Phylogeography and Genetic Diversity of Human Hydatidosis in Bordering the Caspian Sea, Northern Iran by Focusing on *Echinococcus granulosus* Sensu Stricto Complex

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Abstract

Background: Human *Echinococcosisis* a cyclo-zoonotic infection caused by tapeworms of the *Echinococcus granulosus* sensu stricto complex. The detection of mitochondrial genome data of genus *Echinococcus* can reflect the taxonomic status, genetic diversity, and population structure genetics.

Methods: Totally, 52 formalin-fixed paraffin-embedded (FFPE) tissue samples from patients with histologically confirmed CE were collected from Mazandaran province, Iran in the period of Mar 1995 to May 2018. All extracted DNAs from (FFPE) tissue samples were subjected to amplify by polymerase chain reactions method targeting cytochrome c oxidase subunit 1 (*cox1*) gene. All PCR amplicons were sequenced to phylogenetic analysis and genetic diversity.

Results: Molecular analysis showed that 50(96.1%) and 2 (3.84%) isolates were identified as G1 andG3 *E. granulosus* genotypes, respectively. DNA sequence analyses indicated a high gene diversity for G1 (Haplotype diversity: 0.830) and G3 genotypes (Hd: 1.00). Based on multiple sequence alignment analyses, 7 (13.46%; G1 genotype) and 2 (3.84%; G3 genotype) new haplotypes were unequivocally identified.

Conclusion: G3 genotype (Buffalo strain) was identified from two human hydatidosis isolates in the region. Present study strengthens our knowledge about taxonomic status, transmission patterns of *Echinococcus* parasite to human and heterogeneity aspects of this parasite in clinical CE isolates of Northern Iran.

Keywords: Human hydatidosis; Genetic diversity; Phylogeography; Iran

Introduction

Cystic *Echinococcosis* (CE) caused by metacestode *E. granulosus* genotypes remains a potential neglected problem global for matters relating to zoonotic disease and the economic loss (1,2). About 58% of the total populations of Central Asian countries including Iran, Kazakhstan, Tajikistan, Turkmenistan, Uzbekistan, Afghanistan, Mongolia, Pakistan, and Western China are potentially at risk for this orphan disease (3).

Many factors contribute to the transmission and development of CE in humans and other animals, the most important of which are age, occupation, location (urban or village), parasite genotype, and definitive host (dogs or other carnivores) populations in the region (4-7). Human beings are the incidental intermediate host of the tapeworm *E. granulosus*, the larvae of which are forming anywhere from the human body (8-10).

Based on phylomolecular analyses of *Echinococcus* mitochondrial markers (e.g., *cox1/nad1*), ten strains which include *E. granulosus* sensustricto (s.s.) (genotypes G1–G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5), and *E. canadensis* (genotypes G6/G7, G8, G10) (11,12). *E. granulosus* (G1) is predominantly addressed as widespread distribution in Iran, where the genotypes G1, G2, G3, G5, G6, and G7 have sympatrically been identified from hyperendemic foci of Iran (13-15). Although, there is no community based large screening studies in Iran, based on hospital records the prevalence rate has been estimated to be 1.18-3 per 100,000 populations (16). One of the neglected hyperendemic regions of CE with various ecosystem zones is situated in bordering the Caspian Sea, Mazandaran Province, Northern Iran where there is no comprehensive phylomolecular data on structure of *Echinococcus* parasite in human hydatidosis. About 79 patients of CE surgeries were performed for ten years from 2005 to 2015 in this region. Identification of genetic features and emergent novel haplotypes of *E. granulosus* isolates in human can explicitly reflect new insights of cross-transmission patterns of *Echinococcus* among canids-intermediate hosts, epizootiology, gene migration of parasite, evolutionary patterns, and circulation of probable drug-resistance alleles (17,18).

We aimed to assess phylogeography and inter–intra genetic variation levels of *Echinococcus* spp., from clinical samples of hospitalized patients in bordering the Caspian Sea, Northern Iran based on sequencing of cytochrome c oxidase I (*cox1*).

