turkey, it is thought that the source of detected seropositivity is natural infection. The results of serological assays may indicate that the virus is circulating in this area. These results pose a greater risk for people living or visiting the region. In this study, we found no relationship between TBEV RNA in ticks and TBE IgG in serum. The difference may be caused by sampling time and size as mentioned above.

Turkey's tick fauna has been shown to include about 32 species and *Ixodes spp*. have been detected mostly in Northern Turkey, where the amount of rainfall during the year is the highest and intensive forests are the predominant vegetation. Nevertheless, in addition to the Black Sea region, *I. ricinus* activity has been shown to be present in the Mediterranean, Aegean and east/southeast Anatolia (Aydin and Bakirci, 2007). Although TBEV has been detected and characterized in sheep, it has not yet been detected in ticks in Turkey.

This study was carried out in Samsun, Sivas and Tokat provinces in the Black Sea Region, which are suitable for the life cycle of *I.ricinus*. It is estimated that the reason TBEV RNA could not be detected as a result of the study may be due to the low number of tick species that are the main vector (*I.ricinus)* of TBEV among the tick species collected from animals in these provinces. Although more *I.ricinus* ticks were collected in the study of Karan et al. (2014) could not detect TBEV RNA. This situation can be interpreted as that the tick species in our study area are not infected with TBEV.

More seroprevalence studies in domestic animal populations with huge geographical coverage and greater sample size should be carried out. Furthermore, a seroprevalence study on people working in close contact with these animals could provide important epidemiological data for risk evaluation, as they could have been exposed to ticks.

This study put forth the first comprehensive screening of domestic ruminant species in Northern Turkey in terms of TBE antibodies and provides updated information on the distribution of TBEV. This study supports previous findings, which indicates that TBEV is distributed in Turkey, more widely than suggested by human TBE cases. The results obtained from this study contain important findings in terms of public health.

Use of laboratory animals Ethics Committee and other decisions of Ethics Committee and Permissions: We designed all study protocols and procedures following the national legislative rules and ethical standards, under validation order by the Samsun Veterinary Control Institute Scientific Ethics Committee, Ministry of Agriculture and Forestry, Republic of Turkey (No: 250720167-2, Date: 25/07/2016).

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