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## Original Article

# Molecular Characterization of *Echinococcus granulosus* Isolated from Clinical Samples of Patients Diagnosed with Hydatid Cysts in Eastern Türkiye

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*Received* 08 Jun 2025
*Accepted* 23 Aug 2025

***Keywords:***
*Echinococcus granulosus;*
Hydatid cyst;
Genotype;
mt-*Cox1*;
Phylogenesis

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***Abstract***

***Background:*** We aimed to investigate the genetic diversity and population structure of *Echinococcus granulosus* using various cyst materials from the parasite obtained from humans in eastern Türkiye.

***Methods:*** We used cyst samples from 61 patients diagnosed with hydatid cysts by the Department of Thoracic Surgery and Department of Radiology at Atatürk University Faculty of Medicine between 2019 and 2023. Microscopic examination of protoscolex in cyst samples was performed, and, if available, the patients' ELISA IgG results were obtained from the hospital automation system. Two primer pairs revealed DNA regions specific to *E. granulosus* (12S rRNA and mt-*COX1*). Sequence analysis was performed on samples found positive for mt-*COX1* PCR, and a phylogenetic tree was constructed from the resulting data.

***Results:*** The rate of scolex in microscopic examination was 31/61 (50.8%), and the ELISA IgG positivity rate was 36/51 (70.6%). As a result of PCR analysis of 12S rRNA and mt-*COX1* gene regions of 61 cyst samples, positivity was determined in 48 (78.7%) samples. As a result of PCR product sequence analysis, it was found that all samples were of the E. granulosus sensu *stricto* (G1, G3) genotype. Lung cyst alone was detected in 25 (40.9%) cases, while only liver cyst was detected in 15 (24.6%) cases. In 13 (21.3) cases, cysts were present in both the lungs and the liver.

***Conclusion:*** The genotype of *E. granulosus* isolates obtained from cyst material from patients diagnosed with hydatid cysts in Erzurum and surrounding provinces was a domestic sheep strain (G1, G3).


DOI: https://doi.org/10.18502/ijpa.v20i4.20461

## Introduction

*Echinococcus granulosus* is one of the most common zoonoses of medical importance, causing diseases in both humans and animals (1). In addition to harming human health, it also causes severe economic losses, especially in developing countries (2). According to WHO data, *E. granulosus* has been detected on all continents and in more than 100 countries (3). Cases are reported from all regions of Turkey (4). The disease is more common, especially in rural areas where livestock breeding is intensive (5). According to the latest taxonomic studies on *E. granulosus*, the existence of five valid species in *E. granulosus* has been accepted: *E. granulosus sensu stricto* (G1, G3; sheep and buffalo genotypes), *Echinococcus equinus* (G4, horse genotype), *Echinococcus ortleppi* (G5, cattle genotype), *Echinococcus canadensis* cluster (G6/7 camel and pig strain, G8 'American' cervid strain and G10 '*Fennos candian*' cervid strain) and *Echinococcus felids* (4,6).

Especially over the last 25 years, developments in molecular biology and biotechnology have led to significant changes in health and basic life sciences (7). Diagnosis and identification of parasites in parasitology were based on morphological structures, pathological effects, host specificity, and geographical origin before biotechnological developments, but these criteria were often insufficient. Nucleic acid-based techniques used in molecular parasitology have increased specificity and sensitivity and have become new and better diagnostic tools used in this field (8).

Determining the predominant strains and strains in the study regions is essential to start control and eradication programs in the endemic areas and achieve success. Although morphological and biological criteria are used to differentiate strains, molecular methods are gaining ground for definitive strain differentiation (9). The G1 genotype has been reported as the most common in human CE cases worldwide and in Türkiye (10). Although the Eastern Anatolia region of Turkey, including Erzurum, is highly endemic for CE, information on the genotype distribution of this species is very limited.

We aimed to investigate the genetic diversity and population structure of *E. granulosus* using various parasite cysts obtained from humans in our region.

## Materials and Methods

### Collection of samples

A total of 61 samples were used, including 53 cyst materials (cyst tissue, cyst fluid) obtained by the Department of Thoracic Surgery of Atatürk University Faculty of Medicine who were pre-diagnosed with KE between 2019-2023, and 8 cyst fluid samples obtained from patients diagnosed and treated with CE in the Department of Non-Vascular Interventional Radiology of Atatürk University Department of Radiology Erzurum, Türkiye. In addition, the results of *E. granulosus* IgG ELISA (NovaLisa, NovaTec Immundiagnostica GmbH, Germany) for these patients, if any, were obtained from the hospital automation system.

