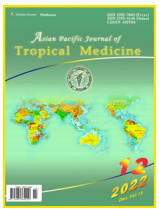

Original Article

Asian Pacific Journal of Tropical Medicine

apjtm.org

doi: 10.4103/1995-7645.364002

Impact Factor: 3.041

Clinical and molecular features of human cystic echinococcosis in Tehran, Iran, 2011-2019

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ABSTRACT

Objective: To evaluate the clinical and molecular features of human cystic echinococcosis in Tehran, Iran.

Methods: In this cross-sectional study, all human cystic echinococcosis patients admitted to Tehran's hospitals from 2011 to 2019 were enrolled, and demographic characteristics, clinical findings, and laboratory data were collected. Formalin-fixed, paraffin-embedded and fresh tissue samples of 175 cystic echinococcosis patients were evaluated for molecular characterization. The samples' isolated DNA was used to amplify cytochrome c oxidase Ⅰ and NADH dehydrogenase subunit Ⅰ genes. Also, the morphological features of fresh samples were examined.

Results: In total, 175 patients with a mean age of 45 (9-98) years were admitted to Tehran's hospitals diagnosed with cystic echinococcosis. Moreover, the highest (26.9%) and the lowest (2.9%) prevalence of cystic echinococcosis cases were in the 16-30 and 1-15 years range, respectively. Male/female ratio was 0.96 (49.1% *vs*. 50.9%). The liver was affected in 92 patients (52.6%), and two or more organs were infected in 7 patients (4.0%). The cysts' diameter varied from 1 to 25 cm, and 96.0% of the patients had a single hydatid cyst. All patients underwent radical surgery and the PAIR technique was applied for all cases. No significant difference was observed between the protoscolex hooks of pulmonary and hepatic cysts. G1-G3 had the highest percentage (99.4%) over other identified G6 genotypes (0.6%).

Conclusions: The sheep-dog cycle plays an important role in transmitting the human cystic echinococcosis infection in Tehran.

KEYWORDS: Human cystic echinococcosis; Clinical features; Genotype; Tehran

1. Introduction

Human cystic echinococcosis (HCE), or hydatidosis, is a significant chronic disease worldwide. Its asymptomatic period can last as long as 20-25 years. This disease is endemic in several areas of North Africa, South America, the Middle East, and China. Also, it is a significant medical, veterinary and economic issue[1]. This zoonotic disease is initiated by accidentally ingesting parasite eggs, and its larval stage (metacestode) can infect different organs of the intermediate host[2].

The final and intermediate hosts are canids and herbivores/omnivores, respectively, and human infection happens inadvertently by ingesting the eggs. It is maintained that human beings are dead-end intermediate hosts, infected by unintentional ingestion of contaminated food or through direct close contact with infected final hosts[1]. Hydatid cysts are principally located in tissues of

Significance

Morphological and molecular features of human cystic echinococcosis in Iran revealed no significant difference between the protoscolex hooks of pulmonary and hepatic cysts. Genetic diversity was observed among the isolates studied. Genotype 1 had the highest percentage among the other identified cases. The main route of transmitting the infection to humans is the sheep-dog cycle.

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How to cite this article: Hamedani NA, Pirestani M, Dalimi A. Clinical and molecular features of human cystic echinococcosis in Tehran, Iran, 2011-2019. Asian Pac J Trop Med 2022; 15(12): 558-567.

Article history: Received 18 June 2022 Revision 15 November 2022
Accepted 18 December 2022 Available online 30 December 2022

the lungs (20%-30%), liver (50%-70%), and other organs, like the brain, spleen, kidneys, muscles, peritoneal cavity, heart, and bones[2].

As the result of close relationship between a large part of the community with animals, contact with the infection sources, and traditional animal husbandry, Iran is an endemic area for cystic echinococcosis in the Middle East[3]. Up to now, ten genotypes (G1-G10) of *Echinococcus* (*E.*) *granulosus* have been found, including sheep strains (G1 and G2), bovine strains (G3 and G5), horse strain (G4), camel strain (G6), pig strain (G7), cervid strains (G8 and G10), and pigs and humans (G9)[4]. These species have several known characteristics: biochemistry, sensitivity to chemotherapeutic agents, significant genetic, morphological differences, parasite life-cycle patterns, and pathogenicity. These differences can be used in drug production, vaccine development and management, and controlling hydatid disease[5].

Studies that mainly use mitochondrial DNA (mtDNA) sequences indicated G1 (89.2%), G6 (8.2%), and G3 (2.3%) in humans as the most frequent *E. granulosus* genotypes in Iran[6]. On the other hand, the meta-analysis study showed a 4.2% (95% *CI* 3.0%-5.5%) HCE prevalence. This prevalence was most frequently reported among females, rural areas, and older patients, with the highest percentage belonging to the southern and southwest[7]. It was estimated that HCE in Iran costs US$93.39 million (for each human case, US$1 539 cost of surgery). Furthermore, 1% of all surgeries in Iran are related to HCE[3,8].

