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Genetic Characterization of *Echinococcus granulosus* from a Large Number of Formalin-Fixed, Paraffin-Embedded Tissue Samples of Human Isolates in Iran

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Abstract. Cystic echinococcosis (CE), caused by the larval stage of *Echinococcus granulosus*, presents an important medical and veterinary problem globally, including that in Iran. Different genotypes of *E. granulosus* have been reported from human isolates worldwide. This study identifies the genotype of the parasite responsible for human hydatidosis in three provinces of Iran using formalin-fixed paraffin-embedded tissue samples. In this study, 200 formalin-fixed paraffin-embedded tissue samples from human CE cases were collected from Alborz, Tehran, and Kerman provinces. Polymerase chain reaction amplification and sequencing of the partial mitochondrial cytochrome *c* oxidase subunit 1 gene were performed for genetic characterization of the samples. Phylogenetic analysis of the isolates from this study and reference sequences of different genotypes was done using a maximum likelihood method. In total, 54.4%, 0.8%, 1%, and 40.8% of the samples were identified as the G1, G2, G3, and G6 genotypes, respectively. The findings of the current study confirm the G1 genotype (sheep strain) to be the most prevalent genotype involved in human CE cases in Iran and indicates the high prevalence of the G6 genotype with a high infectivity for humans. Furthermore, this study illustrates the first documented human CE case in Iran infected with the G2 genotype.

INTRODUCTION

Cystic echinococcosis (CE) or hydatidosis, caused by the larval stage (metacestode) of the tapeworm *Echinococcus granulosus* (Cestoda: Taeniidae) has a global distribution and is one of the most important zoonotic diseases in the world.[1,2] The adult worm infects the small intestine of a wild or domestic Canidae as the definitive host. Human and livestock become infected after ingestion of food contaminated by parasite eggs that after ingestion harbor the hydatid cysts in the liver, lungs, and other internal organs as the intermediate host.

In fact, with a few rare exceptions, human is an aberrant host, because the parasite life cycle cannot be completed.[3] Clinical signs of the condition are generally manifested as pressure on surrounding tissues as a result of pressures exerted by this space-occupying lesion. Cyst rupturing and spillage of the contents may create anaphylactic shock and secondary CE.

Hydatidosis is endemic in some parts of China, Middle East, North Africa, and South America.[4] Iran is an important endemic region of CE where there are various species of the intermediate host for *E. granulosus*.[5] Several studies have reported that hydatid cysts are routinely found in sheep, camels, cattle, and goats in a wide distribution across Iran.[6–9] Adult worms have been recovered from dogs, wolves, and jackals in different geographical areas.[7,10–14] Human CE cases are also routinely documented in medical centers in different parts of Iran, and the rate of human infection is 0.61–2/1,000,000 people in various regions.[7,15] Serological studies on humans showed seroprevalence of CE within 1.2–21.4% of the population in different parts of the country.[7] A recent study reported that the total annual cost of CE in Iran is US$232.25 million, with the cost of the disease conjectured to be about 0.03% of the country's gross domestic product.[16]

There is a high level of genetic variation within *E. granulosus*. During recent decades, based on mitochondrial and nuclear genetic markers, a number of variants have been described within the *E. granulosus* species.[17] These strains/genotypes vary in host range, pathogenicity, maturation patterns of the parasite, epidemiology and sensitivity to chemotherapeutic agents, and prevention and control strategies of hydatid disease.[18] To date, 10 genotypes (G1–G10) have been identified for *E. granulosus*. These genotypes consist of two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camel strain (G6), two pig strains (G7 and G9), and two cervid strains (G8 and G10).[17,19,20] However, some of these distinct strains were originally defined many years ago as separate species or subspecies. Consequently, a taxonomic reappraisal relying mainly on mitochondrial data has proposed that *E. granulosus* species splits to four valid species including: 1) *E. granulosus* sensu stricto (G1–G3 complex), 2) *E. equines* (G4), 3) *E. ortleppi* (G5), and 4) *E. canadensis* (G6–G10).[17,21,22] Moreover, *E. felidis* (lion strain) is closely related to *E. granulosus* sensu stricto and is placed within the *E. granulosus* complex.[23] Recently, based on more complex data containing nuclear sequences and the epidemiological aspects, it was recommended that genotypes G6–G10 should be broken into two distinct species including *E. canadensis* (G8 and G10 genotypes) and *E. intermedius* (G6/G7 genotypes).[24] The validity of the G9 genotype has been controversial.[24,25] All genotypes except G4 and G10 have been reported to infect humans. Most human CE cases in the world have been found to be infected with the G1 genotype of *E. granulosus*.[1,26]