Materials and Methods

Study area and Sample collection

Mazandaran Province is located on a South-East coast of the Caspian Sea between 36.2262° N, 52.5319° E and covers an area of 23,842 km². The area has a temperate climate with 70%-100% relative humidity. The average annual temperature and rainfall ranges from 10–35 °C and 800-1,200 mm, respectively. It has various ecosystems including grasslands, sea and forests the province consists of 15 counties and has a population of around 2,922,432 inhabitants which living in rural and urban areas (19). Fifty-two (n=52 including 30 females) tissue samples (FFPE; Formalin-Fixed, Paraffin-Embedded) of humans were collected from the archives of different hospitals in Mazandaran Province including Amol, Babol, Chalus, Qaemshahr, Neka, and Sari hospitals in which CE patients had undergone surgery between Mar 1995 to May 2018 (Fig. 1).

DNA extraction and PCR procedure

To extract the genomic DNA of human tissue samples, 10 to12 μm thick sections were obtained from FFPE blocks with microtome blades, were transferred to a sterile tube separately for each sample, for deparaffinized, 1 ml xylene was added to sections for 12 min at 37 °C then centrifuged at 1500 g for 5 min, and the supernatant was discarded. According to the protocol (20) for rehydration, sections were followed in 100%, 90%, 80%, and 70% ethanol, after that, the 70% ethanol was omitted and added lysis buffer.
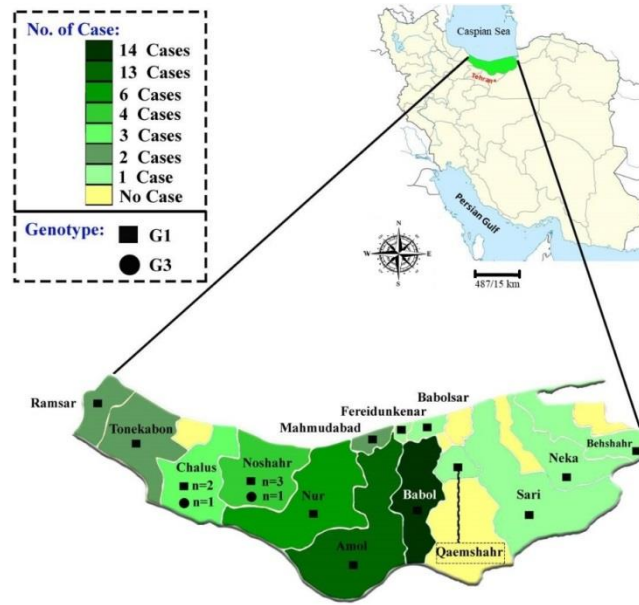
Fig. 1: Map of Iran presenting study counties in bordering the Caspian Sea, Northern Iran

The specimens were digested 24 h, at 56 °C within 400 µL of the lysis buffer with the addition of 40 µL proteinase K (20 mg/ml). Genomic DNA extraction of each sample was done by (WizPrep™ gDNA Mini Kit (Cell/Tissue) according to the manufacturing company (wizbiosolution/ Korea) then the genomic DNA was kept at -20 °C until further use in PCR. The *cox1* gene was amplified with forward and reverse primers JB3-5'-TTTTTT-GGGCATCCTGAGGTTTAT-3', JB4.5-5'-TAAA-GAAAGAACATAATGAAAATG-3', respectively. The PCR reaction was used to amplify the cox1 gene by 35 cycles 94 °C for 5 min as an initial denaturation, 94 °C for 30 sec, 50 °C for 45 sec, 72 °C for 35 sand a final extension at 72 °C for 10 min. The amplification products PCR were run on a 1.5% agarose gel electrophoresis (21).

