

First detection of *Echinococcus multilocularis* in rodent intermediate hosts in Turkey

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SUMMARY

Echinococcus multilocularis is the causative agent of alveolar echinococcosis (AE), a potentially fatal zoonotic disease. Large parts of Turkey are considered as endemic for *E. multilocularis*. The aim of this study was to determine the occurrence of metacestode of *E. multilocularis* in wild rodents in Erzurum, an endemic region for human AE in Turkey. During the sampling period, a total of 498 rodents were trapped in twenty counties of Erzurum Province. Suspected lesions were observed on the livers of 48 rodents, and then partial fragment of mitochondrial 12S rRNA gene was PCR-amplified. Five liver samples exhibited *E. multilocularis* infection. The prevalence of *E. multilocularis* for *Microtus* spp. was 1.3%. All of the infected rodents had fertile metacestodes. Infected rodents were morphologically and molecularly analysed and were confirmed to be *Microtus irani* by the mitochondrial cytochrome *b* gene sequence analysis. This is the first report of the presence of *E. multilocularis* in rodent intermediate hosts in Turkey. Our findings of infected *M. irani* with protoscoleces show that this rodent can act as suitable intermediate host for *E. multilocularis*' life cycle in Turkey. However, there was a complete lack of data on the infection of carnivores from the country. An extensive survey is recommended to determine the prevalence of *E. multilocularis* in definitive hosts in this endemic region.

Key words: *Echinococcus multilocularis*, rodent, intermediate host, Turkey.

INTRODUCTION

Alveolar echinococcosis (AE) caused by the metacestode of *Echinococcus multilocularis* is a serious zoonotic disease. The life cycle of *E. multilocularis* involves various *Microtus* and other arvicolid rodent intermediate hosts and wild [especially red fox (*Vulpes vulpes*)] or domestic canid definitive hosts. Humans can act as accidental intermediate hosts (Davidson *et al.* 2012). The intermediate hosts of *E. multilocularis* become infected by ingesting parasite eggs, and then the metacestodes colonize at internal organs, generally the liver, where lesions develop. If these lesions are not diagnosed and treated early, they can lead to severe health problems and death due to their tumour-like growth (Eckert and Deplazes, 2004).

The parasite's distribution spans the northern hemisphere, including Europe, Asia and parts of North America (Ito *et al.* 2003; Torgerson *et al.* 2010; Deplazes *et al.* 2017). Many rodent species are defined as suitable intermediate hosts for *E. multilocularis* with varying significance in the maintenance of the life cycle in different regions of the world (Vuitton *et al.* 2003; Romig *et al.* 2017). In

central Europe, the most common intermediate hosts of *E. multilocularis* are the common vole (*Microtus arvalis*), the water vole (*Arvicola terrestris*), and the bank vole (*Myodes glareolus*) (Romig *et al.* 2017). In the North America, intermediate host species listed to date include deer mouse (*Peromyscus maniculatus*), meadow vole (*Microtus pennsylvanicus*), southern red-backed vole (*Myodes gapperi*), house mouse (*Mus musculus*) and bushy-tailed woodrat (*Neotoma cinerea*) (Hnatiuk, 1966; Leiby *et al.* 1970; Holmes *et al.* 1971; Kritsky *et al.* 1977; Liccioli *et al.* 2013). In contrast to Europe and North America, a large variety of small mammal species act as intermediate host in this large region. Including more than 274 species of small mammals, many of which are candidate intermediate hosts for *E. multilocularis*, this diversity and excess in intermediate hosts leads increase in the transmission of the parasite (Giraudoux *et al.* 2013). Among small rodent and lagomorph intermediate hosts in Asia, *Microtus* spp., *Ochotona* spp., *Cricetus* spp. and *Meriones* spp. are leading (Vuitton *et al.* 2003).

