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Identification and genotyping of *Echinococcus granulosus* from human clinical samples in Guilan province, north of Iran

Masoumeh Gholami Koohestan [a,b], Reza Saberi [b], Ahmad Daryani [b], Shahabbedin Sarvi [b], Meysam Sharifdini [c], Davood Anvari [d], Seyyed Ali Shariatzadeh [b], Seyed Abdollah Hosseini [b,*], Shirzad Gholami [b,*]

[a] *Student Research Committee, Mazandaran University of Medical Science, Sari, Iran*
[b] *Toxoplasmosis Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran*
[c] *Department of Medical Parasitology and Mycology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran*
[d] *School of Medicine, Iranshahr University of Medical Sciences, Iranshahr, Iran*

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ABSTRACT

Cystic echinococcosis (CE) is a significant health problem in both human and veterinary medicine. It is caused by the tapeworm *Echinococcus granulosus (E. granulosus)*. The objective of this study was to investigate molecular diversity of *E. granulosus* from the paraffin-embedded human (FFPE) tissue samples using sequencing of mitochondrial genes. Thirty-five FFPE tissue samples were collected from different regions of Guilan province, north of Iran. Demographic data were recorded using a questionnaire. Five sections (1 mm) of the tissue were prepared and deparaffined using xylene and ethanol methods. Molecular analysis was performed using the *Nad1* and *Cox1* genes using PCR and DNA sequencing. Totally, 25 cases (71.43%) were women and 10 cases (28.57%) were men. The most affected age group was 21–30 yr old. The most of cysts were isolated from the liver ($n = 19$; 54.29%) and others in the lung ($n = 16$; 45.71%). The *Cox1* and *Nad1* genes were successfully amplified in 16 (45.71%) and 12 (34.28%) DNA samples from FFPE tissue. Sequencing analysis revealed that all samples were *E. granulosus* sensu stricto *complex* (G1 and G3). In this study, *E. granulosus* sensu stricto complex G1 and G3 were identified in human hydatid cysts and showed the presence of sheep/dog cycle in human infection. This finding confirmed and completed previous studies on the geospatial distribution of *E. granulosus* sensu stricto complex G1 and G3 in the southern and coastal areas of the Caspian Sea region.

1. Introduction

Cystic echinocccosis (CE), also known as hydatid disease, is a cosmopolitan zoonotic infection caused by the larval stage of *Echinococcus granulosus* (Chaâbane-Banaoues et al., 2015; Larrieu et al., 2019). The adult form of *E. granulosus* lives in the small intestine of carnivores as a definitive host, and eggs are passed through the feces (Pal et al., 2022). Humans can become infected through the ingestion of food contaminated with the eggs, or through direct contact with an infected definitive host (Casulli et al., 2022). Human CE may affect all organs of the body, but hydatid cysts commonly affect the liver and lungs. Clinical symptoms vary depending

* Corresponding authors at: Department of Parasitology, Toxoplasmosis Research Center (TRC), School of Medicine, Mazandaran University of Medical Sciences, 18th Km of Khazarabad Road, Sari, Mazandaran Province, Iran.
E-mail addresses: hosseini4030@gmail.com (S.A. Hosseini), sgholami200@gmail.com (S. Gholami).

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on the size and anatomical location of the cyst, and the clinical spectrum ranges from asymptomatic to severe or fatal disease (Dietrich et al., 2020; Govindasamy et al., 2023).

According to sequencing of mitochondrial DNA (mtDNA), *E. granulosus* sensu lato has been classified into four species and eight genotype: *E. granulosus* sensu stricto (G1 and G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), and *Echinococcus canadensis* (G6, G7, G8 and G10) (Casulli et al., 2012; Manterola et al., 2021). The G1 and G3 are the predominant genotypes identified in livestock and humans in Iran (Shahabi et al., 2021). Approximately 1% of the surgeries performed in medical centers in Iran are due to hydatid cysts, and the reported prevalence of CE infection in surgical cases ranges from 1.1 to 18.3 per 100,000 population (Fasihi Harandi et al., 2012). It is crucial to determine the genotype of hydatid cysts. A recent study by Debiaggi et al. (2023) discovered significant differences in the clinical aspects of cystic echinococcosis caused by *E. granulosus s.s* and the G6 genotype (Debiaggi et al., 2023).

