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Original Article

Histopathologic Alterations between *Echinococcus granulosus sensu stricto* and *E. canadensis* Genotypes of Human Cystic Echinococcosis Cysts in Shiraz, Iran

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Abstract

Background: We aimed to determine the genotypes of *Echinococcus granulosus sensu lato* (*s.l.*) using DNA regions within the *NADH dehydrogenase subunit-1* (*nad1*) mitochondrial genes in formalin-fixed paraffin-embedded (FFPE) isolates of human cystic echinococcosis cysts (CE cysts) and compare their histopathologic alterations.

Methods: Out of 135 samples, 21 high-quality PCR positive samples were selected for sequencing and were deposited into GenBank database. Moreover, histopathological changes of *E. granulosus sensu stricto* (G1 genotype) and *E. canadensis* (G6 genotype) cases were also compared.

Results: Based on the sequencing results, 16 cases were diagnosed as *E. granulosus s.s.* (G1-G3 genotype) and 5 cases as *E. canadensis* (G6 genotype). Five haplotypes of *E. granulosus* were identified from 21 *nad1* sequences. The histopathological alterations in both genotypes showed laminated layer of CE without inflammatory cells. In a few cases of the G6 genotype, neutrophils in the outer cuticular layer with mild vascular and congestion were observed. Cell debris with multiple areas of necrosis, as well as scanty lymphoplasma cells in the outer cuticular layer were observed in G1-G3 genotype cases. So, the histopathological differences between the two genotypes are not noticeable enough to be differentiated by microscopical observations.

Conclusion: *E. granulosus s.s.* (G1-G3) and *E. canadensis* (G6 genotype) are prevalent among CE patients. In general, five haplotypes were identified by *nad1* genes analysis. The histopathological differences between the two genotypes have not been so big to be differentiated by microscopic observations.

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Introduction

Cystic echinococcosis (CE) is one of the most important zoonotic parasitic diseases of human and animals. The disease is caused by the metacestode of *Echinococcus granulosus sensu lato* (*s. l.*), infecting a wide range of herbivores/omnivores as intermediate hosts. Human is considered as aberrant or accidental host (1).

The disease is endemic in many parts of the world including the Middle East, China, North Africa, and South America. CE has also been reported in different parts of Iran (1–3).

Based on the current evidence, 1–3.6 million disability-adjusted life years (DALYs) have been estimated to be missed due to CE worldwide(4). CE is contributed to 1% of the surgical operations in Iran with an incidence rate of 0.6-1.2 cases per 100000 individuals (5). Therefore, CE is a major public health and economic concern in Iran. Genetic characterization of *E. granulosus s. l.* has important implications for elucidating the epidemiology of this tiny worm, as well as the control of disease in human and other hosts (1,6).

E. granulosus s.l. is currently subdivided into *E. granulosus sensu stricto (s.s.)* (with the genotypic variants of G1-G3), *E. felidis*, *E. equinus* (G4), *E. ortleppi* (G5) and, *E. canadensis* (genotypic variants: G6/G7, G8, and G10). The majority of human *E. granulosus* genotypes are *E. granulosus s.s* (G1–G3) and *E. canadensis* (G6) in Iran (1,3,5,7–9). *E. ortleppi* (G5) has also been reported from camel isolate in Iran (10).

The G1 genotype is frequently found in human isolates worldwide (1,11). *Echinococcus* genotypes are different in many features, including pathogenicity, parasite maturity, potential host-related genetic characteristics, and sensitivity to drugs, epidemiological characteristics, and even morphology (4,8,12).

Various molecular methods have been employed for the identification of *Echinococcus* sp. from different hosts (13). Diagnostic methods such as polymerase chain reaction, restriction fragment length polymorphism (RFLP mitochondrial) analysis, and sequencing of gene regions have been shown advantages for the characterization of Taeniidae worms (14–21). However, *NADH dehydrogenase-1* (*nad1*) and *cytochrome c oxidase-1* (*cox 1*) mitochondrial genes have been more commonly used in different studies. On the other hand, different research has been carried out on formalin-fixed paraffin-embedded (FFPE) of human infected liver and other organs for genetic identification of CE cysts (16,17,22–26). However, none of them investigated histopathological alterations between human CE caused by *E. granulosus s. s.* (G1 genotype) and *E. canadensis* (G6 genotype), so far.