Torgerson *et al.* (2010) suggested that human AE is common in Turkey, and the estimated annual incidence is 100 cases. Deplazes *et al.* (2017) summarized that approximately 500 human cases of AE have been documented in more than 60 studies in Turkey since 1939. The eastern Anatolian region

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of the country is defined with the most common AE cases (Deplazes *et al.* 2017). Investigation of definitive and intermediate hosts of *E. multilocularis* has been neglected in Turkey. The first report of *E. multilocularis* in Turkey was recorded by Merdivenci (1963) from a fox in the northwest of Turkey based on microscopy. The second report was informed by Avcioglu *et al.* (2016) from a fox in Erzurum Province of Turkey, identified using microscopy and PCR. Turkey is an endemic region for human AE, but the situation of the disease in intermediate hosts is unknown. Until now, there have been no reports of *E. multilocularis* in intermediate hosts in Turkey. The purpose of this paper is to describe the first findings of *E. multilocularis* in rodent intermediate hosts in Turkey and to discuss their importance in the transmission of the parasite in Turkey ecosystem.

MATERIALS AND METHODS

Study area

Erzurum is located in the Eastern Anatolia Region (39°52'N, 41°17'E) of Turkey and has the fourth biggest surface area (25 066 km²) in the country (Fig. 1). Erzurum has 20 counties and its total population is 762 000. The province's central districts (Yakutiye, Palandöken and Aziziye) constitute the majority of the total population (411 000). The total population of the other 17 provinces which are in rural areas is 351 000. The majority of the province is elevated and it is situated 1853 m above sea level. Continental climate rules in the province with cold and snowy winters, and warm and dry summers. The average low temperature is -15 °C, while the average high temperature is 27 °C. Average annual precipitation is 453 mm. The majority of region's vegetation cover meadows, and in some parts forests are encountered.

Sample collection

Ethical approval was obtained from the Ataturk University Animal Research Local Ethics Committee (Approval no: 2015/27) for the study. Fieldwork was performed with ethical permits from the Ministry of Forestry and Water Affairs, General Directorate of Nature Protection and National Parks (Approval no: 2015/43002).

Trapping was conducted between February and December 2016, in 20 counties of Erzurum Province. Intensive and active mouse nests in the rural areas of counties were determined and traps were established in these areas. In this way, 49 regions were selected in the rural areas of 20 counties (Fig. 1). An average of 70 traps was set for each of the 49 regions. Consequently, a total number of 3430 traps were placed during the investigation period.

The traps [small Sherman live trap (25 × 10 × 10 cm³)] were set with different baits, including cheese, walnuts, potato chips and cucumber. All traps were checked and trapped animals were collected daily, and then euthanized by cervical dislocation. Sampled animals were labelled with date and place of sampling, and stored at -20 °C until further process.

Laboratory methods

Parasitological and histopathological examinations. Rodents were identified at the genus level using the standard morphological criteria (Corbet, 1978). After dissection, the thoracic and peritoneal cavity, brain and visceral organs, were examined macroscopically for metacestodes of *E. multilocularis*. Suspicious lesions (e.g. white spots) were excised and preserved in 70% ethanol and 10% formalin for molecular and histopathological examinations.

Fluid, drawn from suspicious lesions >6 mm, was examined under the light microscope for the presence of protozoa. In parasite lesions smaller than 6 mm in diameter, histologic examination of tissue sections was performed. For histopathology, pieces of suspicious lesions were fixed in 10% formalin, routinely processed and embedded in paraffin. Five µm thick sections were cut and stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) (Presnell and Schreiber, 1997).

DNA isolation and PCR amplification. Isolation of DNA from suspicious lesions was executed by using Intron DNA extraction kit (G-spin™ Total DNA Extraction Kit; Intron, Korea) according to the manufacturer's instructions. The obtained DNA was stored at -20 °C until the next step. The mitochondrial 12S ribosomal RNA (rRNA) gene was subjected to analysis using classic PCR with species-specific primers Em.nest/for and Em.nest/rev (Dyachenko *et al.* 2008).