E. granulosus genotype is identifiable in laboratories through physiology, morphology, molecular genetics, and biochemistry techniques. Nevertheless, when used together (morphological and molecular approaches), these methods can improve diagnostic sensitivity and declare the extent of variation and nature of *E. granulosus*[9].

The present study aims to identify genotypes of 175 HCE surgery samples in Tehran, the capital of Iran, determined by morphological and molecular techniques.

2. Subjects and methods

2.1. Ethics approval

The ethical approval for the study was obtained from the ethics committee of the Faculty of Medical Sciences, Tarbiat Modares University, Iran (Ref. No. IR.MODARES.REC.1397.181).

2.2. Participants

Altogether, 175 samples [135 formalin-fixed paraffin-embedded blocks (FFPE) tissue and 40 fresh tissues & hydatid fluid] were collected from Tehran hospitals from 2011 to 2019. In this study, all information related to patients was collected, including sex, age, organ involved in cysts, radiology images, and CT scans. All the cysts were classified as cystic lesion, CE1 (active stage), CE2 (active stage), CE3A, B (transitional stage), CE4 or CE5 (inactive/degenerative stage) using the classification instructions currently recommended by the World Health Organization[10].

2.3. Pathological study

All tissues were routinely fixed with neutral formalin 10%, embedded in paraffin, and then 5 μm thick paraffin sections were obtained from samples[11]. These sections were stained with hematoxylin and eosin (H&E) and were used to confirm cystic echinococcosis.

2.4. Morphological analysis

Hydatid fluids were centrifuged for 10 min at 4000×*g* to isolate protoscoleces. Then protoscolices were mixed in polyvinyl-lactophenol and were measured based on small and large hooks. The end-to-end hook and blade lengths were measured, and the ratio of the blade to total length was calculated (blade length/total length×100)[12]. Measurements were made using an Olympus BX50 microscope with a 100× objective and an Optimas image analyzer.

2.5. Molecular analysis

2.5.1. Genomic DNA extraction

DNA was extracted from FFPE (2×100 μm) and fresh (5 mm) samples. After deparaffinization with xylene for 3 min at 60 ℃ (three times), the samples were rehydrated in a descending EtOH gradient. The supernatants were carefully discarded, and the pellets were incubated at room temperature until drying. Next, 200 μL lysis buffer (Tris- HCl 50 mM; EDTA 0.25 mM; NaCl 400 mM; PH: 8) was added and incubated for 30 min at 80 ℃. Then, 700 μL lysis buffer [supplemented with 20 μL proteinase k (20 mg/mL) and 200 μL SDS 10%] was added and incubated overnight at 60 ℃. DNA was isolated by the phenol-chloroform and precipitated by ethanol[24]. Finally, DNA was stored at -20 ℃ for molecular study.

2.5.2. Polymerase chain reaction

For genotyping, the partial length of the *cox1* and *nad1* genes

were amplified by the nested PCR method. A list of primers is shown in Table 1. The temperature profile of the first step nested-PCR *cox1* (using Jb3 and Jb4.5 primers) was: pre-denaturation for 5 min at 94 ℃, denaturation for 30 s at 94 ℃, annealing for 45 s at 52 ℃, extension for 35 s at 72 ℃, and repeat for 40 cycles, and finally, extension for 10 min at 72 ℃. For the second step, the nested-PCR *cox1* (using COX1F and COX1R primers) temperature profile was: pre-denaturation for 5min at 94 ℃, denaturation for 30 s at 94 ℃, annealing for 45 s at 49 ℃, extension for 35 s at 72 ℃, and repeat for 40 cycles, and finally, extension for 10 min at 72 ℃. Two sets of primers were used for *nad1* amplification. For robust amplification of large fragments, the PCR condition was changed by increasing the concentration of primers (40 pm), dNTP concentration (0.25 mM each dNTP), and PCR extension time (90 s)[13]. The first and second PCR programs were run similarly to the first PCR of the *cox1* gene except for the annealing temperature (53 ℃) and extension time (90 s).

Table 1. The list of primers used in this study.

Gene	Primer sequence	Ampliqon size (bp)	Ref
Cox1	First PCR	444	[14]
	Jb3-5′: TTTTTTGGGCATCCTGAGGTTTAT		
	Jb4.5: TAAAGAAAGAACATAATGAAAATG		
	Nested PCR	366	This study
	COX1F: CCYGGRTTTGGTRTWATT		
	COX1R: ATCRTGYAAAAYATTATC		
Nad1	First PCR	1 239	[15]
	ND1F1: TGGAACTCAGTTTGAGCTTTACTA		
	ND1R1: ATATCAAAGTAACCTGCTATGCAG		
	Nested PCR	1 076	[15]
	ND1F2: TATTAAAAATATTGAGTTTGCRTC		
	ND1R2: TCTTGAAGTTAACAGCATCACGAT		

2.5.3. Restriction fragment length polymorphism analysis

According to instructions for genotyping, restriction fragment length polymorphism (RFLP) was performed with Alu1 (Thermo Scientific, USA) for an amplified fragment of the *cox1* gene (159 positive samples). Digested products were electrophoresed on 3% agarose gel. Markedly, the RFLP patterns were visualized with the DNA gel stain (SYBR Safe, Thermo Scientific, USA) utilizing UV transillumination.