We aimed to investigate the genotype characteristics of CE in FFPE tissues of patient's cysts over 12 years using PCR and sequencing methods at Shiraz University of Medical Sciences (SUMS), Southern Iran. So, we investigated and compared the histopathological changes of human CE cases infected with *E. granulosus s. s.* (G1 genotype) and *E. canadensis* (G6 genotype).

Materials and Methods

Geographical location of the study:

Shiraz (29°36'36"N 52°32'33"E), historically known as Pars, is an old city in the south of Iran and the capital of Fars Province. It is located in a green plain at the foot of the Zagros Mountains and at an altitude of 1500 meters above sea level. This city obtains an average of 305.6 mm (12 inch) of rainfall each year. It has an overall pleasant semi-arid climate with hot-summer Mediterranean climate based on the Köppen climate classification.

Collection of CE

A total of 135 FFPE blocks were collected from CE patients who had surgical operation from 2000 to 2011, at Shiraz University of Medical Sciences (Namazi Hospital), Shiraz, Iran. Demographic data, including surgical pathology, file number, age, sex, location, size, and number of cysts were recorded in a data sheet. Pathological slides of all patients were examined under the light microscope and proper sample slide. Then, paraffin blocks were cut into 5 -10 µm thickness sections, and the first sections of the FFPE blocks were removed due to exposure to air. The next sections from each block were considered for transfer to a 1.5 mL micro-centrifuge tube. The sections were transferred to the helminthology laboratory at the Department of Parasitology and Mycology, Shiraz University of Medical Sciences, Shiraz, Iran, for further actions.

DNA extraction and PCR:

The sections were de-paraffinized using 1 ml of xylene for 10 min at 37 °C. Subsequently, samples were centrifuged at 15,000 rpm (18000×g) for 5 min, and the supernatants were removed. DNA was extracted from samples using Qiagen Kit DNase tissue (Qiagen, Germany). A PCR amplification of a 471bp segment of *nad1* was applied to genomic DNA using MS1: (5'-GTAGGTATGTTGG TTTGTTTGGT-3') and MS2: (5'-CCATAATCAAATGGCGTACGAT-3') primers (6). DNA was also recovered from the gel following agarose gel electrophoresis using a Gel DNA recovery kit (Vivantis, Malaysia) and PCR was carried out using recovered DNA in order to enhance DNA quality.

DNA sequence analysis

A panel of 21 well qualities PCR amplicons for the *nad1* gene was subjected to sequencing in two directions, using the same PCR primer set (FAZA Biotech Co. Iran).

Phylogenetic analysis

The sequences of the *nad1* gene (21 cases) were deposited in the GenBank database. Blast software was applied for the identification and comparison of our sequences with other deposited ones in GenBank (http://www.ncbi.nlm.nih.gov). Alignment was carried out using Clustal W and, the aligned sequences were manually refined in BioEdit software (Ver. 5.0.6). A phylogenetic tree for *nad1* sequences was drawn using the sequenced samples and sequences obtained from GenBank with *Taenia saginata* as an outgroup. Phylogenetic analysis was performed using a Bootstrap value of 1000 and Neighbor-joining method based on the Tamura 3-parameter model by Molecular Evolutionary Genetics Analysis software (MEGA v6.0) (27).

Haplotype Analysis

The haplotype network was calculated for mitochondrial DNA sequence datasets genotypes *E. granulosus s.s.* (G1-G3=16), and G6 (n =5) for FFPE isolates. Median-joining haplotype network analysis was carried out on the sequence data by Population Analysis with Reticulate Trees (PopART) software (http://popart.otago.ac.nz) to demonstrate the relationships between the haplotypes (28). In this regard, the Fasta formats were converted to the Nexus format by DnaSP software v.5.10, followed by estimation diversity indices calculation including the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversities (π) and also neutrality indices such as Tajima's D, and Fu's Fs (18,28).