Bidirectional sequencing was performed commercially with an ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, CA) using the ABI PRISM® BigDye terminator cycle sequencing kit. The sequence data were then subjected to BLASTN (RefSeq mRNA) searches of the *E. multilocularis* genome database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). All the sequenced data were edited and aligned using Bioedit 7.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html) following naked eye checking. Nucleotide sequences of *E. multilocularis* isolates obtained from rodents were deposited in GenBank under accession numbers KY863531 to KY863535.

Tissue samples from infected rodents were extracted using Intron DNA extraction kit (G-spin™ Total DNA Extraction Kit; Intron, Korea) following the manufacturer's instructions. The

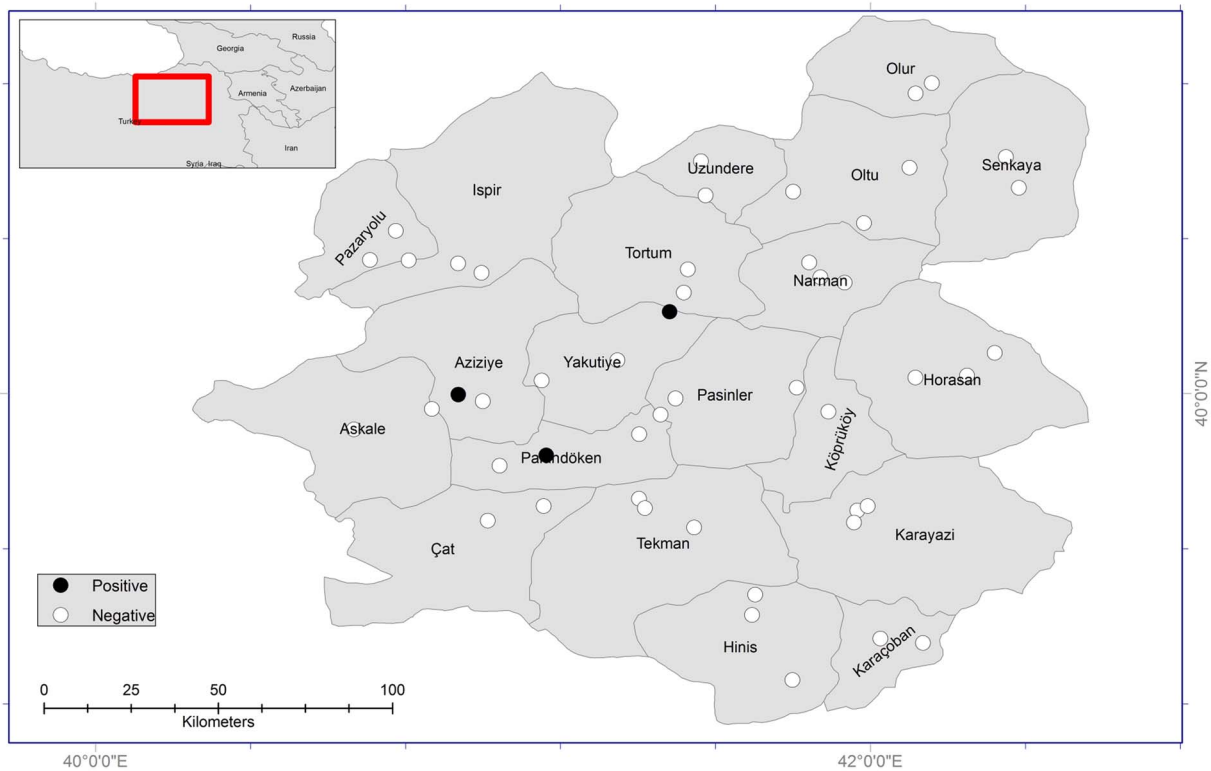


Fig. 1. Study areas, trap locations (circles) and positive findings of *E. multilocularis* (black circles) in Erzurum Province.

mitochondrial cytochrome *b* gene was subjected to analysis using classic PCR with primers L14727-SP and H16498 (Meyer *et al.* 1990; Jaarola and Searle, 2002).