2.5.4. Sequencing, phylogenetic, and haplotyping analysis

To purify the amplicons, a GeneAll Gel Extraction Kit (Korea) was used. A 3130XL ABI (16 capillaries) system was utilized in both directions to sequence the amplicons automatically. Contigs from all samples were edited by Sequencher software (ver. 5.4.5 build 46069)[16]. BLAST algorithms and databases were used by the National Centre for Biotechnology to perform sequence analysis (www.ncbi.nlm.nih.gov). The phylogenetic tree for *nad1* and *cox1* sequences was built with the maximum likelihood algorithm. For this purpose, we used Molecular Evolutionary Genetics Analysis software (MEGA v7.0) (www.megasoftware.net), including sequences representing all genotypes of *E. granulosus* from the GenBank, with Taenia saginata as an outgroup. For this purpose, the maximum likelihood method was used with evolutionary distances obtained by a bootstrap value of 500 and the Hasegawa-Kishino-Yano model[17]. Diversity indices of nucleotide and haplotype were estimated by DnaSP v.6[18]. Median-joining network[19] was inferred based on the sequences of mitochondrial *cox1*, *nad1*, and *cox1–nad1* genes using PopART software (http://popart.otago.ac.nz). Population neutrality indices Tajima's D[20] and Fu's Fs[21], were estimated by DnaSP v.6[21].

Table 2. Patient demographic data, clinical and histological features of cyst.

Variable	Liver (*n*=94)	Lung (*n*=46)	Spleen (*n*=6)	Brain (*n*=1)	Spinal cord (*n*=1)	Heart (*n*=1)	Skin (*n*=1)	Kidney (*n*=3)	Pancreas (*n*=1)	Omentum (*n*=21)	Total (*n*=175)
Sex											
Male	48	19	2	1	1	1	0	2	1	11	86
Female	46	27	4	0	0	0	1	1	0	10	89
Age, years											
1-15	0	4	0	1	0	0	0	0	0	0	5
16-30	25	15	1	0	0	1	1	0	0	4	47
31-45	21	11	4	0	0	0	0	0	0	4	40
46-60	32	6	0	0	0	0	0	1	1	6	46
61-75	11	9	1	0	1	0	0	1	0	5	28
>75	5	1	0	0	0	0	0	1	0	2	9
Cyst dimensions, cm											
1-5	28	14	0	0	1	0	1	0	0	12	56
6-10	48	27	3	1	0	1	0	2	1	6	89
10-15	14	4	3	0	0	0	0	1	0	3	25
16-20	2	1	0	0	0	0	0	0	0	0	3
21-25	2	0	0	0	0	0	0	0	0	0	2
Wall thickness, cm, mean±SD	1.4±0.3	2.3±0.7	1.0±0.2	0.3	0.2	0.5	0.2	1.1±0.5	1.3	1.7±0.6	

