

Molecular detection of *Echinococcus multilocularis* in faecal samples of wild carnivores from an endemic region of Türkiye

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Abstract

The aim of this study was to determine the prevalence of *Echinococcus multilocularis* in fresh faecal samples of wild carnivores from an endemic region of Türkiye and to molecularly confirm its presence. Fresh faecal samples were collected from 38 injured wild carnivores admitted to a wildlife rehabilitation centre in Kars province, Eastern Anatolia. Samples were processed using a sequential sieving and flotation technique and examined microscopically for taeniid-type eggs. Genomic DNA was extracted from faecal material and analysed by species-specific polymerase chain reaction (PCR) targeting *E. multilocularis*. Positive PCR products were sequenced and compared with reference sequences available in GenBank. Taeniid-type eggs were detected microscopically in 7.8% (3/38) of the faecal samples. Molecular analysis confirmed *E. multilocularis* DNA in 2.6% (1/38) of the samples, which originated from a red fox (*Vulpes vulpes*). Sequence analysis revealed 100% identity with previously reported *E. multilocularis* isolates deposited in GenBank. The present study provides molecular evidence for the presence of *E. multilocularis* in wild carnivores from Kars province, an endemic area for human alveolar echinococcosis in Türkiye. Although the detected prevalence was low, the confirmation of *E. multilocularis* in a definitive host indicates ongoing environmental contamination and a potential risk for human exposure. These findings highlight the value of molecular methods for reliable surveillance of *E. multilocularis* in wildlife populations.

Echinococcosis, zoonosis, fox, parasite, wild animals

Alveolar echinococcosis (AE) is a neglected zoonotic disease caused by the larval stage of the tapeworm *Echinococcus multilocularis*. The life cycle of the parasite is maintained primarily by canids, including wolves, foxes, and jackals, as well as by certain felids and hyenas acting as definitive hosts, whereas rodents function as intermediate hosts in the Northern Hemisphere (Romig et al. 2017; Avcioglu et al. 2021a; Woolsey and Miller 2021). Humans and other mammals may be abnormal intermediate hosts. People become infected through hunting wild animals, farming, ownership or close contact with cats or dogs, and consumption of wild plants or water possibly contaminated with helminth eggs (Torgerson et al. 2010; Oksanen et al. 2016). The adult tapeworms localize in the small intestines of definitive hosts without any clinical signs and larvae can localize to the liver, lungs, bone, brain and other organs of intermediate hosts causing organ damage, infiltrative and destructive growth or metastasis and also death (Eckert et al. 2001; Torgerson et al. 2010; Oksanen et al. 2016; Deplazes et al. 2017; Casulli 2020; Avcioglu et al. 2021a; Woolsey and Miller 2021).

The increase in wild carnivore populations, changes in land use, and increased human interaction with wildlife have contributed to the geographical spread and reemergence of AE in many regions around the world (Davidson et al. 2012; Carmena and Cardona 2014).

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Alveolar echinococcosis is highly endemic in Central and Eastern Europe, North America, North Asia including the Baltic countries, China, Russia, Mongolia, Japan, Kyrgyzstan, Türkiye, and Alaska (Eckert et al. 2001; Torgerson et al. 2010; Carmena and Cardona 2014; Deplazes et al. 2017; Avcioglu et al. 2021b). Türkiye is recognized as one of the countries where AE is endemic, with the highest number of cases reported in the Eastern Anatolia region. Kars, Erzurum, and surrounding provinces have been highlighted as high-risk areas based on clinical and epidemiological data (Avcioglu et al. 2016; Avcioglu et al. 2017; Avcioglu et al. 2018; Gürler et al. 2018; Avcioglu et al. 2021b). Evidence exists in Türkiye that interactions between rodents and carnivores sustain the AE cycle, which is important in determining public health control targets in the country (Avcioglu et al. 2016; Avcioglu et al. 2017). Nevertheless, information on the presence and distribution of *E. multilocularis* in wildlife reservoirs in these endemic regions remains insufficient. In particular, molecular data confirming the circulation of the parasite in wild carnivores is extremely limited.

Therefore, this study aimed to investigate the molecular presence of *E. multilocularis* in fresh faecal samples collected from various wild carnivore species admitted to a wildlife rehabilitation centre in Kars and its neighboring provinces, a region in Türkiye where AE is endemic. By integrating coprological examination with species-specific PCR and sequence analysis, this study provides molecular evidence regarding the circulation of *E. multilocularis* in the wild and establishes fundamental data for the development of future epidemiological surveillance and effective control strategies for AE in the region.