Data analysis

The data were analyzed using SPSS software version 18s (Chicago, IL, SA).

Histopathological changes based on CE genotypes:

Several histopathologic slides belonging to *E. granulosus s. s.* (G1 genotype) and *E. canadensis* (G6 genotype) were randomly selected for histopathological studies. The histopathological changes were carefully observed and appropriate photographs were made.

Ethical considerations

The study was approved by the Ethics Committee at Shiraz University of Medical Sciences, Iran (Ethical code: IR.SUMS.REC.1390.S5596).

Results

Histopathological changes of different cases of human CE according to their genotypes are shown in Fig. 1: (a, b, c, d) to Fig. 2: (a, b, c, d). Laminated layer of CE cysts without inflammatory cells has been observed in both genotypes. Neutrophils in the outer cuticular layer with mild vascular and congestion were observed in the G6 genotype. Cell debris with multiple areas of necrosis, as well as scanty lymphoplasma cells in the outer cuticular layer were observed in G1-G3 genotype cases. G6 genotype demonstrated scanty lymphoplasma cells in the outer cuticular layer with mild vascular congestion (dilatation of blood vessels) and severe lymphoplasma cells were finally found in both genotypes. So, the histopathological differences between two genotypes are not noticeable enough to be differentiated by microscopical observations.

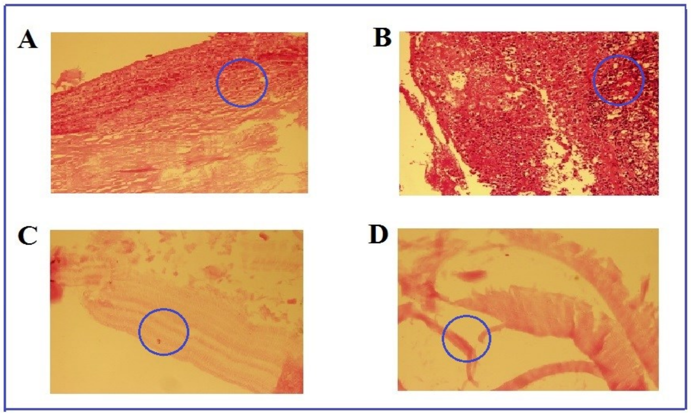

Fig. 1: Histopathological changes of different human CE cases with G6 genotype. Photomicrograph was shown mild and scanty lymphoplasma cells in the outer cuticular layer in case 1 (A), severe lymphoplasma cells and neutrophils in the outer cuticular layer with mild vascular congestion (dilatation of blood vessels) in case 2 (B) and only the laminated layer of hydatid cyst without inflammatory cells in cases 3 and 4 (C,D)

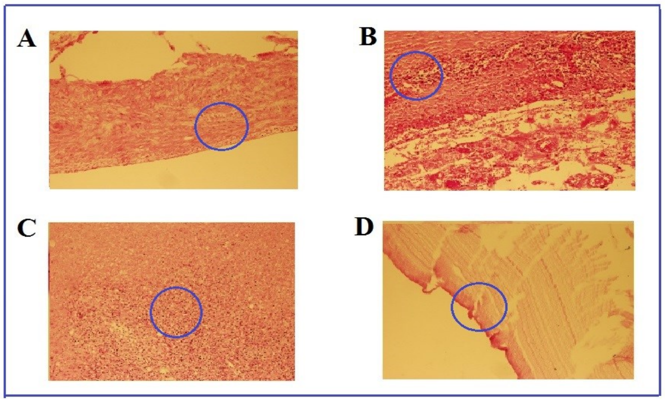

Fig. 2: Histopathological changes of different human CE cases with G1-G3 genotypes. Photomicrograph was shown outer cuticular layer without inflammatory cells, also laminated layer is present without inflammation in case 1 (A), mild and scanty lymphoplasma cells in the outer cuticular layer with mild vascular congestion (dilatation of blood vessels) in case 2 (B), severe lymphoplasma cells and cell debris with multiple areas of necrosis in case 3 (C) and only the laminated layer of hydatid cyst without inflammatory cells in case 4 (D)