Mitochondrial DNA sequencing, haplotype network, and phylogenetic tree
All PCR amplicons were sequenced by targeting *cox1* gene. To show the genealogical relationships of *Echinococcus* mutations, a haplotype network was constructed based on Median Joining algorithm (PopART software) (22). According to the analysis of molecular variance (AMOVA), the diversity indices (Nucleotide diversity (Nd; π) and Haplotype (gene) diversity (Hd) and neutrality indices (Fu's Fs statistic and Tajima's D) were assessed by using DnaSP software version 5.10 (23). The divergence and percent identity among the edited sequences of *Echinococcus* genotypes were drawn using the MegAlign program. To reveal taxonomic status of *Echinococcus* strains, a phylogenetic tree was made based on maximum likelihood algorithm with Kimura 2-parameter (MEGA 5.05 software). Multiple alignments among the DNA sequences and amino-acid sequences were performed based on Clustal W method (BioEdit software, ver. 7.0.5).
This research has been approved by the Deputy of research and technology, Mazandaran University of Medical Sciences Ethics Committee with ethical standards a special code: IR.MA-ZUMS.REC.96.2929.

Results

Demographic data, PCR, sequence analyses, and pairwise distance matrix
Overall, 52 human patients infected with hydatid cysts were collected from 11 counties of Mazandaran Province. Demographic data, identified strain, collected county names, and location of cystectomy organs are fully given in Table 1.

Table 1: Demographic data, identified strain, collected county names and location of cyst (Organs) in *Cystic Echinococcosis* patients (n= 52) from Apr 2017 to May 2018 in Mazandaran province, Northern Iran

Variable Age group(years)	Number of patients (%)	Genotype G1 (%)	Genotype G3 (%)
10–20	1 (1.92)	1 (1.92)	-
>20–30	5 (9.62)	5 (9.62)	-
>30–40	12 (23.08)	12 (23.08)	-
>40–50	10 (19.23)	10 (19.23)	-
>50–70	24 (46.15)	22 (42.30)	2 (3.85)
Sex			
Male	22 (42.30)	22 (42.30)	-
Female	30 (57.70)	28 (53.84)	2 (3.85)
Cyst Location			
Liver	31 (59.62)	29 (55.76)	2 (3.85)
Lung	16 (30.77)	16 (30.77)	-
Kidney	2 (3.85)/	2 (3.85)	-
Ovary	1 (1.92)	1 (1.92)	-
Bile duct	1 (1.92)	1 (1.92)	-
Spleen	1 (1.92)	(1.92)	-
Residence			
City	18 (34.62)	17 (32.69)	1 (1.92)
Village	34 (65.38)	33 (63.46)	1 (1.92)
County names			
Amol	13 (25.00)	13 (25.00)	-
Babol	14 (26.92)	14 (26.92)	-
Babolsar	1 (1.92)	1 (1.92)	-
Behshahr	1 (1.92)	1 (1.92)	-
Tonekabon	2 (3.85)	2 (3.85)	-
Chalus	3 (5.77)	2 (3.85)	1 (1.92)
Ramsar	2 (3.85)	2 (3.85)	-
Sari	1 (1.92)	1 (1.92)	-
FereidunKenar	1 (1.92)	1 (1.92)	-
QaemShahr	1 (1.92)	1 (1.92)	-
MahmudAbad	2 (3.85)	2 (3.85)	-
Neka	1 (1.92)	1 (1.92)	-
Nur	6 (11.54)	6 (11.54)	-
Noshahr	4 (7.69)	3 (5.76)	1 (1.92)

The age ranges of the patients were between 20–68 (average 42) yr old. The amplified PCR products of the *Cox1* gene (444bp) were sequenced for 52 isolates. Overall, 50 (96.1%) and 2 (3.84%) isolates were found to correspond to *E. granulosus* G1 and G3 genotypes, respectively. All of the G3 genotype (n=2; 3.85%) were only found at liver hydatid cyst, while the G1 genotype was characterized by hydatid cysts of liver, lungs, kidney, ovary, bile duct, and spleen (Table 1). Based on multiple sequence alignment analyses, 7 (13.46%; G1 genotype) and 2 (3.84%; G3 genotype) new haplotypes were unequivocally identified (Table 2). Distribution of identified haplotypes of *E. granulosus* G1 and G3 genotypes based on *cox1* registered sequences is given in Table 3.