Several molecular epidemiological studies have been performed on *E. granulosus* isolates in Iran using sequence data of mitochondrial and nuclear genes. Overall, four different

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TABLE 1
Iran reports on *Echinococcus granulosus* genotypes causing human cystic echinococcosis

Geographical origin	Total isolates	Method	*E. granulosus* genotype	References
North	4	CO1 & ND1 sequencing	G1	27
Different provinces	33	ITS1-RFLP	G1 (30 cases), G6 (3 cases)	28
Isfahan (Central)	23	CO1 & ND1 SSCP and sequencing	G1	30
Isfahan (Central)	30	ITS1-RFLP	G1	38
Isfahan (Central)	31	ITS1-RFLP CO1 & ND1 sequencing	G1 (25 cases), G6 (6 cases)	40
Kerman (South east)	1	CO1 & ND1 sequencing	G6	29
Golestan (North)	30	ITS1-RFLP	G1	42
Khuzestan (South west)	5	ITS1-RFLP	G1	41
Ardabil (North west)	9	CO1 & ND1 sequencing	G1 (7 cases), G3 (2 cases)	44
Ilam (West)	4	ITS1-RFLP	G1	36
Tehran (capital), Alborz, Kerman (South east)	125	CO1 sequencing	G1 (68 cases), G2 (1 case), G3 (5 cases), G6 (51 cases)	Present study

genotypes of *E. granulosus* (genotype G1, G3, and G6) have been reported from different livestock[27–36] and dogs (genotype G1, G2, and G3)[37] from Iran. To date, only a few human isolates of *E. granulosus* have been genetically characterized in Iran that indicated G1, G3, and G6 genotypes (Table 1). In each endemic area, the molecular identification of the occurring genotypes in human CE has significant impacts on control strategies. Therefore, the current study was conducted to determine *E. granulosus* genotypes of the causative agents of CE using a high number of human isolates from Iran. The study used partial sequencing of the mitochondrial cytochrome *c* oxidase subunit I (CO1) gene using formalin-fixed paraffin-embedded (FFPE) tissues as a DNA source.

MATERIALS AND METHODS

Collection of samples. Two hundred FFPE specimens were collected from the archives of various pathology departments of three provinces in Iran between 2001 and 2011 (Figure 1). Hospitals chosen in Tehran were central referral hospitals where patients from other parts of the country with hydatidosis were referred for treatment. All specimens had been confirmed histologically by a pathologist as hydatid cysts (observation of laminated layers and/or protoscoleces and/or hooklets) and were transferred to the Laboratory of the School of Medicine, Kerman University of Medical Sciences.

DNA extraction. Tweezers, microtome blades, and other equipment that had direct contact with the FFPE were sterilized. Sterilization of equipment occurred between processing of each new FFPE block, and gloves and the razor blade were changed.

Using a scalpel, excess paraffin was trimmed, and then serial sections of 15-μm thickness were obtained from FFPE blocks using microtome. Because the sample surface was exposed to air, the first sections cut from FFPE blocks were discarded.