To date, numerous investigations have been conducted to identify and characterize *E. granulosus* genotypes in the geographical region of Iran. The findings derived from these studies have consistently demonstrated the presence of G1, G3, G6, and G7 genotypes across various host species (Khademvatan et al., 2019).

Guilan province, located in the north of Iran, is an appropriate area for the transmission of zoonotic diseases due to free and industrial livestock farming, climatic conditions, high humidity, dense vegetation, food culture, and abundance of surface water (Ashrafi and Mas-Coma, 2014). However, there is a limited number of studies on the molecular identification of *E. granulosus* in the definitive and intermediate hosts, especially its human cases in the central regions of northern Iran such as Guilan and Golestan provinces (Mahmoudi et al., 2019; Siyadatpanah et al., 2019). Therefore, this study aimed to determine *E. granulosus* genotypes in FFPE tissue based on sequencing of mitochondrial genes *Cox1* and *Nad1* in the north of Iran.

2. Materials and methods

2.1. Ethics statement

Ethics approval for the current study was obtained from the relevant Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (Approval Number: IR.MAZUMS.REC.1398.667).

2.2. Study area

This study was conducted in Guilan province located in the southern littoral of the Caspian Sea area in northern Iran. The center of the province is the Rasht. Other cities include Astaneh-ye Ashrafiyeh, Astara, Fuman, Talesh, Lahijan, Langarud, Masuleh, Manjil, Rudbar, Rudsar, Shaft, Siahkal, and Sowme'eh Sara. The latest census conducted in 2016 found Guilan to have a population of 2,530,696 people in 851,382 households. According to census, 679,995 people live in the Rasht. The climate of this province is

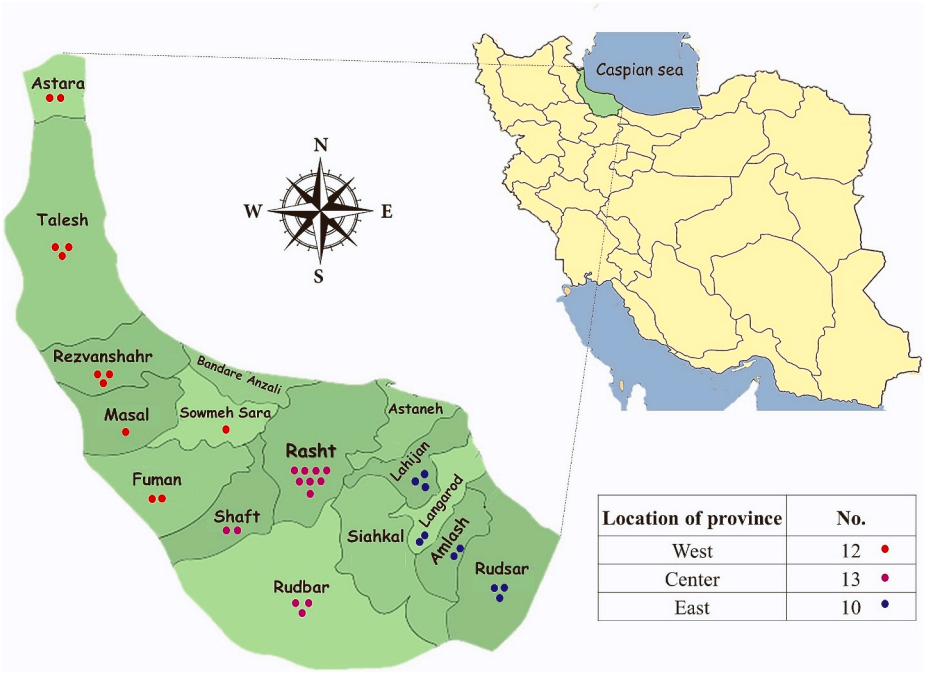

Fig. 1. Geographical location of Guilan Province, Iran.

temperate, which is due to the influence of the mountainous climate of Alborz and the Caspian Sea. Its relative humidity is between 40 and 100% and its average temperature is 17.5 degrees Celsius (Fig. 1). In the animal husbandry industry of this province, due to its many green pastures, sheep are known as the dominant livestock.