Cyst membranes and cyst fluid obtained from humans under sterile conditions were examined microscopically for fertility. Cyst fluids were centrifuged at 2000 rpm for 10 minutes, and a slide-lamellar preparation was prepared from the pellet and examined under a light microscope at 100X magnification for the presence of a protoscolex or a hook. The same procedure was performed with the germinal membrane. Thin sections of the membrane were taken and examined under a light microscope with a preparation between the slide and the coverslip.

### Genomic DNA Isolation

After waiting for the cyst fluids and germinal membrane materials to be deposited for

our research and stored at -80 °C to dissolve at room temperature, the germinal membrane samples were divided into small pieces before extraction. The extraction procedure was performed using the NucleoGene Genomic DNA Tissue Extraction Kit (NUCLEOGENE, Turkey), with a modified proteinase K step per the manufacturer's recommendations. The resulting DNA concentration was measured with a spectrophotometer and maintained at –20 °C.

### Polymerase Chain Reaction

For PCR amplification, two primer pairs were used to amplify DNA from the cyst material. The mitochondrial 12S rRNA gene and cytochrome oxidase subunit (mt-*COX-1*) were amplified using specific primers and PCR conditions previously described by Dinkel and Bowles (Table 1) (11,12). In our study, PCR was performed using 12S rRNA specific to *E. granulosus s.s.* in all samples. For the negative samples in the first PCR study, the second PCR study used mt-*COX1* primers. PCR products were imaged under UV light after electrophoresis on a 1% agarose gel containing ethidium bromide. In each study, *E. granulosus s.s.* gDNA, previously available at the Atatürk University Faculty of Veterinary Medicine Parasitology Laboratory, was used as the positive control, and sterile distilled water as the negative control.

**Table 1:** Primer pairs used in the PCR study

| Target | Primers 5'-3' | Product size (bp) | References |
|---|---|---|---|
| ***Echinococcus granulosus s.s.* 12S rRNA** | E.g.ss1for. GTATTTTGTAAAGTTGTTCTA and E.g.ss1rev. CTAAATCACATCATCTTACAAT | 254 | Dinkel et al (11) |
| ***Echinococcus spp.* mt-*COX1*** | JB3 TTTTTTGGGCATCCTGAGGTT TAT and JB4.5 TAAAGAAAGAACATAATGAAAATG | 446 | Bowles et al (12) |

### DNA Sequencing Analysis and Phylogenetic Tree Creation

The 61 used in the study were first subjected to PCR analysis for the mitochondrial 12S rRNA gene. PCR amplified samples that tested negative in the initial analysis for the mt-*COX1* gene. In our study, of 11 samples with positive mt-*COX1* gene region, the gene products of 6 samples with moderate and robust band profiles were sent to NucleoGene (Istanbul, Turkey) for DNA sequence analysis.

The sequence analysis results were compared with the reference sequence analysis in GenBank by performing BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/). The study and the reference sequences obtained from NCBI GenBank were aligned and compared with the BioEdit program. The phylogenetic tree was created using the neighbour-joining algorithm in MEGA (v.5).

The DNA sequences of our samples were compared with reference genotypes obtained from GenBank, which are known to be *E. granulosus s.s.*, *E. equinus*, *E. ortleppi*, *Echinococcus canadensis* cluster strain, and *E. felids* as reference strains, and strain determination was made based on their compatibility with the standard samples. In the phylogenetic analysis, *Taenia saginata* was used as the outgroup (Fig. 1).