Out of 135 samples, 21 high-quality PCR positive samples were selected for sequencing and the sequences were deposited into GenBank databases under the accession numbers of KF437790- KF437811 (Table 1). Genetic relationships of 21 *E. granulosus* isolates from the university hospitals (Shiraz, Iran) and selected GenBank sequence samples based on phylogenetic analysis of partial *nad1* sequence data is shown in Fig. 3.

The frequency of 21 sequenced samples according to sex is shown in Table 2.

Table 1: The genotype of *E. granulosus* isolates identified by partial mitochondrial *nad1* sequence in a university hospital (Shiraz, Iran) and relevant information on the origins of sequences used for subsequent phylogenetic analyses

Number	Accession Number	Geographic origin	Host	Strain (Genotype)	References
1	KF437790.1	Iran: Fars	Homo sapiens	G1	Present study
2	KF437791.1	Iran: Fars	Homo sapiens	G1	Present study
3	KF437792.1	Iran: Fars	Homo sapiens	G1	Present study
4	KF437793.1	Iran: Fars	Homo sapiens	G1	Present study
5	KF437794.1	Iran: Fars	Homo sapiens	G1	Present study
6	KF437795.1	Iran: Fars	Homo sapiens	G1	Present study
7	KF437796.1	Iran: Fars	Homo sapiens	G1	Present study
8	KF437797.1	Iran: Fars	Homo sapiens	G1	Present study
9	KF437798.1	Iran: Fars	Homo sapiens	G1	Present study
10	KF437799.1	Iran: Fars	Homo sapiens	G1	Present study
11	KF437800.1	Iran: Fars	Homo sapiens	G1	Present study
12	KF437801.1	Iran: Fars	Homo sapiens	G1	Present study
13	KF437802.1	Iran: Fars	Homo sapiens	G1	Present study
14	KF437804.1	Iran: Fars	Homo sapiens	G1	Present study
15	KF437805.1	Iran: Fars	Homo sapiens	G1	Present study
16	KF437806.1	Iran: Fars	Homo sapiens	G1	Present study
17	KF437807.1	Iran: Fars	Homo sapiens	G6	Present study
18	KF437808.1	Iran: Fars	Homo sapiens	G6	Present study
19	KF437809.1	Iran: Fars	Homo sapiens	G6	Present study
20	KF437810.1	Iran: Fars	Homo sapiens	G6	Present study
21	KF437811.1	Iran: Fars	Homo sapiens	G6	Present study
22	MK351886.1	Iran: Gachsaran	*Ovis aries*	G1	GenBank
23	AJ237633.1	Poland	Tasmanian sheep	G2	GenBank
24	MN269994.1	China	Yak	G3	GenBank
25	MZ190836.1	China: Guizhou	Homo sapiens	G5	GenBank
26	LC476690.1	Iran: Tehran	Homo sapiens	G6	GenBank
27	AB235848.1	Japan	Moose	G8	GenBank
28	AF525297.1	Finland: Salla	Reindeer	G10	GenBank
29	AB668376.1	Germany	*Macaca sylvanus*	*E. multilocularis*	GenBank
30	AJ239106.1	Australia: Victoria	Cattle	*T.saginata*	GenBank