2.3. Sample collection

Overall, 35 samples of FFPE tissue were collected from various hospitals, namely Razi Hospital (18 samples), Poursina Hospital (10 samples), Golsar Hospital (5 samples), and Aria Hospital (2 samples). Informed written consent was obtained from all patients and their data was recorded using a questionnaire. All the samples were carefully transported to the molecular parasitology laboratory of Mazandaran University of Medical Sciences.

2.4. DNA extraction and polymerase chain reaction (PCR)

To DNA extraction from tissue samples, first 5 sections (1 mm) of the sample were prepared and deparaffined using xylene and ethanol methods. In a brief summary, the method involved adding 1 ml of xylene to the sample and incubating at the room temperature for 30 min. Within the following, the sampleswere centrifuged at 3000*g* for 3 min, and the supernatants were discarded. Finally, the samples were washed with 100%, 90%, and 70% ethanol (they were centrifuged at 3000*g* for 3 min) (Pikor et al., 2011). In the next step, DNA extraction were performed using DNA extraction kit (Favorgen Company, Taiwan) according to the manufacturer's instructions.

For the amplification of the *Cox1* gene, we used JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAA-CATAATGAAAATG-3′) primers, and for the amplification of the *Nad1* gene, we employed MS1 (5′-CGTAGGTATGTTGGTTTGTTTGGT-3′) and MS2 (5′-CCATAATCAAATGGCGTACGAT-3′) primers (Sharbatkhori et al., 2009). PCR mixture with a final volume of 25 µl consisting 12.5 µL of master mix (Ampliqon Co., Denmark), 10 pmol each of forward and reverse primers, and 4 µl of template DNA were prepared. Next, the temperature program was carried out: primary denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing step at 52 °C and 54 °C for 45 s for *Cox1* and *Nad1*, respectively, and extension at 72 °C for 35 s. A final strand elongation was made at 72 °C for 5 min. The PCR products were confirmed by visualization on 1.5% agarose gel stained with SYBR safe DNA gel stain (Invitrogen,Eugene, Oregon, USA) for imagining of PCR products (Sharbatkhori et al., 2009).

2.5. Sequencing and phylogenetic analysis

PCR products were purified and sequenced using an ABI PrismTM 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) by the Macrogen Company (Seoul, South Korea). The sequences of each samples were edited and aligned with reference sequences by Bio-Edit software. In the next step, the sequences of each samples were blasted for finding regions of similarity between sequences. The phylogenetic tree was created with MEGA (ver; 10) software using the Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. In addition, branch confidence in clades in each tree was assessed using a bootstrap analysis with 1000 replicates (Kumar et al., 2018). Sequences were deposited in GenBank under accession numbers MW464190– MW464194 for *Cox1* and MW460010– MW460014 for *Nad1* genes. To draw the phylogenetic network, G1 ($n = 72$) and G3 ($n = 20$) sequences from Kinkar et al. (2018) and current isolates ($n = 5$) were analyzed. Phylogenetic networks were generated using Network v4.6.1.5 (Bandelt et al. 1999; http://www.fluxusengineering.com, Fluxus Technology Ltd., 2004).

Table 1
Statistical analysis for frequency of hydatid cysts isolates from human in Guilan province based on gender, age, location of hydatid cyst and residence.

Variable		No. of positive	% positive	Chi ^{2}	*P* value
Gender	Female	25	71.43	30.61	<0.001
	Male	10	28.57		
Age	10–20	3	8.57	17.73	0.007
	21–30	11	31.43		
	31–40	8	22.86		
	41–50	3	8.57		
	51–60	5	14.29		
	>61	5	14.29		
Location of cyst	Liver	19	54.29	0.51	0.47
	Lung	16	45.71		
Geographical region	West	13	37.14	0.6	0.74
	Center	12	34.28		
	East	10	28.58		
Residence	Rural	21	56	4.64	0.031
	Urban	14	44		