Materials and Methods

Ethical approval

This study was conducted with the approval of the Animal Experiments Local Ethics Committee of Kafkas University and the Ministry of Forestry and Water Affairs, General Directorate of Nature Conservation and National Parks (approval numbers: KAU-HADYEK/2022–192 and E-21264211-288.04-8314542).

Identification of wild animal species

The characteristics such as species, sex, and age of each wild carnivores brought to University Wildlife Protection, Rescue, Rehabilitation Application and Research Centre (WPRRARC) in Kars were determined, photographed and recorded.

Collection of faecal samples

Fresh faecal samples of 25 red foxes, 5 grey wolves, 3 brown bears, 2 lynxes, 1 Eurasian badger, 1 golden jackal, 1 stone marten (totally 38 samples) were collected directly in sterile faecal containers from the rectums of injured wild carnivores. To inactivate the *E. multilocularis* eggs, faecal samples were first frozen at -80°C for at least 7 days, then at -20°C until examination (Deplazes and Eckert 1996). Various wild carnivore species admitted to the rehabilitation centre were included in the study regardless of their epidemiological role in the life cycle of *E. multilocularis*.

Concentration of faecal samples

To concentrate the parasite eggs, the sequential sieving and flotation method (SSFM) was applied to all faecal samples (Mathis et al. 1996). For this purpose, 12 ml of distilled water was added to 1 g of faecal matter, mixed, and centrifuged at $1,600 \times g$ for 3 min. The same volume of zinc chloride (ZnCl_2 , specific gravity 1.45) was added and centrifuged again at $400 g$ for 30 min. Egg isolation was performed using sequential sieving of the supernatant with 44 and then $21 \mu\text{m}$ mesh sieves and collected in a tube. The supernatant was transferred to a 15 ml falcon tube. In the next stage, the supernatant was left for 30 min to settle to the bottom. The final volume was ensured to be approximately 0.5 ml. To prevent potential cross-contamination between samples, all steps were performed using disposable plastic equipment. Positive samples determined for the presence of taeniid eggs under the microscope were centrifuged at $1,000 g$ for 10 min, and the pellets were preserved in microcentrifuge tubes.

Isolation of DNA and PCR amplification

DNA isolation was carried out from all faecal samples ($n = 38$) using a commercial kit (QIAamp Fast DNA Stool Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted copro-DNA isolates were stored at -20°C until further processing.

All faecal samples ($n = 38$) were screened for the molecular presence of *E. multilocularis* by targeting the mitochondrial 12S ribosomal RNA (rRNA) gene. PCR amplification was performed using the specific primer set Em-nest-for (5'-GTGAGTGATTCTTGTTAGGGGAAGA-3') and Em-nest-rev

(5'-ACAATACCATATTACAACAATATTCCTATC-3'), which amplify a 204 bp amplicon (Dyachenko et al. 2008). The reactions were carried out in a total volume of 25 µl, containing 2.5 µl of 10 × PCR buffer (Eco Tech, Erzurum, Türkiye), 0.5 mM of each dNTP (Bioline, Tennessee, USA), 1.25 µM of each primer, 0.2 U of iTaq DNA polymerase (Thermo, Massachusetts, USA), 2 mM MgCl₂ (prepared from a 25 mM stock), 5 µl of template DNA, and 12.3 µl of nuclease-free water. The final concentration of each primer in the PCR reaction mixture was 1.25 µM, and the final MgCl₂ concentration was 2 mM. To ensure the reliability and reproducibility of the results, all PCR reactions were performed in duplicate. The thermal cycling profile was optimized as follows: an initial denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 40 s, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. Each PCR run included *E. multilocularis*-positive DNA and a negative (no-template) control. Amplified products were analysed using 1.5% agarose gel electrophoresis. The PCR products were stained with ethidium bromide and visualized under UV transillumination.

Sequence analysis

Sanger sequencing of the purified amplicons was performed by Medsantek Inc. (Istanbul, Türkiye). The quality scores of the obtained chromatograms were assessed using Geneious® version 9.1.8 software. Sequence samples were oriented, edited, and aligned using Geneious Prime software. The edited sequences were compared with reference sequences deposited in GenBank using BLASTn to confirm species-level identity (www.ncbi.nlm.nih.gov/BLAST).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Results

The faecal samples were examined in terms of parasite eggs using SSFM. Taeniid-type eggs were detected in 7.8% (3/38) of the examined faecal samples by coprological analysis. The characteristics of wild animal species are summarised in Table 1. Within the fox population, the molecular presence of *E. multilocularis* was determined as 4% (1/25).