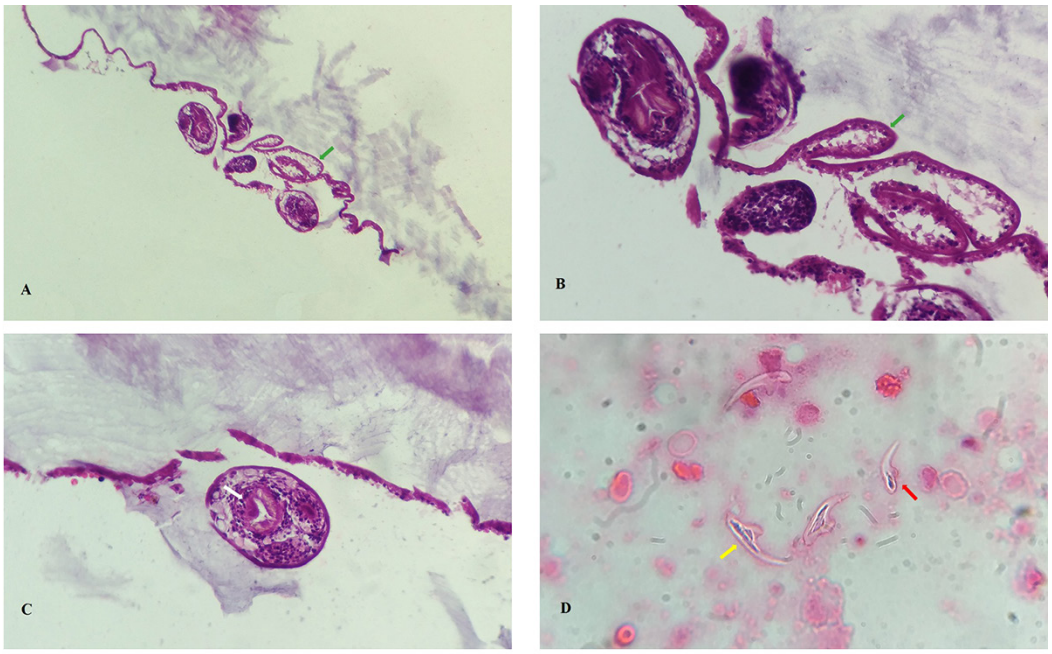

Figure 1. Formalin-fixed, paraffin-embedded *Echinococcus granulosus* cyst stained with H&E. A-C: cross-section of hydatid cyst, the cyst wall, and protoscoleces 100-400× magnification. Arrows indicate the hooklets inside the protoscoleces, D: hooks of protoscoleces 1 000× magnification. Green arrows: germinal layer; white arrow: hooklets inside the protoscolices; yellow arrow: large hook and red arrow: small hook.

2.6. Statistical analysis

Morphometric data were analyzed using SPSS's software package version 22 (SPSS Inc., Chicago, Illinois, USA). Statistical analysis was performed using the student's *t*-test, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Demographic and clinical characteristics

Of 175 hydatid cysts infected patients, 89 were female (50.6%), and 86 were male (49.1%), with an age range of 9 to 98 years (9-98 years for males and 12-81 years for females). Sixty-five percent of these patients lived in urban and rural areas, and the rest were in the city. Organs involved with hydatid cysts include the liver with 52.6%, the lung with 27.4%, and organs such as the brain, spinal cord, heart, and skin with the least involvement (0.6%, respectively) (Table 2). HCEs were classified into cystic lesion (skin and spinal cases) (1.1%), active stage (CE1, CE2) (41.7%), transitional stage (CE3A, B) (42.3%), and inactive/degenerative stage (CE4, CE5) (14.9%).

3.2. Pathological study

The structure of hydatid cysts was investigated in all tissue sections. This composite included the outer acellular laminated membrane, the germinal membrane, and protoscoleces. Markedly, pathology reports have confirmed HCE in all samples. The outer acellular laminated membrane was seen in all sections and the germinal membrane in 159 (90.9%) out of 175 cases. H&E staining revealed that 96 out of 159 cysts contained protoscoleces and were considered fertile. (Figure 1A-1C). The lung cysts had the thickest cyst wall, while the skin and spinal cysts had the least thick one (Table 2).

3.3. Morphological study

Fresh hydatid cysts specimens (40) were used to measure the hooks for morphological analysis. According to the measurement result, no significant difference was revealed between the dimensions of substantial hook blade length and hook length, small hook blade length, and hook length (Figure 1D, Supplementary Table 1). On the other hand, no significant relationship was found

Table 3. Comparison of means of total size containing large hook length, large hook blade length, and small hook length of protoscolices isolated from hepatic and lung hydatid cyst.

Organ	LHL	LBL	LBL/LHL (%)	*P* value	SHL	SBL	SHL/SBL (%)	*P* value
Liver	23.44±1.03	12.18±0.72	51.96	>0.05	18.23±0.66	8.73±0.92	47.88	>0.05
Lung	22.67±1.17	11.66±1.00	51.43		16.85±0.84	8.41±0.61	49.91	

LHL: Large hook length; LBL: Large hook blade length; SHL: Small hook length; SBL: Small hook blade length.

