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Seroprevalence of *Encephalitozoon cuniculi*, *Francisella tularensis* and *Toxoplasma gondii* in Zoonotic Diseases in European Hares (*Lepus europaesus*)

Avrupa Tavşanlarında (Lepus europaeus) Encephalitozoon cuniculi, Francisella tularensis ve Toxoplasma gondii Zoonoz Hastalıklarının Seroprevalansı

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ABSTRACT

Objective: European hares (*Lepus europaeus*) are among the most important animals that are connected with humans in many countries and natural life. Hares are important for public health, since they carry many zoonotic diseases, such as *Encephalitozoon cuniculi, Francisella tularensis* and *Toxoplasma gondii*. The study aimed to determine the seroprevalence of *Encephalitozoon cuniculi*, *Francisella tularensis* and *T. gondii* and the potential zoonotic risk posed by hares that live in provinces of Turkey.
Methods: Blood samples were collected from hares during the official hunting season. Serum samples were examined serologically by enzyme-linked immunosorbent assay for *E. cuniculi*, Sabin-Feldman dye test was used to examine *T. gondii*, while micro-agglutination test was used to examine *F. tularensis*.
Results: Of the total of 42 hares examined, one (2.4%) was found positive for *E. cuniculi*, two (4.8%) were found positive for *T. gondii* and one (2.4%) was found positive for *F. tularensis*.
Conclusion: Anti-*T. gondii* and anti-*E. cuniculi* antibodies were serologically detected in hares for the first time in Turkey. Furthermore, this is the first study reporting the seropositivity of *F. tularensis* infection in hares.
Keywords: Hare, zoonotic, *Encephalitozoon cuniculi, Francisella tularensis*, *Toxoplasma gondii,* serology

ÖZ

Amaç: Avrupa tavşanları (*Lepus europaeus*) pek çok ülkede bulunan ve doğal yaşamda insanlarla bağlantısı olan en önemli hayvan türlerinden biridir. Tavşanlar; *Encephalitozoon cuniculi, Francisella tularensis*, *Toxoplasma gondii* gibi birçok zoonotik hastalığı taşıdığı için halk sağlığı açısından oldukça önemlidir. Bu çalışmanın amacı, bu tip enfeksiyonların yaygınlığını ve Türkiye'de tavşanların oluşturduğu potansiyel zoonotik riski göstermektir.
Yöntemler: Kan örnekleri, tavşanlardan resmi av sezonunda toplanmıştır. Serum örnekleri; *E. cuniculi* için enzim bağlantılı immüno sorbent testi, *T. gondii* için Sabin-Feldman boya testi ve *F. tularensis* için ise mikroaglütinasyon testi kullanılarak, serolojik olarak incelenmiştir.
Bulgular: Yapılan analiz sonucunda 42 tavşandan 1'inde (%2,4) *E. cuniculi* testi pozitif, 2'sinde (%4,8) *T. gondii* testi pozitif ve 1'inde (%2,4) *F. tularensis* testi pozitif olarak tespit edilmiştir.
Sonuç: Bu çalışmada, Türkiye'de ilk kez yabani tavşanlarda anti-*T. gondii* ve anti-*E. cuniculi* antikorları serolojik olarak tespit edilmiştir. Bu ayrıca, tavşanlarda *F. tularensis* enfeksiyonunun seropozitifliği ile ilgili olarak bildirilen ilk çalışmadır. Çalışmada yer alan üç farklı zoonotik hastalığın sıklığı, diğer Avrupa ülkelerinde bildirilen bulgular ve diğer referanslı çalışmalarla da tutarlıdır.
Anahtar Kelimeler: Tavşan, zoonoz, *Encephalitozoon cuniculi, Francisella tularensis, Toxoplasma gondii*, seroloji

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Wild animals as hosts and reservoirs have been known to play an important role in spreading of some zoonotic diseases. Indeed, throughout history, it is known that zoonotic diseases caused by various bacteria, viruses, and parasites in wild animals constitute a major public health problem, affecting the world population (1). Hare has been involved in the epidemiology of important pathogens, posing threat especially for public health (1-3). Therefore, the importance and the attention given to zoonosis is increasing in the fields of veterinary medicine and public health.