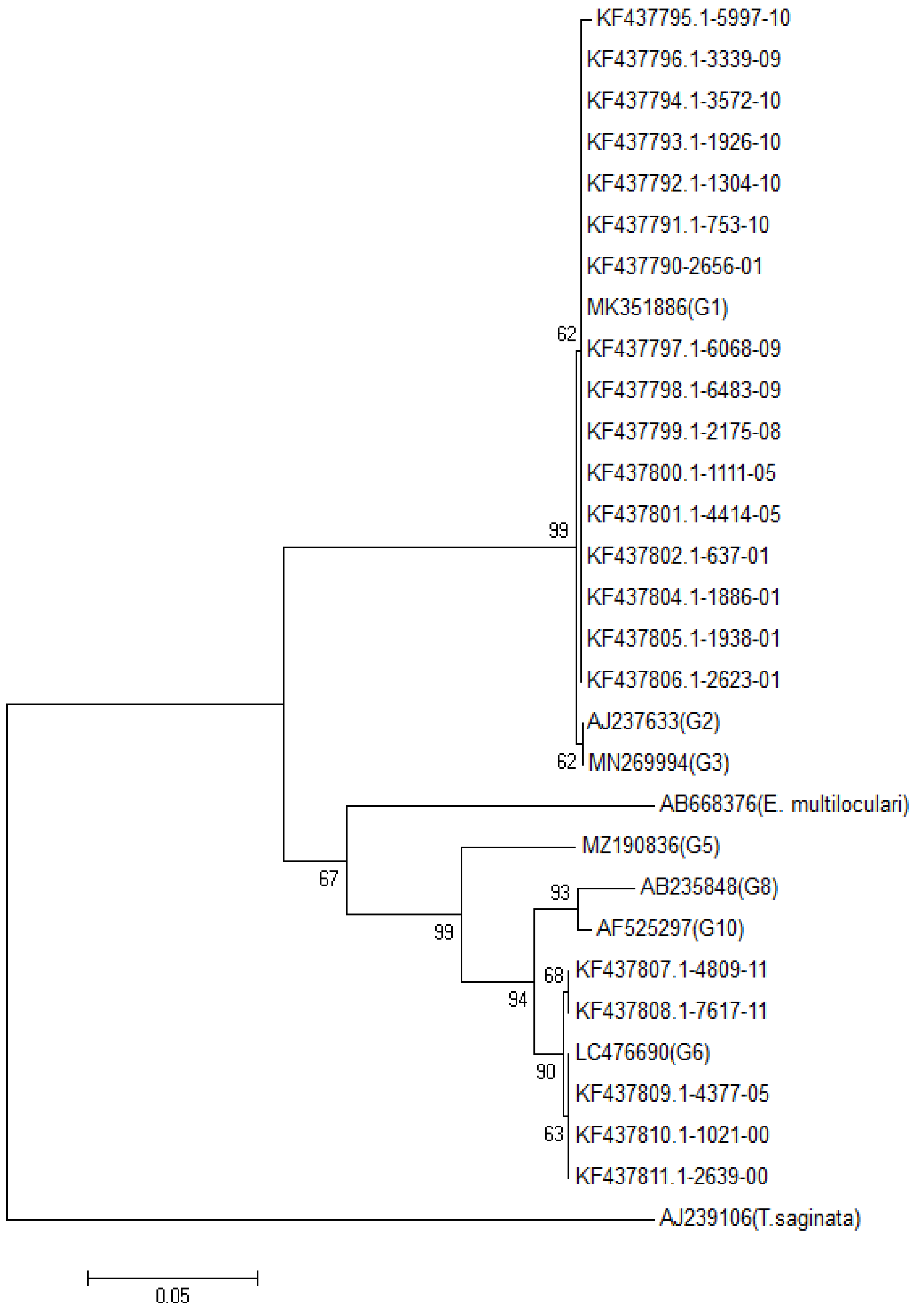

Fig. 3: Genetic relationships of 21 *E. granulosus* isolates and selected GenBank sequence samples based on phylogenetic analysis for partial *nad1* sequence

Out of 21 sequenced samples, 14 cases were categorized as liver cysts, followed by lung (6 cases) and epigastric cysts (1 case). The mean diameter of the sequenced cysts are shown in Table 2. No statistically significant association was found between age and sex of patients with cyst size and cyst location.