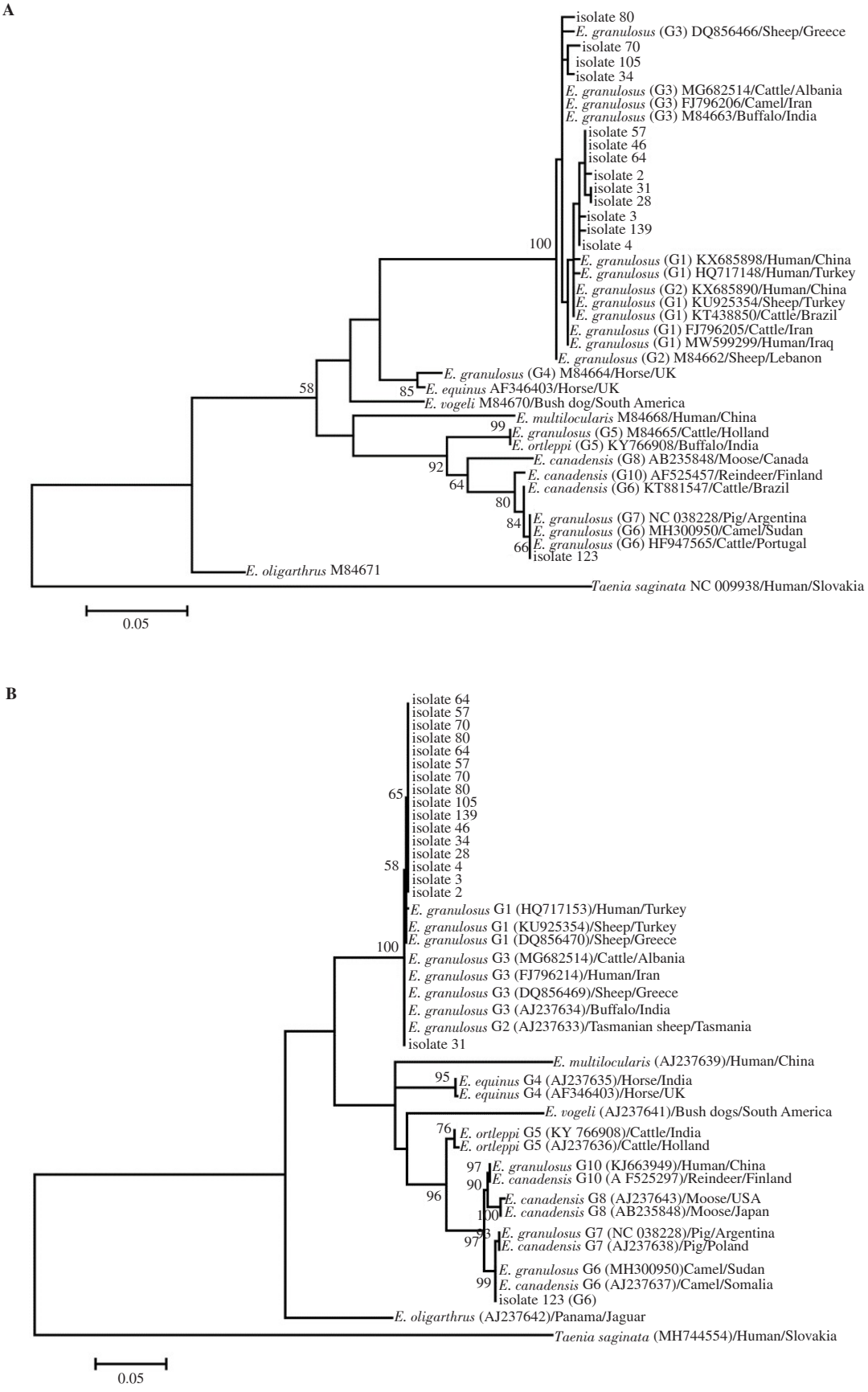

Figure 2. Phylogenetic tree of *Echinococcus* spp. based on sequences of the (A) cytochrome c oxidase Ⅰ gene (*cox1*) and (B) NADH dehydrogenase subunit Ⅰ gene (*nad1*). Bootstrap values obtained from 500 replicates are indicated on branches in percentage, and only bootstrap values >50% are displayed.