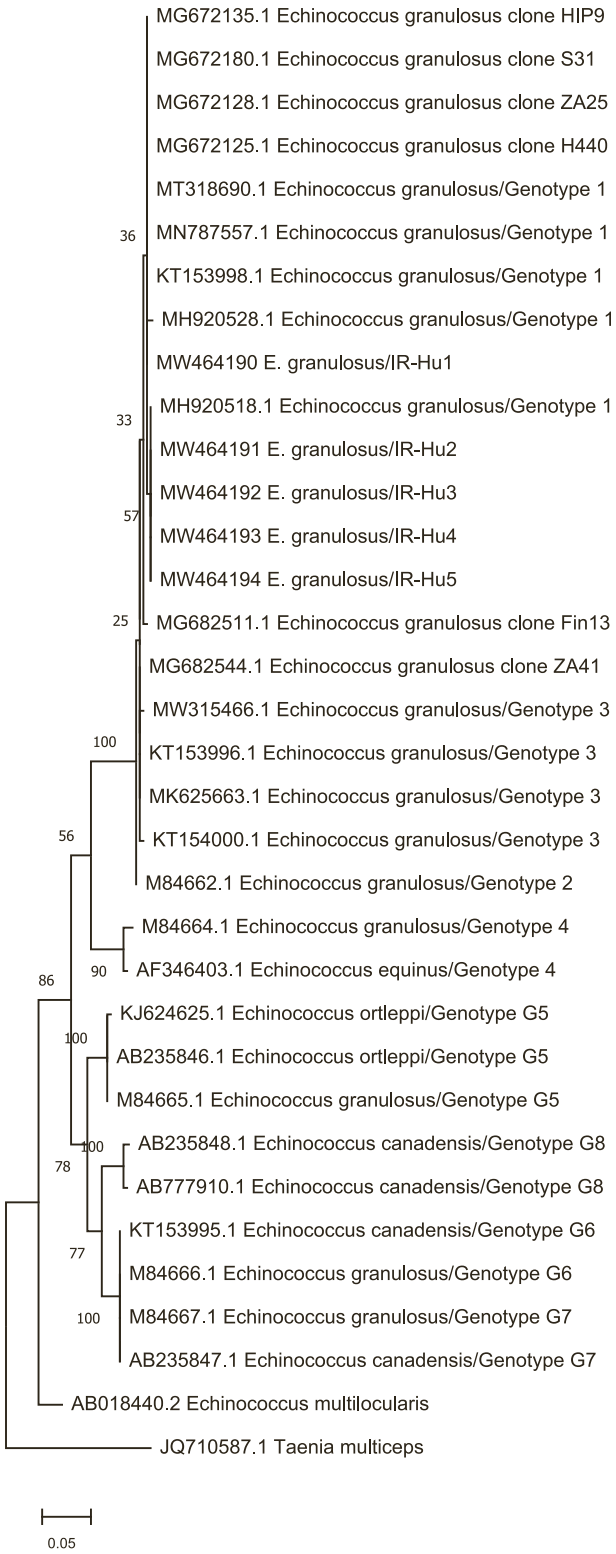

Fig. 2. A distance-based Maximum likelihood method cladistic tree of *Echinococcus granulosus* G1 genotype based on the *Cox1* gene. *Taenia multiceps* was considered an out-group branch (Accession No: JQ710587).

2.6. Statistical analysis

The results were analyzed using the SPSS (ver.19) software package (SPSS Inc., Chicago, IL, USA). The Chi squared test was used to evaluate differences between the frequency of hydatid cyst and variables such as gender, age, location of hydatid cyst, geographical location and residence in urban and rural areas. *P*-value <0.05 was considered statistically significant.