After bidirectional sequencing, the sequences of cytochrome *b* were edited and visually optimized using SEQUENCHER, v. 4.1.4 (Gene Codes Corporation). The sequences were cut an 1140 bp fragment and aligned in BioEdit, v. 7.2.5 (Hall, 1999) with all previously obtained from GENBANK sequences. Finally, the sequences containing 1140 bp was blasted using Bioedit 7.0. (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Statistical analyses

Data were subjected to 2×2 cross tabulation to evaluate association of breed, gender, location with positivity using χ^2 test (Fisher Exact Test if necessary). Relative risks were reported at 95% confidence interval. Statistical significance was declared at the *P* value of 0.05.

RESULTS

A total of 498 rodents [females 205 (41.2%) and males 293 (58.8%)] were trapped on 49 trapping sites (catch rate: 14.5%, 498/3430). Among counties, Aziziye ($n = 31$, 6.22%), Palandoken ($n = 43$, 8.63%) and Yakutiye ($n = 8.63$, 8.63%) municipalities are within Erzurum Metropolitan, consisting of

23.49% of the samples. Other municipalities were 50–200 km away from city centre, and consisted of 76.51% of the samples ($n = 381$).

Based on morphological criteria, rodents were represented with 391 (78.5%) *Microtus* spp., 93 (18.7%) *Apodemus* spp., 12 (2.4%) *Mesocricetus* spp. and 2 (0.4%) *Crocidura* spp. (Table 1). *Microtus* genus was determined from the most prevalent rodent in the study area. Counties neighbouring the Black Sea region was high in *Apodemus* spp. and those within city centre and plato were rich in *Microtus* spp. ($\chi^2 = 229.02$, $P < 0.0001$). *Microtus* spp. was the predominant both in rural and urban areas. However, *Apodemus* spp. was more predominant in rural than urban areas ($\chi^2 = 19.30$, $P < 0.0002$).

Macroscopic examination of visceral organs showed liver lesions in 48 of 489 (9.6%) animals. No lesion was found in the brains of any of the rodents. Five rodents were molecularly confirmed to be infected with *E. multilocularis*, leading to a prevalence of 1.3% in *Microtus* genus. All infected rodents were confirmed to be *Microtus irani* by the mitochondrial cytochrome *b* gene sequence analysis.

The positivity was not specific to counties ($\chi^2 = 16.91$, $P < 0.60$) and gender ($\chi^2 = 3.14$, $P < 0.0762$). There was no species by positivity association ($\chi^2 = 1.38$, $P < 0.71$), the positivity rate of being 0 for *Apodemus* spp. ($n = 93$), *Crocidura* spp. ($n = 2$) and *Mesocricetus* spp. ($n = 12$), and 5 for *Microtus* spp. ($n = 391$). All infected rodents were seen in

Table 1. *Echinococcus multilocularis* infection in captured rodents in 20 counties from Erzurum Province