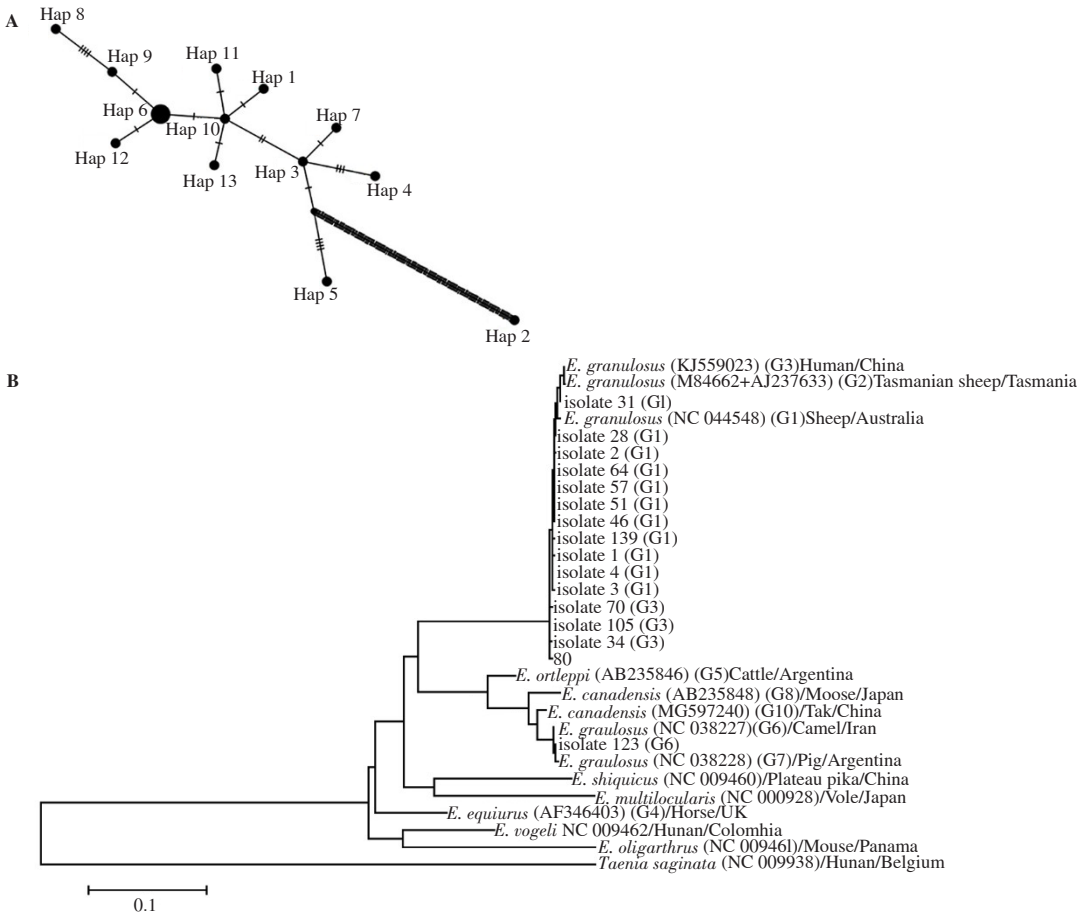

Figure 3. Median-joining network of *Echinococcus granulosus* G1/G3/G6 haplotypes based on the *cox1–nad1* (1 442 bp) mitochondrial genes (A); Phylogenetic tree of *Echinococcus* spp. based on sequences of concatenated cytochrome c oxidase Ⅰ (*cox1*) and the NADH dehydrogenase subunit Ⅰ (*nad1*) (B). Bootstrap values obtained from 500 replicates are indicated on branches in percentage, and only bootstrap values >50% are displayed.

between the mean total size and hook blade of protoscolices isolated from the liver and lung (P>0.05) (Table 3).

3.4. Molecular study

The *cox1* and *nad1* genes were amplified in 159 (90.9%) out of 175 samples (40 fresh samples and 119 FFPE samples). The length of amplicons was ~360 bp and ~1 100 bp for *cox1* and *nad1*. According to the RFLP results, 99.4% of samples (158) were G1-G3, and one sample was G6 genotype (0.6%). The results of RFLP were confirmed by sequence analysis. Of the 16 isolates, 12 belonged to G1, 3 belonged to G3, and 1 sequence belonged to G6.

3.4.1. Sequencing, phylogenetic, and haplotyping analysis

All sequences were submitted in GenBank with accession numbers of MZ572943-MZ572958 for *nad1* and MZ927656-MZ927671 for *cox1*. The multiple alignments of *cox1* sequences of all G1 isolates were grouped into seven patterns according to the single nucleotide polymorphisms of isolates compared with reference sequences for the G1 genotype (HM636641, KT438850, FJ796205, HQ717148, and DQ856467) (Supplementary Table 2). Four isolates were classified into four patterns showing more than three substitutions relative to the G3 sequence DQ856466 and M84663. One sample showed 99% identity to the G6 sequences of MH300950 and HF947565. The alignment of *nad1* sequences of G1 isolates showed two patterns, of which all isolates showed more than six nucleotide substitutions compared to G1 reference sequences (Supplementary Table 3). Fifteen isolates had 99% identity with published G1 sequence MG672290. Four samples showed 99% identity to G3 genotype sequences MG682522/KY766902 and KJ559023, and all cases showed five nucleotide substitutions. One specimen showed 100% identity with the G6 reference (MH300950).

Phylogenetic analysis of *cox1* showed that all G1 and G3 genotypes form a cluster with the reference sequences. Phylogenetic tree of *cox1* supported the alignment of 12 haplotypes clustered as G1-G3 complex with 100% bootstrap value; one isolate clustered in the G6-G10 complex (Figure 2A). In the phylogenetic tree of *nad1*, two patterns clustered with the G1-G3 complex and one isolate with the G6-G10 complex (Figure 2B). The phylogenetic tree of concatenated sequences revealed that all G1 and G3 isolates are located in the G1-

G3 complex. This analysis showed 11 haplotypes clustered with sequences representing genotypes G1-G3 and one with G6-G10 (Figure 3).

The diversity and neutrality index of *cox1*, *nad1*, and *cox1–nad1* sequences are presented in Table 4. Overall, the *cox1–nad1* haplotype diversity (Hd) and nucleotide diversity (π) were 0.95 and 0.02, respectively, while Tajima's D (-2.39) and Fu's Fs (-0.09) were negative and insignificant for the entire population.