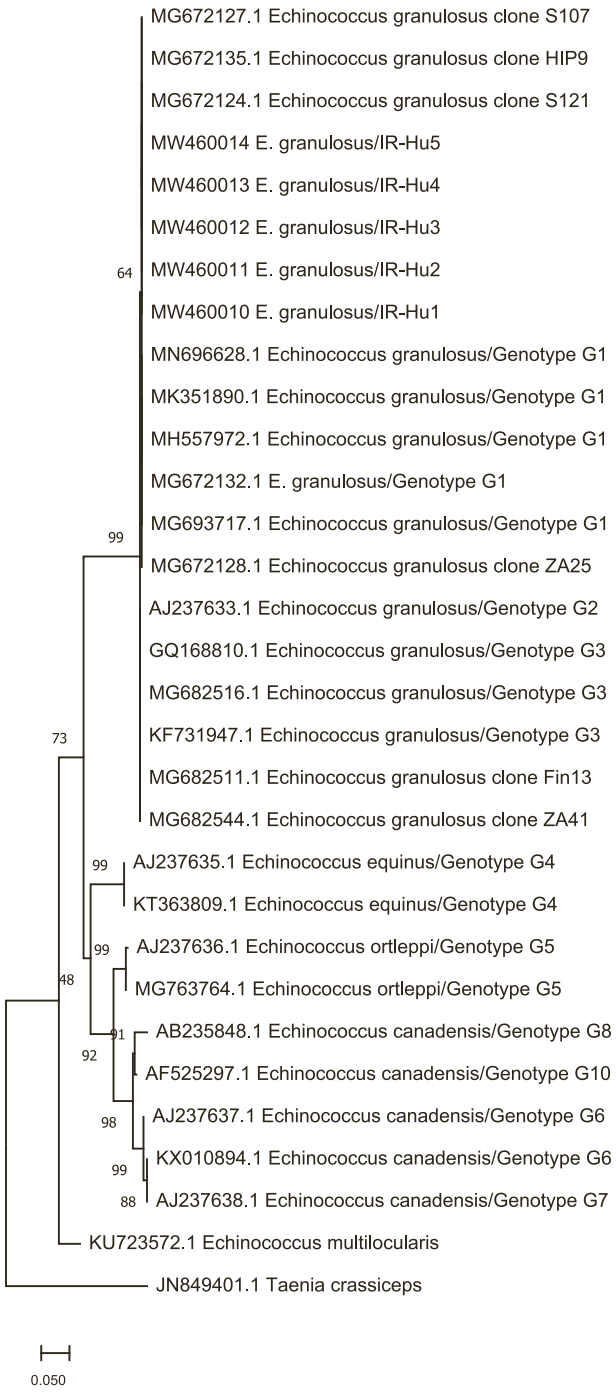

Fig. 3. A distance-based Maximum likelihood method cladistic tree of *Echinococcus granulosus* G1 genotype based on the *Nad1* gene. *Taenia crassiceps* was considered an out-group branch (Accession No: JN849401).

3. Results

The demographic analysis revealed that the majority of human hydatid cysts were more prevalent in women (25/35; 71.43%), particularly in the age range of 21–30 yr old (31.43%). The minimum age recorded among the patients was 16 yr, with two cases being 18 yr old. There was a significant difference in the frequency of hydatid cysts based on gender ($P < 0.001$) and age ($P = 0.007$). Regarding the location of the cysts, the majority of cysts were identified in the liver ($n = 19$, 54.29%), while the remaining were detected in the lung ($n = 16$; 45.71%). Nevertheless, no statistically significant difference was observed between the two locations ($P = 0.47$).

The analysis of data based on geographical area revealed the highest number of human hydatid cyst cases ($n = 13$, 37.14%) was observed in the western part of the province. However, no statistically significant difference was found in this regard ($P = 0.74$). Out of the total 35 samples, 21 (56%) were obtained from rural areas, while 14 (44%) were from urban areas. Interestingly, a statistically significant difference was observed between these two areas ($P = 0.031$) (Table 1).

Molecular analysis revealed that 16 samples were positive for *Cox1* gene and 12 samples were positive for *Nad1* gene. The sequences obtained in this study exhibit a high similarity of 99–100% to the recorded sequences of *E. granulosus* sensu stricto complex G1 and G3, which have been reported in various regions of Iran (including Mazandaran, Fars, and Hamedan provinces) as well as in other countries, particularly in Asian countries such as Turkey, Uzbekistan, Pakistan, and Kyrgyzstan. Based on the phylogenetic analysis, human samples were *E. granulosus* sensu stricto G1 and G3 complex genotypes (Fig. 2. and Fig. 3). The phylogeny network showed that G1 and G3 sequences were divided into two separate haplogroups with 16 and 7 distinct haplotypes, respectively. Furthermore, the findings indicated that the samples in this study were categorized into haplotype 1 ($n = 4$) and haplotype 7 ($n = 1$) of *E. granulosus* sensu stricto G1 (Fig. 4). The phylogenetic network analysis revealed a clear distinction between G1 and G3 genotype groups based on mitochondrial data. There were no sequences found between G1 and G3, indicating their distinctiveness.