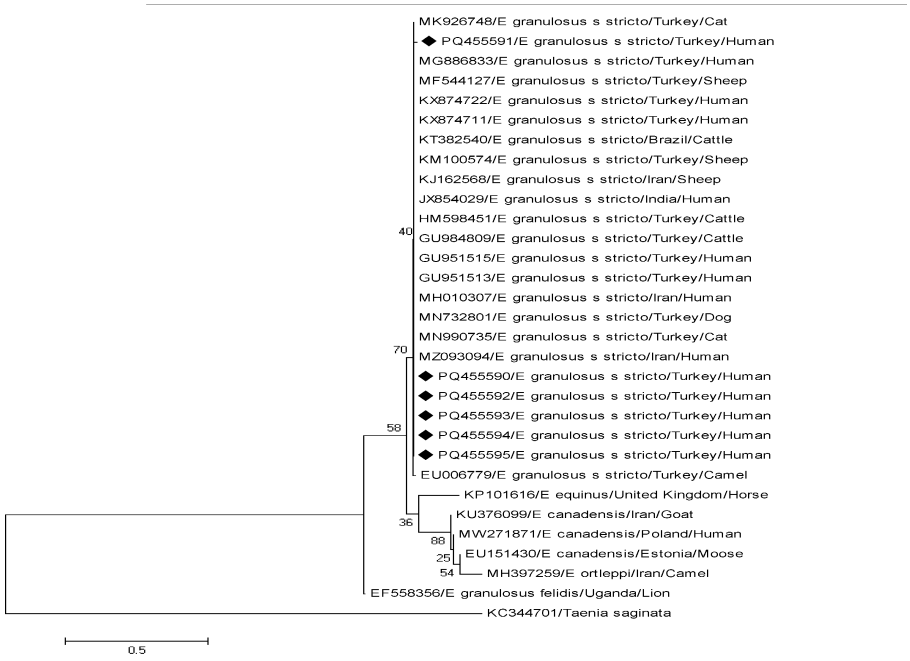


**Fig. 1:** A phylogenetic tree was constructed from the alignment of partial *E. granulosus COX1* gene sequences obtained from the current study and GenBank references. *E. granulosus* strains were aligned with BioEdit software, and phylogeny was constructed using the neighbour-joining algorithm method using MEGA (v.5) software. The GenBank accession numbers of reference *E. granulosus* strains are included in the phylogeny. *E. granulosus* strains detected in the current study are marked with "♦". *T. saginata* was used as an outgroup

### *Ethics Committee Approval*

The Ethics Committee approval required for this research was obtained from the Atatürk University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee in line with decision No. 3 dated 02.05.2023.

### *Statistical Analysis*

All data were tested with SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

## Results

The study examined the hydatid cyst material of 61 patients. The distribution of the patients by gender shows that 31 (50.2%) were female and 30 (49.8%) were male. Of the 61 cases, 10 involved children and 51 involved adults. The minimum age in the study group was 9, the maximum was 81, and the mean age was 29.8 ± 16.4 years.

Only a lung cyst was detected in 25 (40.9%) cases, and only a liver cyst was detected in 15 (24.6%) cases. 11 (18.0%) patients had cysts in both lungs and liver. Table 2 shows the age and gender distributions of the patients, the organs with cyst involvement, and the incidence rates. The provinces where the patients in our study lived are shown in Fig. 2.

**Fig. 2:** Erzurum, Erzincan, Ağrı, Ardahan, Iğdır, Kars, and Bayburt cities, where the patients in our study group live in Türkiye