Table 2: Distribution of different genotypes of 21 sequenced CE samples according to sex and age of host and size of cysts in Shiraz, Iran

Strain	Sex		Mean size of cyst	Mean of age
	Females	Males		
G1-G3	8	8	6.75 cm	31.93
G6	3	2	6.60 cm	40.80
Total	11	10	6.67 cm	36.36

Haplotype analysis

Five haplotypes of *E.granulosus* were identified from 21 *nad1* sequences. Haplotype network of FFPE samples with three genotype G1-G3 (red circle; n=16) and G6 (purple circle; n=5) was shown in Fig. 4. This network was employed to demonstrate the phylogenetic network, the size of the circles indicates, the number of sequences in each haplotype, and each color represents a specific genotype. The G1-G3 with three haplotypes Hap_1 (n=6; KF437790.1, KF437792.1, KF437802.1, KF437804.1, KF437805.1, and KF437806.1), Hap_2 (n=9; KF437791.1, KF437793.1, KF437794.1, KF437796.1, KF437797.1, KF437798.1, KF437799.1, KF437800.1, and KF437801.1), and Hap_3 (n=1; KF437795) is presented in Fig. 4. Two haplotypes were identified for the G6 genotype including Hap_5 (n=2; KF437807.1 and KF437808.1) and Hap_6 (n=3; KF437809.1, KF437810.1, and KF437811). Statistical values associated with haplotype diversity of the 21 analyzed nucleotide sequences are given in Table 3.

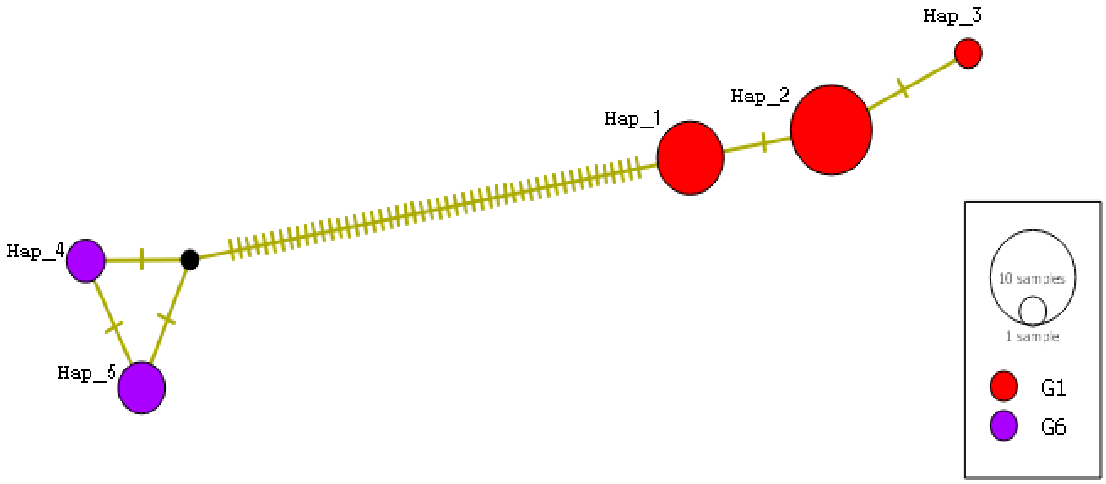

Fig. 4: Haplotype networks of 21 *Echinococcus granulosus* isolates (G1, n = 16, G6, n =5) based on *nad1*. Circle sizes are proportional to the corresponding haplotype frequencies. Hatch marks represent the number of mutations.

Table 3: Diversity and neutrality indices of *Echinococcus granulosus* population calculated from the nucleotide sequence of mitochondrial *nad1* gene

Population indices	
Number of sequences	21
Number of Haplotypes	5
Haplotypes Diversity +SD	Hd: 0.762+ 0.063
Nucleotide Diversity +SD	Pi: 0.05215+0.01364
Tajima's D	1.23904
Fu's Fs statistic	12.706

Discussion

The present study investigated the genotype specifications of CE cysts in paraffin-embedded tissues using PCR and sequencing, demographic characteristics ofCE cysts and histopathologic alterations of different genotypes cysts. The status of the disease is different in different georaphical areas even in genotypes of the parasite which can be interpreted as risk factors which itself can change the epidemiology of the disease in the endemic areas (29).