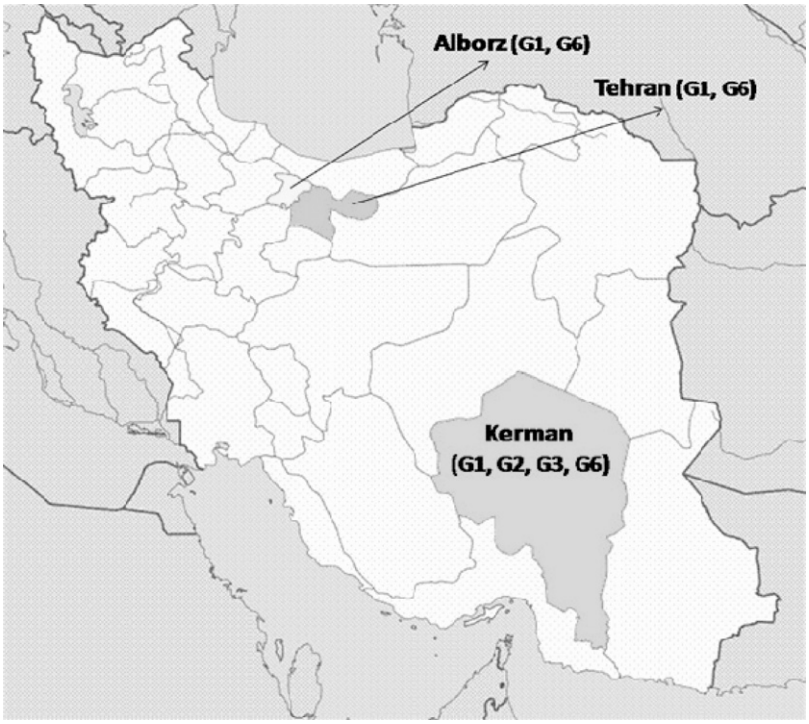

FIGURE 1. Map of Iran displaying geographical origin of human cystic echinococcosis samples and distribution of four different genotypes of *Echinococcus granulosus* in this study.

A total of 7–8 sections from each FFPE block were transferred to a sterile 1.5 mL microcentrifuge tube, after which 1,000 μL of xylene was added for 10 minutes to deparaffinize the samples. The tubes were capped and vortexed vigorously for 10 s. Centrifuging at full speed for 2 min at room temperature allowed the supernatant to be removed. This procedure was repeated once. After deparaffinization, rehydration in 100%, 90%, 80%, and 70% ethanol followed. Thereafter, the 70% ethanol was removed, and tissue lysis solution was added (QIAamp DNA FFPE Tissue Kit, Hilden, North Rhine-Westphalia, Germany). The genomic DNA was extracted using the "DNA Mini Kit" from QIAGEN, Hilden, North Rhine-Westphalia, Germany. The QIAamp DNA FFPE Tissue Kit is optimized for purification of DNA from FFPE tissue sections. The extraction procedure was performed according to the manufacturer's instructions. The obtained gDNA samples were stored at –20°C until further use.

Mitochondrial polymerase chain reaction. The DNA was used for the polymerase chain reaction (PCR) amplification of the CO1 gene. A 400-bp fragment of the CO1 gene was amplified by PCR using forward JB3 (5′-TTTTTTGGGCATCCT GAGGTTTAT-3′) and reverse JB4.5 (5′-TAAAGAAAGA ACATAATGAAAATG-3′) primers.[45]

Polymerase chain reactions (50 μL) were performed using 3.5 mM MgCl2, 250 mM of each of the dNTPs, 25 pmol of each primer, 2 U Taq polymerase, and 4 μL (50–100 ng/mL) of the DNA template, under the following thermal profile: 5 min at 94°C as an initial denaturation step, followed by 35 cycles of 30 s at 94°C, 45 s at 50°C, 35 s at 72°C, and a final extension step of 10 min at 72°C. The amplicons were electrophoresed on 1% (w/v) agarose gel containing ethidium bromide.