**Table 2:** Some characteristics of the patients in our study and the results of the analysis

| Patient number | Gender | Age(yr) | Cyst localiza-tion | Protoscolex | Serology (ELISA) | PCR | Genotype |
|---|---|---|---|---|---|---|---|
| **1** | F | 22 | Lung+ liver | + | + | + | G1, G3 |
| **2** | M | 20 | Lung | - | - | - | - |
| **3** | M | 15 | Lung + liver | - | + | + | G1, G3 |
| **4** | M | 28 | Lung + liver | - | | - | - |
| **5** | F | 29 | Lung + liver | + | - | + | G1, G3 |
| **6** | F | 46 | Liver | + | + | + | G1, G3 |
| **7** | M | 12 | Lung + liver | - | - | + | G1, G3 |
| **8** | M | 58 | Retroperitoneal location | + | + | + | G1, G3 |
| **9** | F | 37 | Lung+ liver+ renal | + | + | + | G1, G3 |
| **10** | F | 23 | Lung | + | + | - | - |
| **11** | M | 21 | Lung | + | - | + | G1, G3 |
| **12** | M | 14 | Lung + liver | - | + | + | G1, G3 |
| **13** | M | 24 | Lung | + | + | + | G1, G3 |
| **14** | F | 14 | Lung | - | + | - | - |
| **15** | F | 27 | Lung | - | + | + | G1, G3 |
| **16** | F | 70 | Liver | + | | + | G1, G3 |
| **17** | F | 20 | Lung | + | + | + | G1, G3 |
| **18** | M | 41 | Liver | + | + | + | G1, G3 |
| **19** | M | 53 | Lung | + | - | + | G1, G3 |
| **20** | F | 24 | Liver | + | - | + | G1, G3 |
| **21** | F | 29 | Lung | + | + | + | G1, G3 |
| **22** | F | 59 | Liver | + | | + | G1, G3 |
| **23** | M | 40 | Lung | - | + | + | G1, G3 |
| **24** | M | 12 | Lung | - | - | - | - |
| **25** | F | 20 | Liver | + | + | + | G1, G3 |
| **26** | M | 32 | Liver | + | + | + | G1, G3 |
| **27** | F | 14 | Lung + liver | + | - | + | G1, G3 |
| **28** | F | 38 | Liver | + | - | + | G1, G3 |
| **29** | F | 21 | Liver | + | + | + | G1, G3 |
| **30** | M | 57 | Liver | + | + | + | G1, G3 |
| **31** | F | 17 | Vertebral local-ization | - | - | - | - |
| **32** | F | 18 | Spleen + liver | + | + | - | - |
| **33** | M | 36 | Liver | + | + | + | G1, G3 |
| **34** | M | 24 | Lung | - | | + | G1, G3 |
| **35** | F | 27 | Lung | - | + | + | G1, G3 |
| **36** | M | 9 | Lung | - | | + | G1, G3 |
| **37** | M | 46 | Liver | - | + | + | G1, G3 |
| **38** | F | 47 | Lung | - | + | - | - |

**Table 2:** Continued…

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **39** | M | 26 | Lung | - | + | - | - |
| **40** | M | 43 | Liver | - | + | + | G1, G3 |
| **41** | M | 15 | Lung | - | + | - | - |
| **42** | F | 18 | Lung + liver | - | + | + | G1, G3 |
| **43** | F | 81 | Clavicle + renal | - | + | + | G1, G3 |
| **44** | M | 20 | Lung | + | + | - | - |
| **45** | M | 27 | Subdiafragmatik + lung | - | | - | - |
| **46** | F | 14 | Lung + liver | - | + | + | G1, G3 |
| **47** | M | 17 | Lung | - | | + | G1, G3 |
| **48** | M | 16 | Lung + liver+ spleen | + | | + | G1, G3 |
| **49** | M | 12 | Lung + liver | - | + | + | G1, G3 |
| **50** | F | 12 | Lung | + | - | + | G1, G3 |
| **51** | F | 18 | Lung | - | - | + | G1, G3 |
| **52** | M | 33 | Lung | + | - | + | G1, G3 |
| **53** | M | 29 | Lung | - | + | + | G1, G3 |
| **54** | F | 58 | Lung + over | - | - | + | G1, G3 |
| **55** | M | 30 | Lung | - | - | + | G1, G3 |
| **56** | F | 46 | Mediasten + liver | - | + | + | G1, G3 |
| **57** | F | 19 | Lung + liver | + | + | + | G1, G3 |
| **58** | F | 31 | Liver | + | + | - | - |
| **59** | F | 64 | Liver | + | + | + | G1, G3 |
| **60** | M | 30 | Lung + epi-didim | + | 3 | + | G1, G3 |
| **61** | F | 13 | Lung | - | + | + | G1, G3 |

According to PCR analysis of the cyst material, 48 (78.7%) samples were positive and 13 (21.3%) were negative. In parasitological examination of the patients' cyst material, protoscoleces were detected in 31 (50.8%) patient samples. On the other hand, 30 (49%) patient specimens were evaluated as unfavourable for protoscolex (Table 3).