Genus Counties	<i>Microtus</i> spp. N _P /N _C (%)	<i>Apodemus</i> spp. N _P /N _C (%)	<i>Mesocricetus</i> spp. N _P /N _C (%)	<i>Crocidura</i> spp. N _P /N _C (%)	Total N _P /N _C (%)
Yakutiye	2/39 (5.1)	0/2 (0.0)	0/1 (0.0)	0/1 (0.0)	2/43 (4.7)
Palandöken	2/38 (5.3)	0/4 (0.0)	0/1 (0.0)	0/0	2/43 (4.7)
Aziziye	1/29 (3.4)	0/0 (0.0)	0/2 (0.0)	0/0	1/31 (3.2)
Aşkale	0/26 (0.0)	0/7 (0.0)	0/1 (0.0)	0/0	0/34 (0.0)
İspir	0/26 (0.0)	0/3 (0.0)	0/0	0/0	0/29 (0.0)
Pazaryolu	0/9 (0.0)	0/16 (0.0)	0/0	0/0	0/25 (0.0)
Pasinler	0/20 (0.0)	0/0 (0.0)	0/4 (0.0)	0/0	0/24 (0.0)
Köprüköy	0/14 (0.0)	0/2 (0.0)	0/1 (0.0)	0/0	0/17 (0.0)
Horasan	0/14 (0.0)	0/0 (0.0)	0/0	0/0	0/14 (0.0)
Karayazı	0/31 (0.0)	0/3 (0.0)	0/0	0/1 (0.0)	0/35 (0.0)
Karaçoban	0/13 (0.0)	0/0 (0.0)	0/0	0/0	0/13 (0.0)
Hınıs	0/17 (0.0)	0/4 (0.0)	0/0	0/0	0/21 (0.0)
Tekman	0/37 (0.0)	0/0 (0.0)	0/1 (0.0)	0/0	0/38 (0.0)
Çat	0/25 (0.0)	0/0 (0.0)	0/1 (0.0)	0/0	0/26 (0.0)
Tortum	0/13 (0.0)	0/7 (0.0)	0/0	0/0	0/20 (0.0)
Narman	0/24 (0.0)	0/6 (0.0)	0/0	0/0	0/30 (0.0)
Uzundere	0/1 (0.0)	0/5 (0.0)	0/0	0/0	0/6 (0.0)
Oltu	0/13 (0.0)	0/17 (0.0)	0/0	0/0	0/30 (0.0)
Olur	0/2 (0.0)	0/10 (0.0)	0/0	0/0	0/12 (0.0)
Şenkaya	0/0	0/7 (0.0)	0/0	0/0	0/7 (0.0)
Total	5/391 (1.3)	0/93 (0.0)	0/12 (0.0)	0/2 (0.0)	5/498 (1.0)

NP, number of *E. multilocularis* positive rodents; NC, number of captured rodents.

urban [$\chi^2 = 16.45$, $P < 0.0001$, logit Odd ratio value of 37.30 (2.04–679.76, 95% CI) and relative risk ratio of 1.05 (1.01–1.09, 95% CI)].

One of the five positive rodents contained liver with two metacestode lesions (15–13, 10–9 mm in diameter) (Fig. 2A and B). Protoscoleces were found after examining the cysts vesicle fluid under a light microscope (Fig. 2C and D). The other four positive rodent livers contained metacestode lesions were smaller than 5 mm in diameter.

Five metacestode lesions were histopathologically examined after H&E and PAS staining, and multicystic lesions were detected as shown in Fig. 2E and F. Histopathologically, metacestode lesions were consisted of numerous small vesicles with well-developed germinal and PAS-positive thin laminated layers. Most of the vesicles contained protoscoleces, presenting the features of larval *E. multilocularis* (Fig. 2C and D). All cysts sampled from the infected rodents had protoscoleces.

DISCUSSION

Echinococcosis is a serious veterinary public health concern in most areas of the world. Cystic echinococcosis (CE) occurs throughout Turkey, whereas AE predominantly occurs in the Eastern Anatolian Region of the country (Deplazes *et al.* 2017). Occurrence in definitive hosts is used to define endemicity for areas of Europe and North America (Romig *et al.* 2017). Because of investigation of definitive and intermediate hosts of *E. multilocularis* has been neglected in Turkey, human AE data were

considered the most reliable source of information in Turkey. Large parts of Turkey are considered as endemic for *E. multilocularis*. In definitive hosts, there is one record of an infected red fox (Merdivenci, 1963) from the European part of the country. Despite the large number of human cases particularly in the eastern part of Turkey, there was an absence in data of animal infection from the Asian part of the country (Romig *et al.* 2017). Recently, Avcioglu *et al.* (2016) recorded *E. multilocularis* infection from one out of ten red foxes, which confirmed microscopically and molecularly, in Erzurum Province from the Asian part of the country. To date, there are no data available on *E. multilocularis* in intermediate hosts in Turkey. In the present study, *E. multilocularis* metacestode was found in the liver of five out of the 498 rodents. This is the first report describing the presence of *E. multilocularis* in rodent intermediate hosts in Turkey.