DNA sequencing and phylogenetic analysis. All amplicons were sequenced by an ABI-3730XL capillary machine (Macrogen Inc., Seoul, Gyeonggi-do, South Korea). Nucleotide sequence analysis was undertaken by the basic local alignment search tool (BLAST). Sequence data were analyzed using BLAST databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov), whereas alignments were conducted using the software packages ClustalX and BioEdit. The CO1 nucleotide sequences of representative isolates were submitted to the National Center for Biotechnology Information GenBank. Phylogenetic trees and pairwise calculations were obtained by using the Molecular Evolutionary Genetics Analysis (Mega5) software package (Figure 2). The differences among all of the different sequence types of CO1 were obtained using pairwise comparisons. The dendrogram was drawn by using the sequences obtained in this study and reference sequences available for the *E. granulosus* sensu stricto (G1, G2, and G3 genotypes) and *E. granulosus canadensis* (G6 and G7 genotypes) in GenBank. *Taenia saginata* (accession no. NC009938) was applied in the model as the outgroup.

The evolutionary history was inferred employing the maximum likelihood (ML) method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 70 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All

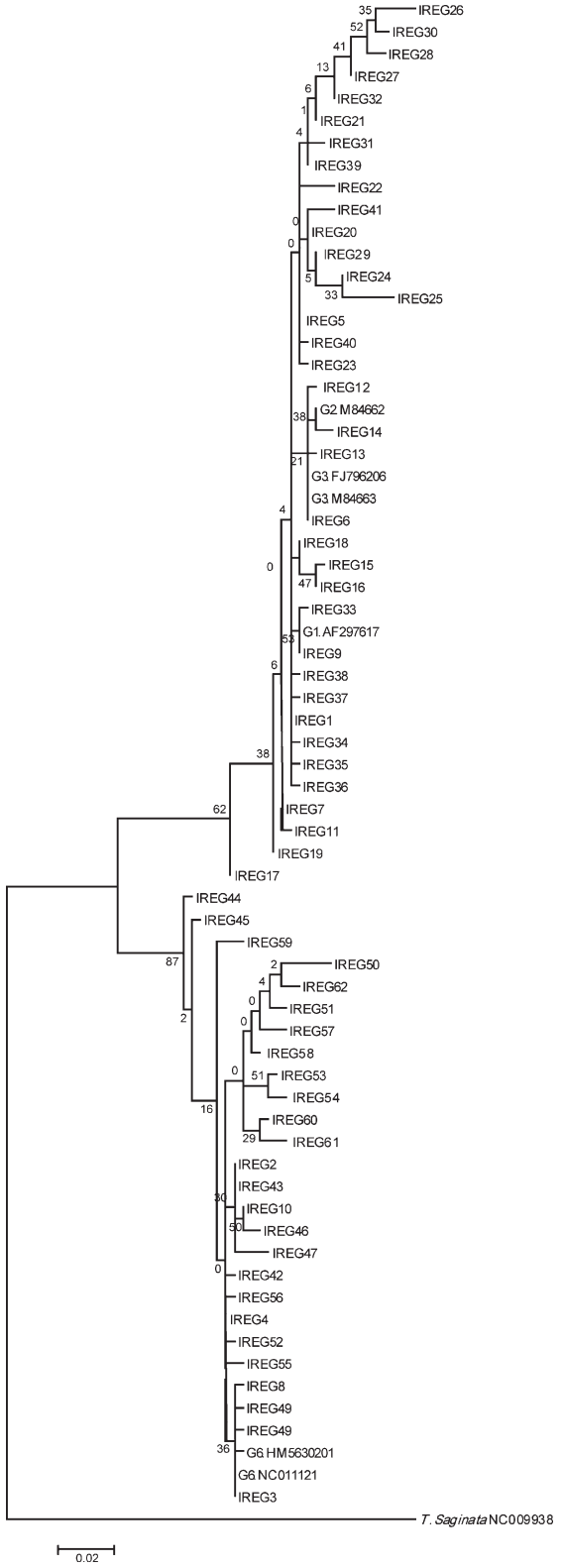

FIGURE 2. Genetic relationships of *Echinococcus granulosus* isolates from human cystic echinococcosis (CE) in three provinces of Iran and reference sequences for *E. granulosus* G1, G2, G3, G6, and G7 genotypes. *Taenia saginata* was applied as the out group. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model in MEGA5 software. The tree with the highest log likelihood (–1773.5798) is shown. The percentage of trees in which the associated taxa clustered is indicated next to the branches.