Table 1. The characteristics of wild animal species.

Host	Number of animals examined	Locations (Number of host)	Microscopic examination	Molecular diagnosis
Eurasian lynx (<i>Lynx lynx</i>)	2	Erzurum, Kars	Negative	Negative
Grey wolves (<i>Canis lupus</i>)	5	Kars (3) Ardahan (2)	Negative	Negative
Brown bear (<i>Ursus arctos</i>)	3	Erzincan (1) Erzurum (2)	Negative	Negative
Eurasian badger (<i>Meles meles</i>)	1	Ardahan	Negative	Negative
Golden jackal (<i>Canis aureus</i>)	1	Erzurum	Negative	Negative
Stone marten (<i>Martes foina</i>)	1	Kars	Negative	Negative
Red fox (<i>Vulpes vulpes</i>)	25	Kars (15) Ardahan (1) Erzurum (9)	<i>Taenia</i> sp. (3 Sample)	<i>E. multilocularis</i> (1 Sample, Kars)

Molecular analysis revealed the presence of *E. multilocularis* DNA in 2.6% (1/38) of the samples. The positive sample was subsequently bidirectionally sequenced by a commercial company (Medsantek Inc., Istanbul, Türkiye) using the species-specific PCR primers targeting the 12S rRNA gene region. The sample, confirmed as *E. multilocularis* by sequence analysis, was deposited in GenBank under accession number PV569553. BLAST analysis demonstrated 100% nucleotide identity with previously reported *E. multilocularis* isolates, including ON840193 (Kars, Türkiye), ON840152 (Erzincan, Türkiye), and ON840130 (Ardahan, Türkiye).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (−428.80)

is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 204 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Fig. 1).