**Table 3:** Comparison of protoscoleces, ELISA, and PCR results in hydatid cyst samples

| | Protoscolex (+) | Skoleks (-) | ELISA IgG (+) | ELISA IgG (-) |
|---|---|---|---|---|
| **PCR (+) N=48** | 27 | 21 | 29 | 13 |
| **PCR(-) N=13** | 4 | 9 | 8 | 2 |
| **N=61** | 31 | 30 | 37 | 15 |

When the patients' serology results were checked in the hospital automation system where the samples were obtained, it was found that serological studies had been performed in

51 of the 61 patients. Of these 51 patients, 36 (70.6%) had *Echinococcus* ELISA IgG (+), whereas 15 (29.4%) had *Echinococcus* ELISA IgG (-). In the remaining 10 patients, no serological studies were performed.

Protoscolex-positive cyst fluids were used directly for DNA extraction, and germinal membranes were used instead of cyst fluids in protoscolex-negative cyst samples. PCR amplification was performed using appropriate primers to amplify the mitochondrial 12S rRNA and mt-*COX1* (S) gene regions in all samples. As a result of the study, 254 bp and 446 bp bands were obtained. The 446 bp band image obtained using the mt-*COX1* primer is shown in Fig. 3.

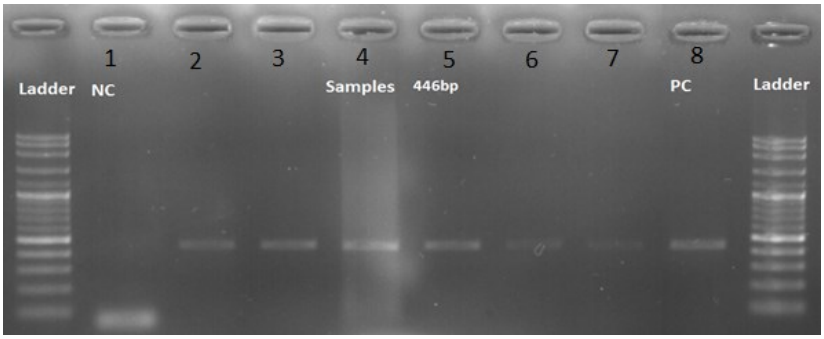


**Fig. 3:** Mt-*COX1* PCR products of *E. granulosus* were 1: Negative control, 2-7: Positive samples, 8: Positive control, M: 100bp DNA

Overall, 37 of 61 hydatid cyst samples and fluids were determined to be positive for 12S rRNA by PCR analysis. The 24 negative samples were put into PCR analysis regarding mt-*COX1*, and 11 positives were determined. The six specimens with strong positivity under UV light were subjected to sequence and phylogenetic analyses. As a result of PCR analysis of 12SrRNA and mt-*COX1* gene regions of 61 clinical samples, positivity was determined in 48 (78.7%) samples. Despite repeated PCR applications, DNA band images could not be obtained in 13 PCR-negative samples. Using the primers from our study, we obtained a 446-bp PCR product. By sequencing these PCR products, we obtained a partial *COX1* gene sequence of approximately 400 bp. We uploaded these sequences to GenBank (NCBI) and received accession numbers. We presented these accession numbers in the phylogeny. Our sequences have been added to NCBI and are available worldwide. As a result of phylogenetic analysis of 6 samples sequenced for the mt-*COX1* gene region, they were found to belong to the E. *granulosus sensu stricto* (G1, G3) genotypes, together with the reference strains. As a result of the pairwise comparison between mt-*COX1* sequences and some previously published *E. granulosus* isolates obtained from GenBank, it was seen that the isolates obtained were 100% identic with Turkish (MG886833, KX874722, MK926748, HM598451, MF544127, GU951515, KM100574, HM598451, GU984809), Iranian (MH010307, KJ162568, MZ093094), Brazilian (KT382540) and Indian (JX854029) isolates (Fig. 1).

## Discussion

CE has a cosmopolitan spread and poses a significant public health problem in some regions. In addition to causing health problems in humans, it is a disease that causes severe economic damage with treatment costs and production losses in farm animals (13). Identifying *Echinococcus* species and genotypes in intermediate and definitive hosts where CE is endemic is necessary to understand the parasite's transmission dynamics and to establish

effective control programs (4,14). Many case reports published in our province on human CE have shown that Erzurum is a highly endemic region for CE (15-17).