TABLE 2

Frequency distribution of *Echinococcus granulosus* genotypes in FFPE tissues from 125 human CE identified by partial CO1 sequence analysis, in three provinces of Iran

Province (total isolates)	G1 no. (%)	G2 no. (%)	G3 no. (%)	G6 no. (%)
Kerman (48)	20 (41.7)	1 (2.1)	5 (10.4)	22 (45.8)
Tehran (70)	42 (60)	–	–	28 (40)
Alborz (7)	6 (85.7)	–	–	1 (14.3)
Total (125)	68 (54.4)	1 (0.8)	5 (4)	51 (40.8)

FFPE = formalin-fixed paraffin-embedded; CE = cystic echinococcosis; CO1 = cytochrome *c* oxidase subunit I.

positions containing gaps and missing data were eliminated. There were a total of 336 positions in the final data set.

RESULTS

The PCR amplification was successfully performed on 182 of the isolates. No amplification was observed in the negative controls of any PCR sets. The DNA sequencing was successfully done on 125 of 182 amplicons for the CO1 gene. Overall, 56%, 40%, 3.2%, and 0.8% of isolates indicated the G1, G6, G3, and G2 genotypes, respectively. The frequency of genotypes in each province is indicated in Table 2. In total, 62 representative profiles were differentiated and designated as haplotypes IREG1 to IREG62 for CO1 (Table 3). A total of 36 and 26 haplotypes belonged to *E. granulosus* sensu stricto and *E. granulosus canadensis* (G6 genotype), respectively (Table 3). The sequences from CO1 (336 bp) of *E. granulosus* larvae were identified and submitted to GenBank under accession nos. KF443137 to KF443198. The frequency distribution of each haplotype among 125 *E. granulosus* isolates and relevant accession nos. are shown in Table 3. A total of 61 segregation sites were observed within 62 haplotypes obtained from 125 isolates in this study. Upon pairwise comparison, the differences among all haplotypes of CO1 ranged from 0.00% to 12.8%. Overall, the level of nucleotide diversity in *E. granulosus* sensu lato was 18.32%.

Phylogenetic analyses of CO1 data for haplotypes 1–62, including representative sequence data for G1, G2, G3, G6, and G7 genotypes of *E. granulosus* and *T. saginata* (as an outgroup) (see Table 3) were conducted using ML. A consensus tree constructed using ML is shown in Figure 2.

DISCUSSION

In this study, four genotypes of *E. granulosus* including G1, G2, G3, and G6 were inferred to exist in three provinces in Iran (Figure 1). This information was derived from the study of 125 FFPE tissue samples using mitochondrial sequencing of partial CO1.

The FFPE tissue samples are a precious source of retrospective studies all over the world. However, DNA extraction from FFPE tissue samples is not as simple as would be from fresh or alcohol preserved materials, because formalin has inhibitory effects on PCR reactions. Although some commercial specialized kits for extracting DNA from FFPE tissues are available, many isolates have not yielded valid results when using the PCR protocols. Therefore, researchers operating in the countries where hydatid cysts are endemic prefer to use a fresh protoscoleces/germinal layer of human hydatid cyst rather than the FFPE or alcohol preserved isolates. Thus, there are limited studies of *E. granulosus* using FFPE tissues as the DNA source.