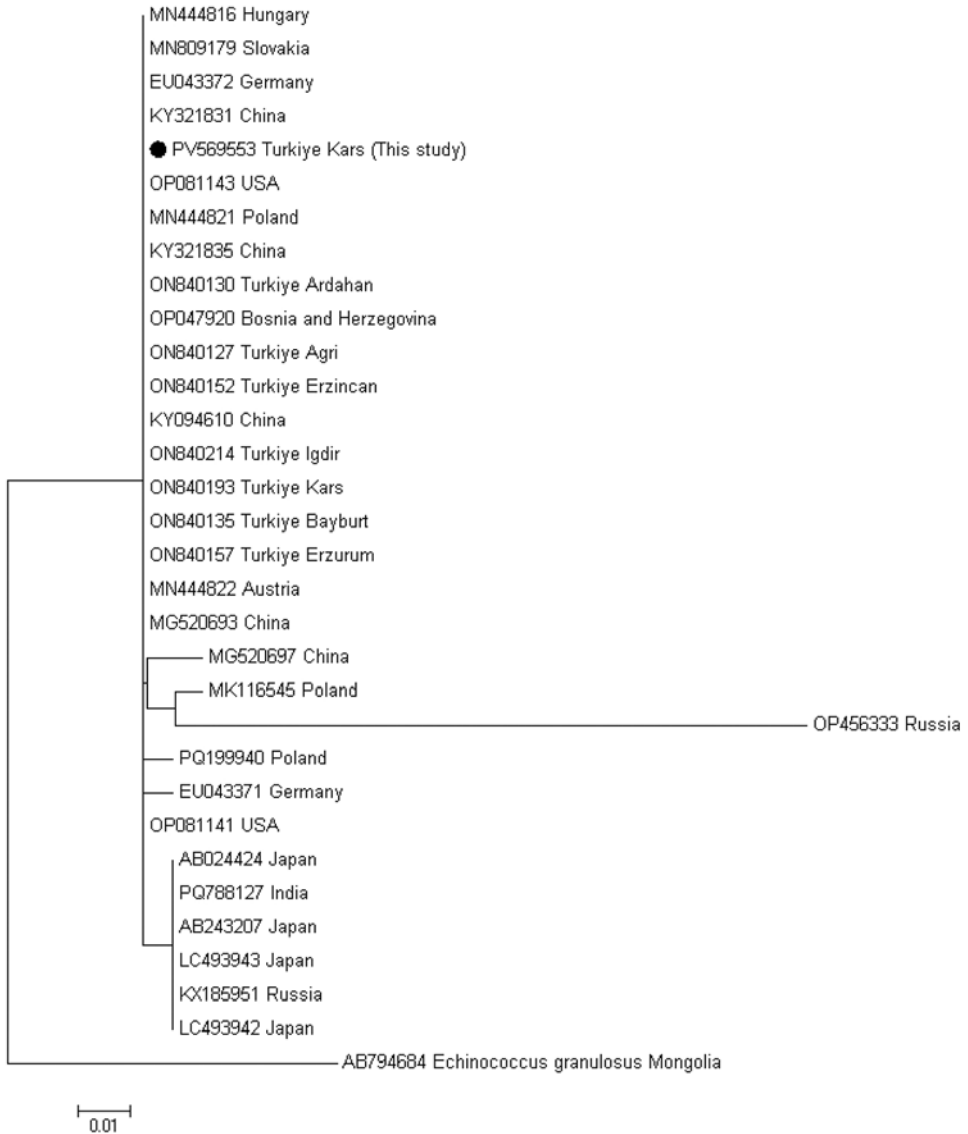


Fig. 1. Maximum likelihood phylogenetic tree based on partial 12S rRNA gene sequences of *Echinococcus multilocularis* isolates. The sequence generated in the present study (GenBank accession number: PV569553) was included in the phylogenetic analysis. Bootstrap values are shown at the branch nodes.

Discussion

Monitoring *E. multilocularis* in definitive hosts is crucial for understanding transmission dynamics and assessing the zoonotic risk to humans. It has been reported that *E. multilocularis* is most prevalent in red foxes, and that wild canids, including foxes, coyotes, and wolves, are frequently infected. Wild carnivores increase the risk of disease by entering areas where local people live, garbage accumulation areas and domestic animal shelters such as pens and stables for hunting (Eckert et al. 2001; Davidson et al. 2012; Carmena and Cardona 2014; Deplazes et al. 2017).

Although coproparasitological methods allow for the detection of taeniid-type eggs, they are insufficient for species-level identification due to the morphological similarities among various taeniid species. In contrast, polymerase chain reaction (PCR)-based molecular approaches offer high sensitivity and specificity. These methods enable the accurate identification of *E. multilocularis* DNA in faecal samples, providing greater reliability for epidemiological surveillance (Mathis et al. 1996; Trachsel et al. 2007). Consistent with previous findings (Avcioglu et al. 2021b), the discrepancy between the three samples that tested positive by SSFM and the single sample confirmed by PCR may be attributed to the presence of other *Taenia* spp. eggs, which are morphologically indistinguishable from those of *Echinococcus*.

Across Europe, the prevalence of *E. multilocularis* in fox populations varies considerably, with reported rates of $\leq 1\%$ in Denmark, Norway, Finland and Sweden, $\leq 10\%$ in Austria, Belgium, Croatia, Hungary, Italy, the Netherlands, Romania and Ukraine, $> 10\%$ in Czechia, Estonia, France, Germany, Latvia, Liechtenstein, Lithuania, Poland, Slovakia, and Switzerland (Wahlström et al. 2015; Oksanen et al. 2016; Gürler et al. 2019). Although data on the distribution of *E. multilocularis* in Türkiye remain limited, the number of studies addressing this parasite has increased in recent years.

In Eastern and Northeastern Anatolia, several studies have confirmed the presence of *E. multilocularis* in both wild and domestic hosts. In Erzurum province which shares similar geographical characteristics with Kars, *E. multilocularis* was detected in 10% of examined foxes ($n = 10$) (Avcioglu et al. 2016) and in a lynx (*Lynx lynx*) (Avcioglu et al. 2018). Furthermore, the prevalence of adult *E. multilocularis* in red fox carcasses was reported to be 42%, while environmental faecal contamination reached 10.5% (Avcioglu et al. 2021b). Additionally, an infection rate of 8.7% was identified in dogs based on faecal examinations (Akyuz and Avcioglu 2025).

In Thrace, the prevalence of *E. multilocularis* was reported as 0.5%, whereas a higher prevalence of 3.8% was observed in Central Anatolia (Gürler et al. 2018). Moreover, studies conducted in Türkiye have reported the prevalence of *E. multilocularis* in rodents to range from 1.3% (Avcioglu et al. 2017) to 2.0% (Gürler et al. 2023). These findings demonstrate the molecular presence of *E. multilocularis* among wild carnivores and rodents, indicating the persistence of the parasite within the local sylvatic cycle.

A large-scale study conducted on dogs in Northeastern Anatolia reported an overall prevalence of 8.7%, with province-specific rates ranging from 1.9% to 14.4%, including a prevalence of 10.4% in Kars province (Akyuz and Avcioglu 2025). The relatively lower prevalence observed in domestic dogs in the present study may be associated with differences in host ecology and sample size. Notably, *E. multilocularis* has been shown to occur more frequently in urban environments, thereby increasing the potential risk of transmission to humans (Avcioglu et al. 2021b; Simoncini and Massolo 2024).