4. Discussion

Echinococcosis is of particular importance in Iran due to its significant public health impact and prevalence in the country (Siyadatpanah et al., 2019). In this study, our objective was to identify the genotypes of *E. granulosus* isolates based on mitochondrial genes in north Iran. Demographic analysis demonstrated that most of the human hydatid cyst were isolated of women. It seems, women are more likely than men to be infected with the parasite due to spending more time on household chores, caring for pets at home, gardening, cleaning and washing vegetables and fruits (Youssefi et al., 2016). In the case of the age variable, more infection may be observed in older individuals (age range of 21–30 yr old) due to prolonged exposure to risk factors and routes of transmission. The higher proportion of cases in adults may also be attributed to the fact that cysts require time to develop and manifest symptoms (Garcia and Procop, 2016; Siyadatpanah et al., 2019).

In the present study, most of cysts were isolated of the liver (54.29%) and other in lungs (45.71%). According to literature review, hydatid cysts can develop in various organs, with the liver being the most commonly affected. This is due to several factors including the liver's anatomy, rich blood supply, route of infection, filter function, and immune response. The liver's size and blood supply make it more susceptible to the spread of the parasite and the development of cysts. The primary route of infection is through the ingestion of contaminated food or water, and the larvae can travel to different organs via the bloodstream. The liver's role as a filter for the blood may increase the likelihood of larvae being trapped and developing into cysts. Additionally, the liver's immune response to the infection may not effectively eliminate the parasite, allowing the cysts to persist and grow (Ciftci et al., 2021; Crippa et al., 1999; Govindasamy et al., 2023). The frequency of hydatid cysts was higher in the rural regions compared to urban regions. This difference

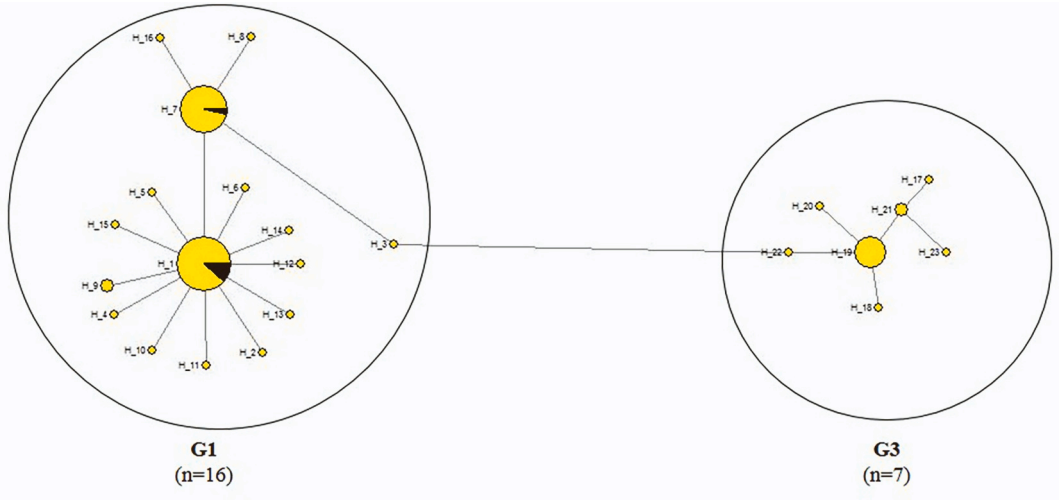

Fig. 4. Phylogenetic network of *Echinococcus granulosus* sensu stricto G1 and G3 sequences. The current samples are marked with black color.

may be attributed to the close proximity between humans and infected dogs in rural areas, inadequate sanitation and hygiene practices, and limited access to healthcare (Sarkari et al., 2020).