Toxoplasma gondii was discovered in 1908. Since then, it is still a significant zoonotic parasite that has a worldwide distribution which affects humans as well as warm-blooded animals (4). Hares are stated to be exceptionally susceptible to the primary infection (3,5,6). Therefore, the animals are potential reservoirs for *T. gondii* transmission, and the detection of *T. gondii* hosted by rabbits is a public health concern, since human consumption of rabbit meat continues to increase. For this reason, the importance of *T. gondii* infections in hares is on the rise (3,7,8). On the other hand, as far as we know, there are very few studies on the prevalence of toxoplasmosis regarding some wild animals, but concerning the aforementioned infection, there is no data on hare in Turkey.

Tularemia was first reported as an outbreak in a military garrison and rural community in 1936 in the Lüleburgaz district of Turkey. Since then, the disease has been recorded from different regions of Turkey, but the majority of epidemics were reported in the western Black Sea and the Marmara Regions (9,10). Recently, in different regions of Turkey, there has been an increase in reports of tularemia epidemics. Therefore, in 2005, tularemia was included in the list of Communicable Diseases Notification System (Group C) by the Ministry of Health of the Republic of Turkey (11,12). Hare is a common host of *Francisella tularensis*, therefore, infection in humans can occur especially by contacting infected animals during hunting activities, as well as through the consumption of contaminated food and water (1,2,13).

Encephalitozoon cuniculi is a microsporidian parasite commonly found in rabbits that can infect humans, causing encephalitozoonosis. It is an opportunistic pathogen for humans and often causes an asymptomatic disease in wild and domestic rabbits as well. Encephalitozoonosis causes renal and central nervous system diseases in rabbits (14-16).

To date, in Turkey, encephalitozoonosis has been reported in domestic rabbits, but the situation has not yet been investigated in hares, which were identified as European hares (*Lepus europaeus*) according to molecular and morphometric analysis (17). As described above, hares are a potential source of these zoonotic diseases that seriously threaten public health. Therefore, the aim of the present study was to investigate the seroprevalence of *F. tularensis, T. gondii* and *E. cuniculi* in hunted hare in the Central Anatolia region of Turkey.

METHODS

Study Area and Samples

This study was conducted in Ankara and Çankırı provinces at the Central Anatolia region of Turkey (Figure 1). Especially within the districts in the north of the Northern provinces which are located in the transition zone between Central Anatolia and the Western Black Sea regions because the climate characteristics of the Black Sea region and the Inner Anatolian region differ from each other. Blood samples were collected directly from the heart shortly after hares (*Lepus europaeus*) were shot by hunters in the hunting areas. Each blood sample was taken into tubes containing EDTA, and labeled, placed on ice, and were transferred to the Protozoology and Entomology Laboratory of Ankara University and the Zoology Laboratory of Çankırı Karatekin University. Then, the blood samples were centrifuged at 2.000 rpm for 10 minutes in order to separate the sera. Serum samples were stored at -20 °C until serological analysis was performed. Previously collected serum samples from hares in Ankara province by Orkun and Karaer (2017) between 2013 and 2016 were used for serological analysis. The study did not require an Animal Ethics Statement in accordance with the regulations due to samples being obtained from hunted hares during the official hunting season in wildlife (HADMEK: 2021/61).

Serological Assays for Diagnosis of the Diseases

Encephalitozoon cuniculi

The sera of hare were screened for *E. cuniculi*-specific IgG antibodies with a commercial ELISA kit (Express Biotech International-USA) following the manufacturer's instructions while containing positive and negative controls (18). When the difference between sample optical density (OD) and negative-control OD (Δ) was greater than, or equal to 0.300, the sample was evaluated as positive.

Toxoplasma gondii

Anti-*T. gondii* IgG antibodies levels in sera samples were assessed by SFDT (4), and the assessment was carried out in the toxoplasmosis laboratory at the Public Health General Directorate. Briefly, sera samples were inactivated at 56 °C for 30 min. and later, were diluted at 1/4, 1/16, 1/64, 1/256 and 1/1024 titers. Titers 1/16 and above were considered as positive.

Francisella tularensis

The microaglutination test (MAT) was used for the serological diagnosis of *F. tularensis* (19). The application took place in the zoonosis laboratory at the Public Health General Directorate. Briefly, it was based on the observation of antigen antibody reaction in micro plates with the help of dyed antigens. Positive and negative controls and sera were diluted with saline with serial dilutions (1/10, 1/20, 1/40, 1/80, 1/160, and 1/320). *F. tularensis* MAT stock dyed antigen containing 0.005% safranin-O was used as the test antigen. When agglutination in the form of lace was seen, antibody titer was considered positive.

Statistical Analysis

The data obtained in our study is presented in the form of numbers and percentages in the Table 1.