Table 4. Diversity and neutrality indices for *Echinococcus granulosus* (G1, G3, and G6) populations in Tehran.

Indices	*cox1* (366 bp)	*nad1* (1076 bp)	*cox1–nad1* (1 442 bp)
No. of isolates	16	16	16
No. of segregating sites	44	123	168
Parsimony informative sites	7	2	9
No. of haplotypes	12	4	13
Haplotype diversity (Hd)	0.94	0.44	0.95
Nucleotide diversity (π)	0.16	0.02	0.06
Tajima's D (*P*-value)	-1.96	-2.40	-2.39
Fu's Fs	-2.70	20.46	-0.09

4. Discussion

Currently, there is valuable evidence available on the significance of specifying *Echinococcus* strains due to genotypic diversity. It is especially crucial in local areas, like Iran, where one of the above intermediate host species is present. The design and implementation of control programs for developing an explicit knowledge of the disease epidemiology and control depend on this information. In particular, which transmission cycles are hazardous to human health[22].

This study investigated aspects of cystic echinococcosis in 175 patients undergoing surgery in Tehran hospitals. Of all cysts examined surgically in the present study, 41.7% were univesicular (CE1 and CE2), 42.3% contained daughter cysts (CE3A, B), and 14.9 % were degenerative (CE4 and CE5). In the study conducted on HCE in Chile[23] and Tunisia[24], 48.5% and 30% were univesicular, respectively. The daughter and degenerative cysts contained 33.1% and 18.4% of cases in Chile, 38.3%, and 31.7% in Tunisia, respectively. This study found most hydatid cysts in the liver (52.6%) and lungs (27.4%). In studies of HCE in Kyrgyzstan[25] and Tunisia[24], HCE appeared more common in females than males, probably reflecting behavioral differences between the genders. In general, women have the highest chance of contact with sources of infection, such as dogs, soil, and vegetable[26]. The present study showed no significant difference in cystic echinococcosis in males and females. Demographic information showed that 65% of the patients lived in Tehran's rural areas, where there are traditional livestock breeding centers and agricultural farms. Eighteen percent owned dogs, all women were housewives, and 51% of men were farmers. The presence of stray and herd dogs in these areas and patients' professions are probably risk factors for cystic echinococcosis transmission.

Researchers have used different criteria for describing *Echinococcus* isolates, such as total numbers, length, and the blade length of rostellar hooks[27]. Evidently, in several cases, applying morphology to distinguish among the *Echinococcus* forms is of practical value[28]. Since the larval hook is transferred to the adult stage and is finally found in the definitive host, it is plausible to use larval hook characteristics to identify the source of infection and help identify the transmission patterns of different strains[12]. This study measures morphological criteria, including large hook blade length, large hook length, small hook blade length, and small hook length. According to hook morphology, samples were of the 'sheep' strain. In addition, no significant differences were observed between isolates. The hooks' morphology results were similar to other studies conducted in Iran[29], Jordan[30], Spain[31], and Tunisia[24], except for the SHL length, which was smaller in the Tunisian isolates.

However, due to the morphological diversity problems caused by the host[12] and also the absence of morphological differences, for ease of diagnosis among the *E. granulosus* genotypes and strains[32], molecular methods and DNA analysis are required. Based on molecular and morphology criteria, two independent strains of *E. granulosus* are available in Iran (camel and sheep)[9].

Genotypic variation in 159 isolates of *E. granulosus* from Iran was evaluated utilizing DNA characterization. The occurrence of two 'camel' and 'sheep' genotypes of *E. granulosus* was indicated in the present study. Thus, previous findings on a limited number of isolates gathered from different regions of Iran are confirmed[33].

In the continuation of the planning process among FFPE cysts and fresh samples of 175 CE patients' cysts, 90.9% of samples had amplified due to high-quality DNA. For genotyping, we conducted RFLP with Alu1 for an amplified fragment of the *cox1* gene (159 positive samples) and sequenced for *nad1* and *cox1*. Possibly formalin and calcification of hydatid cysts in the non-amplified isolates elevated DNA degradation. A noteworthy point worth mentioning in this study was the amplification of the *nad1* gene in all isolates. Markedly, it refers to the primers used in the present research. This point is contrary to the findings by Moradi et al., who were only able to amplify this gene in 85% of isolates[34].

The sequencing and phylogenetic analysis detected three genotypes of *E. granulosus* (G1, G3, and G6) as causing factors of HCE in Iran treated surgically. The predominant one is the G1 genotype, while G3 and G6 are in the following ranks. In phylogenetic tree of *cox1*, high genetic diversity of sequences makes the G1 isolates

subclustered by the reference. It is worth mentioning that there was a low difference between the isolates of this study and the reference sequences. Unlike *cox1*, phylogenetic analysis of *nad1* showed that most isolates are placed in the G1-G3 complex at a close distance from the reference. This phenomenon indicates low genetic diversity between isolates.