A new PCR protocol was introduced by Schneider and other[46] for the characterization of *E. granulosus* complex in FFPE tissues. They found the G7 genotype in 92% and 33% of Austrian and Yugoslavian patients, respectively, whereas the G1 genotype was found in all 20 of the Turkish patients investigated. In a comprehensive molecular survey of occurrence of *E. granulosus* in FFPE tissue samples in Turkey, only 41.6% (29 of 70) of the total blocks could be genotyped.[45] However, in this study about 70% (125 of 180) of FFPE blocks were successfully characterized. The lone previous Iranian study of human CE in FFPE tissue samples investigated 30 samples, but the method used was the restriction enzyme analysis of ITS1 region which cannot precisely differentiate genotypes within *E. granulosus* sensu lato.[42]

The *E. granulosus* G2 genotype has been reported in dogs in the Lorestan Province, western Iran.[37] No previous study has reported an incident of this genotype occurring in the intermediate host in Iran. However, in the current study, the G2 genotype occurred in one human CE isolate from Kerman Province (south-eastern Iran). Therefore, this is the first identification of this genotype in a human host in Iran.

The E. granulosus G3 genotype has been isolated from humans in various countries including Italy, Romania, Turkey, India, Tunisia, and Brazil.[48–55] For the first time in Iran, Sharbatkhori and others[30] reported the occurrence of the G3 genotype in camels from the Isfahan Province (central Iran). This was a global first for the identification of the G3 genotype in this host. Later, this genotype was reported by other researchers to be hosted in buffalo, sheep, cattle, and camels from different parts of the country.[29,35] In a recent study in

TABLE 3

The frequency distribution of 62 haplotypes among 125 *Echinococcus granulosus* isolates from human CE in Iran and relevant genotypes and accession numbers

CO1 haplotypes	Total isolates	Genotype	Accession no.	CO1 haplotypes	Total isolates	Genotype	Accession no.
IREG1	32	G1	KF443137	IREG12	1	G3	KF443148
IREG2	14	G6	KF443138	IREG13	1	G3	KF443149
IREG3	8	G6	KF443139	IREG14	1	G2	KF443150
IREG4	4	G6	KF443140	IREG15	1	G1	KF443151
IREG5	4	G1	KF443141	IREG16	1	G1	KF443152
IREG6	3	G3	KF443142	IREG17	1	G1	KF443153
IREG7	2	G1	KF443143	IREG18	1	G1	KF443154
IREG8	2	G6	KF443144	IREG19	1	G1	KF443155
IREG9	2	G1	KF443145	IREG20	1	G1	KF443156
IREG10	2	G6	KF443146	IREG21–IREG41	21	G1	KF443157–KF443177
IREG11	1	G1	KF443147	IREG42–IREG62	21	G6	KF443178–KF443198

CE = cystic echinococcosis; CO1 = cytochrome *c* oxidase subunit I.

TABLE 4

World reports on *Echinococcus granulosus* G6 genotype (camel strain) in human

Country	G6 genotype/ total isolates	Frequency of G6 genotype (%)	Reference
Peru	1/20	5	57
Peru	1/5	20	39
Chile	1/20	5	43
Argentina	4/9	44.4	62
Argentina	21/66	31.8	63
Argentina	15/41	36.6	64
Argentina	8/26	30.7	65
Muritania	2/2	100	66
Egypt	30/31	96.8	67
Sudan	5/5	100	68
Kenya	1/178	0.5	69
Kenya (Turkana)	10/59	16.9	70
Turkey	2/29	6.9	54
Russia (Altai region)	2/8	25	71
Mongolia	16/50	32	72
China (Xinjiang)	2/47	4.2	73
Nepal	2/2	100	74
India	1/32	3.1	48
Iran	3/33	9.1	28
Iran	6/31	19.3	40
Sudan	5/5	100	75
Iran	51/125	40.8	Present study

north-western Iran, 22.2% of human isolates (2 of 9 cases) belonged to the G3 genotype, whereas the rest were of the G1 genotype.[44] Similarly, in the current study, four human CE isolates originally from Kerman Province belonged to the G3 genotype.