Microtus spp. was the most frequently captured species (78.5%) and the dominant rodent in the study area. The study area, at an elevation of 1400–2400 m, have open grasslands such as meadows, and pastures is a suitable habitat for the voles of genus *Microtus*. In general, the subdivision of the genus *Microtus* into subgenera and species groups is very complicated and controversial (Zagorodnyuk, 1990; Nowak, 1999). Because of the morphological similarities between species, rodents were identified at the genus level in this study. Only five positive rodents were molecularly analysed and were confirmed to be *M. irani*. The main

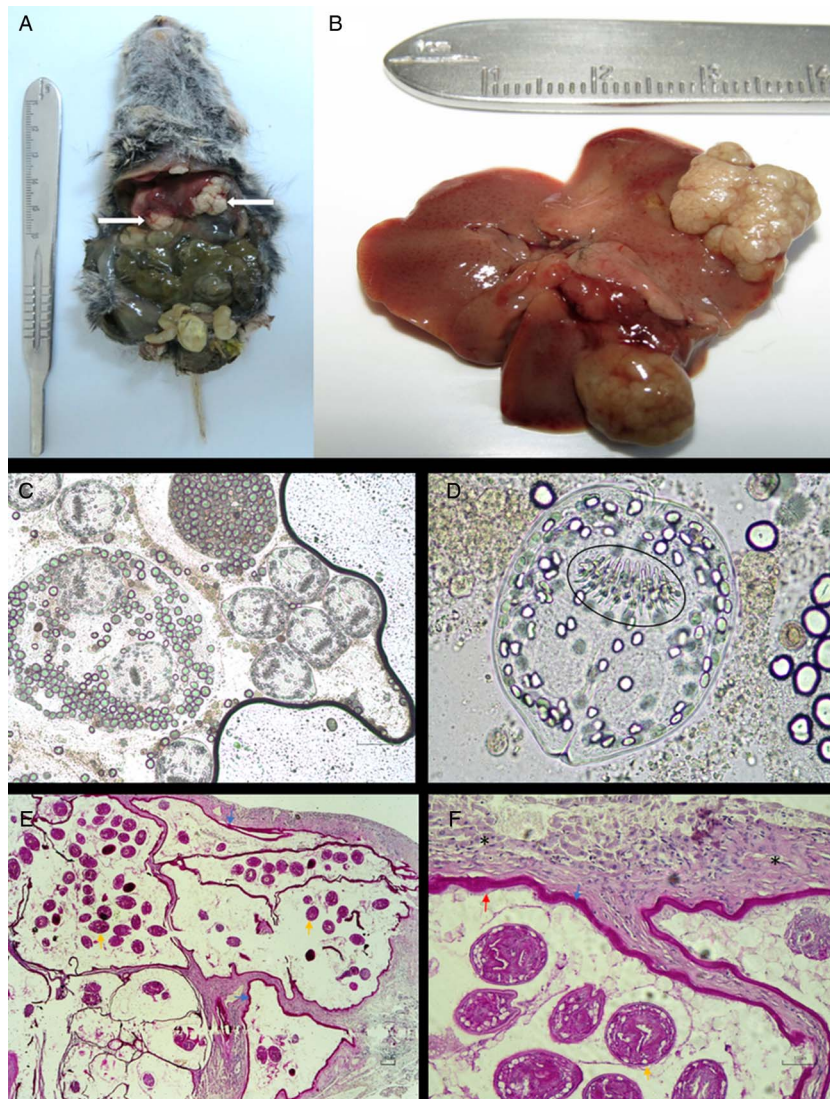


Fig. 2. (A) Metacestodes (arrows) of *E. multilocularis* in the liver. (B) The liver containing multiple metacestodes. (C) Protoscoleces in the cysts vesicle fluid. (D) Rostreller hooks (in the black circle). (E) Histological sections of the liver (H&E stained). Section through cysts containing protoscoleces (yellow arrows) bordered by the homogenous laminated layer (blue arrows). (F) The cellular inner germinal layer (red arrow), liver parenchyma (asterisks).

intermediate hosts of *E. multilocularis* are *M. arvalis*, *A. terrestris* and *M. glareolus* in Europe (Romig *et al.* 2017) and *Microtus* spp., *Ochotona* spp., *Cricetus* spp. and *Meriones* spp. (Vuitton *et al.* 2003) in Asia. In this study, *M. irani* is determined as an intermediate host in *E. multilocularis* life cycle in Turkey.