The most globally prevalent genotype is the G1 genotype, probably because of the extensive range of intermediate hosts, facilitating more circulation in the environment[35]. It can be the reason for the considerable genetic diversity observed in the G1 genotype[35], supported by findings of the current work in seven haplotypes. The most prevalent genotype in our samples was G1, followed by G3, consistent with previous research on human CE in Iranian[36] and other regions worldwide. In neighboring countries of Iran, Turkey[37], and Iraq[38], the sheep strain (G1) and the less critical G3 strain have significant roles in the transmission cycle of CE. At the same time, in its eastern neighbor, Pakistan, the G3 genotype plays a prominent role in the development of CE[39]. Global subpopulations (haplotypes) of G1 are pointing to regurgitated expansion via animal trade. The Middle East is the main center of domestication and the possible origin of the *E. granulosus* G1-G3 complex. It is believed that the parasite has been distributed to other parts of the world from this area. Domestication history, complex trade routes, and livestock movements largely determine geographic phylogeny patterns. It is also a determinant factor for genetic subpopulations.

The G1 has a high (>90%) share of CE infections in Central/Eastern Asia, Africa, and South America. The insignificant percentage of CE caused by G3 is meaningful in Africa, South America, and Central/Eastern Asia. Meanwhile, CE, with the origin of G3, is slowly increasing in countries such as Turkey, Iran, and Italy. This data shows with moving from the Middle East to Europe and South America, the prevalence of G3 is slowly increasing, and it has become the dominant genotype in South Asia; meanwhile, in East Asia, the G1 genotype is still dominant. According to Kinkar et al., the G3 genotype is distributed from Iran to Italy and India via domestic animal trade, and the G1 genotype[35] was spread from Turkey to other regions.

Genotype 6 was detected in only one HCE in this study. Our findings agree with this argument: despite the rank of G6 as the second most prevalent factor causing HCE following the *E. granulosus* sensu stricto (G1-G3 complex) in the world, human health is trivially influenced due to its low incidence in *E. granulosus* endemic regions[35]. Nevertheless, it is the primary factor for the incidence of HCE in some areas where *E. granulosus* sensu stricto rarely causes animal infection. According to previous works, Genotype 6 shows a higher prevalence than G1 in some regions of Birjand[40] and Kerman[41] in eastern and south-eastern Iran, where camel-rearing is commonplace.

In the present study, high haplotype diversity and low nucleotide diversity values indicate minor differences between haplotypes. The haplotype network also shows that most haplotypes have single nucleotide differences. Combining high haplotype and low nucleotide diversity are signs of rapid population expansion from a small effective population to a larger population. Tajima's D and Fu's Fs tests were used in this study to detect population expansion. The negative values of the Tajima's D test indicate a bias towards an excess number of rare alleles and a sign of recent population expansion. On the other hand, the negative values of the Fu's Fs test indicate extensive haplotypes, an indication of recent population expansion or genetic hitchhiking[42].

Another research limitation was identifying *E. granulosus* genotypes based on partial *nad1* mitochondrial genes by sequences of inadequate length for separation of the G1-G3 complex[35]. We used *nad1* primers in our work for amplifying a larger fragment. Thus, *Echinococcus* genotypes could be easily differentiated. Short mitochondrial sequences as the optimum alternative for amplifying low quantity DNA, reasonably low-quality DNA in formalin-exposed FFPE tissues, and extensive utilization for phylogenetic investigation and genotyping of *E. granulosus* present a foundation for comparison of our results.

In conclusion, it was concluded that after examining the morphological criteria (LHL, LBL, SHL and SBL), no significant difference was observed between the isolates. 90.85% of FFPE samples in our study were amplified using specific primers. The amplified fragments were used for genotyping and haplotyping of *E. granulosus*. Further, we conducted genotyping and RFLP with Alu1, which expresses the predominance of genotype G1-G3, especially genotype G1. The genotype G1 predominance could prove that the sheep-dog cycle is the primary route for transmission of human infection. According to this study and based on the predominance of sheep (G1) genotype *E. granulosus* in Iran, control programs should target the dog-sheep cycle. There is also a clear need for research into the development of diagnostics and prevention programs.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

The authors received no extramural funding for the study.

Acknowledgements

This study is result of M.Sc. thesis that has been supported by Vice-Chancellor for Research of Tarbiat Modares University.

Authors' contributions

M.P. conceptualized the study; N.A. and M.P. carried out the investigation and developed the methodology and M.P. were responsible for project administration; N.A. and A.D. provided the resources; supervision was done by M.P.; M.P. and A.D. validated the data and A.D. and M.P. were responsible for data visualization; roles/writing original draft were by N.A. and M.P.; writing-review and editing were carried out by M.P. and A.D.

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