In our study, the 12S rRNA and mt-*cox1* gene regions were targeted for PCR-based genotyping. This study detected PCR positivity in 48 (78.7%) of 61 human isolates. In 13 samples with negative PCR results, either no tape or tape of inferior quality was obtained. In some examples, the reasons for not receiving a high-quality tape are: it can be shown that the antiparasitic drugs administered to the patients preoperatively damage the germinal membranes of the cysts, or that the extraction kits used in the study do not work with 100% efficiency. The incidence of protoscolex in microscopy was 49%. The 21 specimens reported as protoscolex-negative were also found to be PCR-positive. However, although the protoscolex was positive in 4 samples, the PCR was negative. It was thought that this situation may be due to the low amount of DNA for non-amplified samples. The incidence of protoscolex in cyst fluid has been reported as 31-34% in the literature, and it has been noted that the amount of material to be examined and its storage under appropriate conditions may affect microscopic examination (18).

In our study, 37/52 (70.6%) patients were ELISA-positive for *Echinococcus* IgG serology, as recorded in the hospital automation system. The literature shows that the sensitivity of ELISA *Echinococcus* IgG positivity tests ranges from 64.8% to 100%. Therefore, the serology results obtained in our study are compatible with the literature (7). Among the *E. granulosus* genotypes, *E. granulosus s.s.* is the causative agent of nearly 90% of genotyped human CE cases worldwide, with G1 being the most frequently described (19-21). However, some studies have been reported in Pakistan (22) and India (23), showing the common strain of *E. granulosus s.s.* genotype is represented by G3. However, infections caused by *E. granulosus* s.s. G3 genotype have been described less frequently (3,24). The G1 genotype has been reported as the most common in human CE cases worldwide and in Turkey (4,10,25-27).

In Hatay province of Turkey, phylogenetic analysis of 11 PCR-positive samples revealed that the isolates were included in the *E. granulosus s.s.* (G1-G3) complex (28). In recent molecular studies, G1 and G3 genotypes have been shown in *E. granulosus s.s.* species (29).

In our study, samples with negative mt-*COX1* PCR and moderate and strong band profile positive with mit 12S rRNA primer were taken to DNA sequence analysis, and all samples were identified as *E. granulosus s.s.* (G1, G3) according to the BLAST algorithm. In our study, the presence of *E. granulosus s.s.* in all samples is in harmony with the general literature in Turkey. Our city and the surrounding provinces are generally settlements established on plateaus at 1400-2000 m above sea level. In these settlements, people are widely engaged in sheep breeding as a source of livelihood.

In our study, the absence of different species or genotypes of *E. granulosus s.s.* (G1, G3) was interpreted as being due to the small sample group. It is thought that different genotypes/species can be identified through extensive studies using more samples.

Many factors support the life cycle of *E. granulosus s.s.* The disease is primarily endemic in areas where livestock is the primary source of income, traditional livestock practices are practised, illegal and home slaughter is common, and the population of stray dogs is high.

In addition, due to a lack of public health education, people continue to feed dogs infected offal, and the consumption of raw, unwashed vegetables contaminated with parasite eggs is among the factors that increase the prevalence of the agent. Fertile cysts in sheep have also been reported to be important in the global prevalence of the disease (14,30). Many of the risk factors mentioned above also apply to our region. Because animal husbandry is

common in our area, the stray dog population is also high.

In our study, cyst involvement was primarily localised in the lung at a rate of 67.2% in 41 cases (25 single lungs + 16 with lung and other organ involvement) and secondly in the liver at a rate of 49.2% in 30 cases (15 single liver + 15 with liver and other organ involvement). When our data were compared with those reported in many studies, the lung cyst incidence was higher in our study (29,31). This result is because the samples we used in our study mostly came from the thoracic surgery clinic, so it does not reflect the actual incidence rates. This is because the chest surgery clinic usually performs lung cyst surgery. In contrast, other treatment methods, such as the puncture, aspiration, injection, and respiration (PAIR) technique and antiparasitic medication, may be used before surgery for liver cysts.

## Conclusion

The genotype of the isolates was domestic sheep strain (G1, G3). Data that can contribute to the control of CE, a significant public health problem in our region, early diagnosis, effective treatment, eradication, vaccine, and drug development studies have been obtained. More comprehensive studies in our area and across the country will help establish effective control programs in the fight against CE.

## Acknowledgements

Our study was supported by the number TSA-2023-10274 within the scope of Ataturk University Scientific Research Projects. The Institution that funded the research had no role in the design, data collection, analysis, or manuscript preparation.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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