Reports of the worldwide prevalence of *E. multilocularis* infection have varied between >1% and more than 83% in various rodent species (Eckert *et al.* 2001; Gottstein *et al.* 2001; Eckert and Deplazes, 2004). The infection rate (1.3%) reported in this study is in accordance with the previous records throughout the world. Differences can be based on a wide spectrum of sensitive intermediate hosts, the number of examined hosts and the diagnostic methods used (Eckert *et al.* 2001). For Turkey, which is an endemic region for *E. multilocularis*, this rate seems to be low, but other endemic

regions also have the similar situation. As quoted by Oksanen *et al.* (2016) pooled that the prevalence of *E. multilocularis* in intermediate hosts on 371 studies in Europe was estimated to be 1.1–6%. Eckert (1998) reported that the average prevalence of *E. multilocularis* in rodents is usually low in central Europe, but higher prevalences have been determined in certain foci. Similarly, in our study the prevalence was found to be 1.3% for the whole study area, whereas the infection was more common in certain regions such as Palandöken 5.3%, Yakutiye 5.1% and Aziziye 3.4% (Table 1). Deplazes *et al.* (2004) pointed out the crucial significance of anthropogenic food resources for the urbanization of foxes. They have adapted to synanthropic life and increasingly use anthropogenic food sources within urban settings. In this study, all positive rodents captured from Erzurum's central districts (Yakutiye, Palandöken and Aziziye),

which could be explained by the synanthropic life of foxes.

The differing findings in the studies might be related to the identification method based on gross and histopathologic examinations (Barabasi *et al.* 2011) or PCR (Hofer *et al.* 2000; Burlet *et al.* 2011). In this study, cysts were found in one of the 498 examined liver samples by gross examination. Positivity increased to five after PCR analysis conducted on 48 suspicious liver lesions. This finding indicated the complexity of distinguishing small immature cysts, especially in lesions smaller than 5 mm in diameter (Torgerson and Deplazes, 2009). On the other hand, parasite DNA can be easily detected by species-specific PCR from these small suspicious lesions. These data showed that early infection in intermediate hosts can be reliably diagnosed by PCR (Deplazes *et al.* 2003).

The presence of protoscolexes in infected rodent species indicates that they can serve as competent intermediate hosts of *E. multilocularis*. Burlet *et al.* (2011) reported high *E. multilocularis* prevalence in *Arvicole* spp. in Switzerland, and also indicated that the potential infectivity of these voles may be much lower (12 with protoscolexes from 129 infected). In a study by Hofer *et al.* (2000), only 2/19 *Arvicola* spp. positive for *E. multilocularis* had protoscolexes. Similar figures were reported by Stieger *et al.* (2002) where 26/81 positive *Arvicola* spp. contained protoscolexes, and Reperant *et al.* (2009) where 2/31 positive *Arvicola* spp. contained protoscolexes. In addition, seven with protoscolex from 13 infected voles in Romania (Barabasi *et al.* 2011), one with protoscolexes from five infected *M. gapperi* in North America (Liccioli *et al.* 2013), three with protoscolexes from eight infected *A. amphibius* in Sweden (Miller *et al.* 2016) were reported. Our histological observations showed that all infected *M. irani* (5/5) had fertile metacystodes, suggesting that this rodent species could act as a competent intermediate host and thus contribute to the transmission of *E. multilocularis* in Turkey.

Our findings of all infected *M. irani* with protoscolexes show that this rodent can act as suitable intermediate hosts for *E. multilocularis* in Turkey. It is recommended that molecular analysis should be conducted in addition to gross and histological examinations to determine the presence of *E. multilocularis* in rodents. An extensive survey should be conducted to investigate the prevalence of *E. multilocularis* in definitive hosts (especially fox and dog) in Erzurum Province.

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CONFLICTS OF INTEREST

None.

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