The environmental survival of *E. multilocularis* eggs is highly dependent on climatic factors such as temperature, humidity, and precipitation (Danson et al. 2003; Simoncini and Massolo 2024). Experimental and field studies have demonstrated that *E. multilocularis* eggs are highly sensitive to heat and desiccation (Veit et al. 1995). In this context, the

high altitude and predominantly cold and humid climate of the Kars region likely provide an optimal environment for egg persistence. In contrast to regions characterized by high solar radiation and arid climates which restrict parasite distribution (Botero-Cañola et al. 2019), the prolonged snow cover and low average temperatures in Northeastern Anatolia may enhance the survival of *E. multilocularis* eggs in the environment, thereby increasing the risk of transmission to both intermediate hosts and humans.

Although *E. multilocularis* was previously considered primarily a rural parasite due to the habitat preferences of foxes, it has increasingly been detected in urban and peri-urban areas, likely as a result of fox adaptation to synanthropic life (Deplazes et al. 2004; Avcioglu et al. 2021b). The synanthropization of red foxes and the establishment of *E. multilocularis* within urban environments is a globally recognized phenomenon. In northeastern Anatolia, in the central regions of Erzurum, *E. multilocularis* has been shown to be more prevalent than in rural areas (Avcioglu et al. 2021b). The detection of *E. multilocularis* in a red fox admitted to a rehabilitation centre in Kars is consistent with this expanding urban transmission pattern. Furthermore, the abundant water bodies and high humidity levels of the Kars plateau likely facilitate environmental egg survival while supporting dense intermediate host populations, thereby intensifying zoonotic risk at the human–wildlife interface.

The positive sample identified in this study was bidirectionally sequenced targeting the 12S rRNA gene region. The obtained sequence (GenBank accession number: PV569553) showed 100% nucleotide identity with previously reported *Echinococcus multilocularis* isolates, including isolates from northeastern Türkiye as well as several reference sequences originating from Europe and Asia within the analysed 204 bp region. No nucleotide variation was detected among these sequences within the analysed fragment. However, because the phylogenetic analysis was based on a relatively short 204 bp fragment of the mitochondrial 12S rRNA gene, subtle genetic differences among geographically distinct isolates cannot be excluded, and future studies employing longer mitochondrial regions or multilocus genetic markers are warranted to better resolve phylogeographic relationships.

The species-specific primer set (Em-nest-for/Em-nest-rev) employed in the present study has been widely used for the sensitive and specific molecular detection of *Echinococcus multilocularis*, including previous studies conducted in Türkiye (Avcioglu et al. 2016; Avcioglu et al. 2017). Therefore, the obtained sequence provides reliable confirmation of species identity.

The main limitation of this study is the relatively small sample size and the inclusion of only injured animals admitted to a rehabilitation centre. Therefore, the results may not fully represent the entire wild carnivore population in the region. In addition, although the species-specific PCR assay used in the present study is regarded as a highly sensitive and specific tool for the molecular detection of *Echinococcus multilocularis*, false-negative results cannot be completely excluded, particularly in samples containing a low parasite burden. Furthermore, faecal samples may contain substances that inhibit PCR amplification, potentially affecting the detection of parasite DNA. Moreover, because the intermittent shedding of gravid proglottids and eggs by infected definitive hosts has been reported, faecal samples collected during non-patent or low-shedding periods may not contain detectable parasitic material. Consequently, the actual prevalence of *Echinococcus multilocularis* in the investigated wildlife population may have been underestimated. Therefore, the findings of the present study should be interpreted in light of these methodological limitations.

In conclusion, this study demonstrates the molecular presence of *Echinococcus multilocularis* in wild carnivores in Kars province, a region where alveolar echinococcosis is endemic in Türkiye. Following sequential sieving and flotation of faecal samples, species-specific PCR analysis detected *E. multilocularis* DNA in one of the examined samples.

Despite the limited number of positive samples, this finding indicates that the parasite is circulating in the region and may pose a potential risk of environmental contamination, particularly in areas close to human settlements. The discrepancy between the detection of taeniid eggs by microscopic examination and the identification of a single positive sample by molecular analysis highlights the importance of molecular methods for reliable species-level discrimination.

The relatively small sample size and the inclusion of only injured wild carnivores admitted to the rehabilitation centre require cautious interpretation of the results. Consequently, further large-scale and systematic studies are needed to better elucidate the regional distribution of *E. multilocularis* and to support the development of effective public health control measures.

Conflict of interest

The authors declare no competing interests.

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