RESULTS

In the official hunting season in wildlife, blood samples were obtained from hares in 11 districts of Ankara and Çankırı provinces (Figure 1) in the Central Anatolian Region of Turkey (Table 1). In this study, 42 sera were taken from hares and were used in the serological screening of the three zoonotic diseases. In these samples, seropositivity rates were 1 (2.38%) for *E. cuniculi*,

2 (4.76%) for *T. gondii*, and 1 (2.38%) for *F. tularensis*. Totally, 4 (9.52%) of these 42 hares were found to be positive (Table 1).

DISCUSSION

With regards to various domestic and wild animals, there are many studies conducted to determine the seroprevalence of *Toxoplasma, Francisella* and *Encephalitozoon* infections using serological techniques. In Turkey, infections caused by the protozoan parasite *T. gondii* are widely prevalent in humans, domestic-animals and some wild animals. In hares, there are few studies on the prevalence of *F. tularensis*, however, as far as we know, there is no study on *E. cuniculi* and *T. gondii* infections. Besides, this study is also the first to detect serologically anti-*E. cuniculi* and anti-*T. gondii* antibodies in hares. It would contribute to the literature by providing up to date information about these diseases which are found in wildlife.

Figure 1. The distribution of rabbits whose blood was taken during hunting season

Although there are many reports about the importance of encephalitozoonosis in rabbits since 1922, the number of these reports are very limited and the related studies are done with minimal numbers of hares (20,21). Compared to the results of the previous reports, the disease has a high prevalence in domestic rabbits, while with regards to the hare, it had a relatively low prevalence. Namely, the prevalence of *Encephalitozoon* spp. infection in domestic rabbits was detected as 15.0% in Egypt (22), 16.5% in Nigeria (23), 52.0% in The United Kingdom (24), 53% (18) in Turkey and 75.4% in Italy (25). On the other hand, in a recent study, Bártová et al. (21) notified that 1.2%, 3.2%, and 4.2% of hares in the Czech Republic, in the Slovak Republic and in Austria respectively, were detected positive for *E. cuniculi* through serology testing and the total prevalence was found to be 1.42%. However, in Western Australia, Thomas et al. (26) reported that 25% of *E. cuniculi* in hares were found seropositive. The results of our study are similar with the previous studies which had low seroprevalence.

Francisella tularensis was first reported to be found in a rodent in California in 1911, and since then, the tularemia infection caused by *F. tularensis* and *F. tularensis* subspecies *holarctica* has been reported in many parts of the world. The transmission of *F. tularensis* to humans can be directly through contaminated water and soil, as well as the meat and fur of an infected hare. In addition, from 1932 until today, tularemia cases have been reported in several studies, thus, the disease is still a public health problem in Turkey (9,10).

In the Central Anatolia region of Turkey, the first epidemic was occurred in the Yağmurdere village of the Ayaş district of Ankara. A total of 16 cases of tularemia were reported in 1997. It was stated that the epidemic was transmitted by water (27). In the following years, the second epidemic was reported from the

Table 1. Distribution of the zoonose diseases found in hares from 11 districts of two provinces in the Central Anatolian Region of Turkey. Titers of the positive samples of Toxoplasma gondii were considered as 1/16 and 1/64. *Francisella tularensis* antibody titer was found as 1/40

Location			***Encephalitozoon cunculi***		***Francisella tularensis***		***Toxoplasma gondii***	
Province	District	N	+	-	+	-	+	-
Ankara	Akyurt	2	0	2	0	2	0	2
	Bala	2	0	2	1	1	0	2
	Gölbaşı	1	0	1	0	1	0	1
	Kazan	1	0	1	0	1	0	1
	Kızılcahamam	6	0	6	0	6	1	5
	Nallıhan	4	0	4	0	4	0	4
	Polatlı	7	1	6	0	7	0	7
	Sincan (Temelli)	7	0	7	0	7	0	7
Çankırı	Bayramören	1	0	1	0	1	0	1
	Kurşunlu	7	0	7	0	7	0	7
	Yapraklı	4	0	4	0	4	1	3
Total		**42**	**1**	**41**	**1**	**41**	**2**	**40**
%			**2.38**		**2.38**		**4.76**	

N: Sample number (+): Seropositive, (-): Seronegative

Çankırı province in the Central Anatolia region. Ulu Kılıç et al. (28) reported that a waterborne tularemia outbreak had occurred in Kadıözü, a village of Çerkeş, which was located in the North-west part of Central Anatolia of Turkey. In addition, experts stressed that the spread of tularemia towards non-endemic regions posed a significant threat to public health. Until now, tularemia was reported to be water-borne in published outbreak reports in Turkey, in addition to the findings which indicate that widespread and large outbreaks were caused by contaminated water sources (29). Therefore, infected rodents and hares have been found responsible for the contamination of water (1,30).