In the current study, 135 paraffin blocks were positive for CE by PCR. Many studies have been done to extract DNA from FFPE tissues (30, 31). Due to several factors including application of formaldehyde in the processing of FFPE samples, DNA extraction is difficult and DNA is not extracted from all samples as has been shown earlier(20–23,30,31). In accordance with these findings, a comprehensive molecular study demonstrated that only 29 samples out of 70 FFPE tissues could be successfully characterized in Turkey (31). In a survey, out of 50 formalin-fixed paraffin-embedded tissue, only 18 samples had a band using the *cox 1* gene (19). In the present study, the *E. granulosus s.s.* genotype was found to be the most prevalent, attributing to a prevalence of 76.19% (16 cases). The characterization of humans and animals CE isolates from different geographical areas of Iran revealed that *E. granulosus s.s.* (G1–G3) was widely distributed throughout the country, among which G1 was found to be the most frequent genotype. The dog–sheep cycle of CE is widely distributed in Iran, where sheep, cattle, goats, and camels are intermediate hosts (9,22,32,33). Evaluation of 19 isolates obtained from patients referred to Baqiyatallah Hospital in Tehran, demonstrated that all cases were *E. granulosus s.s.* (9).

In the present study, G6 was the second prevalent genotype (5 cases: 23.81%). The G6 has been demonstrated as the second source of human infection in Iran (30,34). The highest rate of human G6 genotype has been reported in eastern parts of Iran (2). Furthermore, the majority of cyst isolates from humans and animals were assigned to be G1-G3 complex (*E. granulosus s.s.*), whereas some cysts from camels were attributed to the G6-G10 complex or *E. canadensis* (35). Mohaghegh et al. assessed 61 formalin-fixed paraffin-embedded tissue samples using the mitochondrial *cox1* gene and real-time PCR and the high-resolution melting curve (HRM), where the HRM analysis showed that out of 40 *E. granulosus* human isolates, 35 (87.5%), 4 (10%), and 1 (2.5%) of the isolates were classified as G1, G3, and G6 genotypes, respectively (36). G6 has a higher affinity for the human brain than G1 (8). Shirmen et al. have shown the *E. canadensis*, G6 genotype in the brain in Mongolia (37). In addition to the brain, the G6 genotype has been reported from other organs such as the lung and the liver (38,39). The G2 genotype has been documented in human and dog isolates from north Khorasan, Kerman, and

Lorestan provinces (22,40,41), while this genotype was not found in evaluated specimens of our study. The G3 genotype was also confirmed in buffalo, cattle, sheep, and camels in Iran (34).

There is some evidence about the cyst size-genotype relationship. A study has reported a CE size of 10 cm for the G1 genotype in the liver, whereas the size of the G6 genotype was recorded 18 cm in this organ (42). This issue is in agreement with our results, in which the sizes of G6 cysts were bigger than G1-G3 ones. The study showed that G1-G3 and G6 genotypes are more common genotype in the southern part of Iran.

This study did not show much difference in the host pathology of the CE genotypes. However, it could be interesting to work on this subject with a larger sample to find the probable differences.

Our findings for haplotypes are in line with findings of other studies in Iran and a number of other countries including China, Pakistan, and Jordan (33,39,43,44,45). One of the limitations of this study was keeping the samples in preservatives, including formaldehyde and paraffin which can reduce the quality of DNA samples.

Conclusion

E. granulosus s.s. (G1-G3) and *E. canadensis* (G6 genotype) are prevalent among CE patients. In general, five haplotypes were identified by *nad1* genes analysis. The histopathological differences between the two genotypes have not been so big to be differentiated by microscopic observations. Genotyping studies and the use of histopathology may lead to a histopathological classification of genotypes in the future.

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Conflict of interests

The authors declare that they have no competing interests.

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