Epidemiological investigations aimed at identifying the genotypes of this parasite are critical due to various factors including host specificity, human susceptibility to different genotypes, pathogenicity, and the clinical course of the disease. (Romig et al., 2015; Siracusano et al., 2012). In the current study, sequence analysis based on the mitochondrial genes revealed all the tissue samples were infected with *E. granulosus* sensu stricto complex G1 and G3. Numerous molecular studies have been performed to identify genotypes in Iran, which shows the G1 genotype is predominant in Iran (Khalkhali et al., 2018; Shariatzadeh et al., 2015; Siyadatpanah et al., 2019).

Several studies have been conducted on human echinococcosis in Iran. In a study by Pezeshki et al. (2013) in Ardabil province, the presence of G1 and G3 genotypes in human cysts was identified (Pezeshki et al., 2013). Another study by Gholami et al. (2012) revealed the presence of G1 and G3 genotypes in hydatid cysts from sheep, cattle, and camel isolates in Golestan province (Gholami et al., 2012). Gorgani-Firouzjaee et al. (2019) also identified the G1 in human hydatidosis cases in Mazandaran province (Gorgani-Firouzjaee et al., 2019). In a study conducted in Guilan province, Nematdoost et al. (2021) determined the genotypes of 57 animal isolates, including cattle, sheep, and goat, as well as human hydatid cyst samples. The results showed that the genotypes isolated from different livestock were G1, G3, and *E. ortleppi*. All human hydatid cysts were *E. granulosus* s.s., which is consistent with the findings of the present study (Nematdoost et al., 2021). Sharbatkhori et al. (2009) used the SSCP method on *Nad1* and *Cox1* genes and performed DNA sequence analysis on sheep, cattle, goat, camel, and human isolates. They introduced the G1 and G3 genotypes, which consistent with the findings of this study (Sharbatkhori et al., 2009). In a study by Vahedi et al. (2014) in East Azerbaijan Province, it was demonstrated that all human hydatid cysts studied were identified as the *E. granulosus* sheep strain (G1) (Vahedi et al., 2014). These findings indicate that the G1 is the predominant genotype in cases of echinococcosis in Iran. Overall, these studies highlight the importance of understanding the genotypes and distribution patterns of hydatid cysts in different regions.

In addition, numerous genotyping studies have been conducted in different parts of the world on the nuclear and mitochondrial genes of *E. granulosus*. This indicates the special importance of studying the intraspecific and phylogenetic diversity of this parasite from medical and veterinary perspectives (Busi et al., 2007; Kinkar et al., 2018). Mitochondrial genes are more efficient than nuclear genes due to their rapid evolution in gene sequences and the presence of very high amounts of gene copies to establish phylogenetic relationships between closely related species (Busi et al., 2007). Mitochondrial genes of *E. granulosus* due to short genomic sequences such as *Nad1*, *Cox1*, 12srRNA, 16srRNA, etc., are easily sequenced and provide the necessary information for genotyping and phylogenetic analysis (Knapp et al., 2022). Since the mitochondrial genome is affected by many mutations, it is better to study the nuclear and mitochondrial genomes together in phylogenetic studies.

The principal limitations of this study was the low sample size. Another challenge encountered was the extraction of DNA from paraffin blocks, which was mitigated through deparaffinization procedures and troubleshooting.

5. Conclusion

This study identified the presence of *E. granulosus* sensu stricto complex G1 and G3 in human hydatid cysts, and this result indicates the presence of sheep/dog cycle in human infection of this region. Given the presence of favorable conditions for the transmission cycle in the southern and coastal areas of the Caspian Sea region, it is important to recognize it as a potential health threat. Consequently, the implementation of control and prevention programs is strongly recommended.

Authors' contributions

Sh.Gh conceived the work and designed the protocol with assistance of S.A.H, A.D, SH·S, and M.Sh, performed formal analysis supervised by M.Gh.K, D.A, and S.A.Sh performed data curation and analysis. M.Gh.K, R.S and S.A.H drafted the manuscript that was reviewed and edited and approved by all authors.

Ethical considerations

This study was approved by the ethical principles and the national norms and standards for identification and genotyping of *Echinococcus granulosus* from human clinical samples in Guilan province, north of Iran (IR.MAZUMS.REC.1398.667).

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

All data generated or analyzed during this study are included within the paper.

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