Ozsan et al. (31) reported that *F. tularensis* could not be isolated in rodents (n=1.191), hare (n=89) and turtles (n=56) in Ankara, Konya, Urfa and Nevşehir provinces, but antibodies were detected at 1/40 titers from a living rodent (Citellus). However, during the investigation of the tularemi outbreak in Edirne Demirköy, Gürcan et al. (32) reported that one of the rabbits (n=25) which belonged to a patient had antibody at low titers. On the other hand, studies in European brown hare conducted in various European countries showed that the prevalence of *F. tularensis* ranged from 0% to 7%. That is, 6.5% in the Czech Republic (2), 6% in Austria (33), 5.1% in Hungary (34), 4.5% Austria (35) while no seropositivity was detected in Germany (36). In our study, the rate of seropositive (2.38%) hares were found to be lower than these previous serological studies.

Hare gets infected by ingestion of food or water contaminated with *T. gondii* oocysts. In this way, infected rabbits can be a potential source of *T. gondii* for carnivores and humans (3). The previous studies have shown that *T. gondii* infection has a high seroprevalence rate in many species of warm-blooded animals including humans in many parts of the world (37,38), In Turkey, toxoplasmosis is widely prevalent in animals and the rate of seropositivity in humans is one third (39). In a recent study, Karakavuk et al. (40) notified that the prevalence of *T. gondii* was determined to be molecularly 89.6% in wild birds.

Although it is recommended to use serological methods together in serological screening (41-43), SFDT is considered as gold standard for the detection of anti-*T. gondii* antibodies (44). In several European countries, the prevalence of *T. gondii* was tested with different serological methods such as MAT, IFAT, ELISA, DAT and SFDT (45-48). The results in the Brown hare (*Lepus Europaeus*) were reported in different rates such as, 5.7% in Greece (45), 6% in Slovakia (46), 9% in France (47), 13% in Austria, 21% in the Czech Republic (46) and 46% in Germany (36), while no seropositivity was detected in Sweden (48). In the present study, when compared with the results of other countries, the low seropositivity rate of *T. gondii* (4.8%) was found similar to the prevalence rates which were found in Greece and Slovakia.

This present study detected anti-*E. cuniculi* and anti-*T. gondii* antibodies serologically in hares for the first time in Turkey. In addition, it is the first study which reports the seropositivity of *F. tularensis* infection in hare since 1976.

CONCLUSION

As a result, the frequency of these three different zoonotic diseases were found to be consistent with other results reported from various European countries. However, as relationships between humans and the wildlife become more frequent, the occurrence of these infections can increase among humans. Therefore, in order to prevent the spread of these diseases, it is important to raise awareness about them. Moreover, the prevalence of zoonotic diseases in hares or other wild animals may differ according to the existing climate structure, geographical characteristics and the degree of climate change. In addition, the potential zoonotic risk effects of these diseases may vary from country to country depending on the socio-economic and cultural structures of the relevant countries and societies. For this reason, it should be known that these infections carried by wild animals, especially hare, even at low rates, are considered to be important for public health and therefore, precautions should be taken.

* Ethics

Ethics Committee Approval: Your petition on ''Seroprevalence of Encephalitozoon cuniculi, Francisella tularensis and Toxoplasma gondii in Zoonotic Diseases in European Hares (Lepus europaesus)'' was discussed at the Animal Experiments Center Ethics Committee (HADMEK) meeting dated 20.01. According to the organisation, it has been decided by HADMEK's decision no. 61 that interventions that are not subject to HADYEK permission (procedures with extinct animals or tissue, slaughterhouse materials, waste fetuses) are within the scope of interventions.

Informed Consent: Your petition on ''Seroprevalence of Encephalitozoon cuniculi, Francisella tularensis and Toxoplasma gondii in Zoonotic Diseases in European Hares (Lepus europaesus)'' was discussed at the Animal Experiments Center Ethics Committee (HADMEK) meeting dated 20.01. According to the organisation, it has been decided by HADMEK's decision no. 61 that interventions that are not subject to HADYEK permission (procedures with extinct animals or tissue, slaughterhouse materials, waste fetuses) are within the scope of interventions.

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