In a recent study on 32 CE patients from North India, the G3 genotype of *E. granulosus* was the most common (53.1%) followed by the G1 (40.62%), G5 (3.1%), and G6 (3.1%) genotypes.[48]

However, in the current study and many other global studies, G1 was the most common *E. granulosus* genotype (54.4%), followed by the G6 (40.8%), G3 (4%), and G2 (0.8%) genotypes. Previous studies have indicated the presence of the G6 genotype in different hosts such as sheep, goats, cattle, camels, and humans in Iran.[28,29,32,40,56] However, the high prevalence of the G6 genotype in this study is not in accordance with previous human CE studies in Iran (Table 1), because most of these studies indicated the G1 genotype as the only genotype found in humans. However, the sample sizes used within some of the previously mentioned studies were very low. On the other hand, the only human CE isolate in the previous study conducted in Kerman Province was of the G6 genotype, confirming as with this study that there is a higher prevalence of the G6 (45.8%) compared with the G1 (41.7%) genotype in this province.[29] Globally, many studies identified the G1 genotype as the most common or the only genotype causing human CE, whereas the G6 genotype has indicated no or low infectivity to humans.[55,57–61] However, the G1 and G6 genotypes of *E. granulosus* genotypes are most commonly associated with human infection worldwide and in Iran. Table 4 summarizes the identification of the G6 genotype in humans across the world. As inferred from this table, the most human reports of the G6 genotype were from some African countries such as Mauritania, Egypt, and Sudan. In Egypt, the G6 genotype has been associated with high infectivity.[67] In South America, a high prevalence of the G6 genotype has been found in Argentina where goats are considered as the reservoir of the camel strain in the region.

The G7 genotype (swine strain) has been isolated from humans in different countries such as Austria, Yugoslavia, Poland, Slovakia, Romania, Ukraine, and Turkey.[44,47,76–79] In a recent study from Poland, all of the 30 human CE isolates identified belonged to the G7 genotype, implying that this genotype has considerable infectivity for humans.[80] However, lack of reporting on the G7 genotype in Iran is not surprising because the consuming of pork is forbidden for Muslims, and there is no pig breeding in Iran.

Comparison of molecular data with geographical origins in this study have indicated that 62.3% and 36.7% of total FFEP tissues from the Tehran and Alborz (was integrated in Tehran province before July 2010) provinces indicated the G1 and G6 genotypes. Sharbatkhori and others[30] found that all 34 sheep isolates investigated from Tehran Province indicated the G1 genotype, in concordance with the findings of the highest prevalence of G1 genotype in humans in this study.

The G2 and G3 genotypes were only found in the Kerman Province. The identification of the G3 genotype in Kerman confirms results of a previous study that reported the G3 genotype in sheep, cattle, and camel hosts in this province.[29] On the other hand, the mentioned study found the G1, G3, and G6 genotypes in 75.7%, 13.5%, and 10.8% of 58 livestock isolates, respectively. This is not in concordance with our result, with a higher prevalence of the G6 genotype (45.8%) than even the G1 genotype (41.7%) and a low prevalence of the G3 genotype (4%) in human CE. It seems that the camel-dog cycle has a more important role compared with the sheep-dog cycle in the link between *E. granulosus* and human infection in this region.

To the best of our knowledge, this study illustrates the first identification of the *E. granulosus* G2 genotype from human CE patients in Iran. In conclusion, the results of the current study using a remarkably large sample size of FFPE tissues confirmed the presence of G1 and G2 (sheep strain), G3 (buffalo strain), and G6 (camel strain) genotypes of *E. granulosus* in the country, with a higher prevalence of the G6 genotype (40.8%) in human hosts compared with findings of previous studies in the country. The high prevalence of the G6 genotype emphasizes the zoonotic potential of this strain. As the camel strain has a shorter maturation period in the definitive host, the results from this study may have significant implications for the control procedures of human hydatidosis in Iran.

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