Table 2: Diversity indices of *E.granulosus* G1 and G3 genotypes based on cytochrome c oxidase subunit 1 mitochondrial sequence in Mazandaran Province, Northern Iran

Parasite		Diversity indices N Nh (%) Hd± SD Nd (π) No. of polymorphic sites				Neutrality indices Tajima's D* Fu's Fs statistic**	
E. granulosus sensu stricto complex	*E. granulosus* G1	50	7 (13.46)	0.830± 0.029	0.00470	-0.27403	-0.193
	E. granulosus G3	2	2 (3.84)	1± 0.500	0.01351		
	Total	52	9			*ND	ND

*ND: Not determined. * *P*<0.01. ** *P*<0.02.

N: Number of isolates; **Nh:** Number of haplotypes; **Hd:** Haplotypes (gene) diversity; **Nd:** Nucleotide diversity.

Table 3: Haplotype distribution of *E. granulosus* G1 and G3 genotypes in human FFPE tissue samples based on partial cytochrome c oxidase subunit 1(cox1) mitochondrial sequence in Mazandaran Province, Northern Iran

Haplotype	Genotype	Accession number
MAZ04	G3	MH920521
MAZ09	G3	MH920524
MAZ02	G1	MH920515
MAZ05	G1	MH920518
MAZ07	G1	MH920520
MAZ11	G1	MH920522
MAZ12	G1	MH920525
MAZ13	G1	MH920526
MAZ15	G1	MH920528

No Indel mutations (deletions/insertion) identified in *E. granulosus* G1 and G3 genotypes. Within the 444bp consensus position of G1 and G3 genotypes, 7 and 5 variable (polymorphic) sites were detected, respectively. The pairwise distance matrix between aligned sequences of G3 genotype provided an intra-diversity of 1.4% and percent identity of 98.6% while an intra-divergence of 0.3%-1.6% and high percent identity of 98.6%-99.7% were calculated for G1 genotype (Fig. 2). The inter-divergence level between G1 and G3 genotypes was estimated 1.1% to 2.2% (Fig. 2).
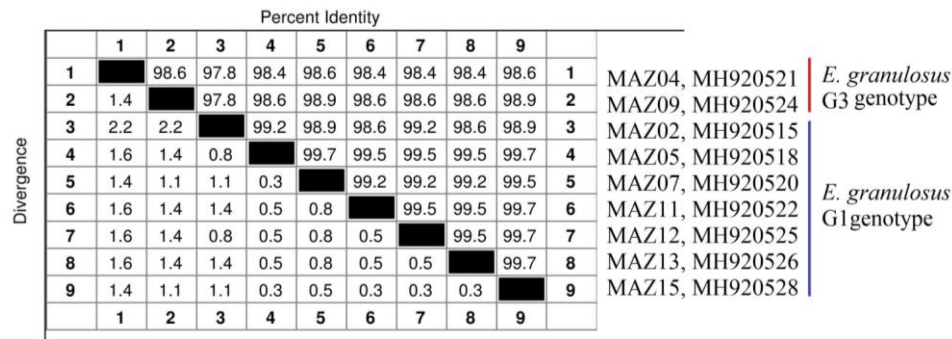

Fig. 2: The sequence pairwise distances including intra and inter-divergence levels and percent identity of *E. granulosus* G1 and G3 genotypes among the analyzed sequences

Diversity indices, neutrality indices, and multiple sequence alignment

The diversity indices, neutrality indices, and Hn of G1 and G3 genotypes are given in Table 2. DNA sequence analyses of *E. granulosus* G1 and G3 genotypes indicated high genetic diversity of *Cox1* (G1: Hd: 0.830 and G3: Hd: 1.00). The negative indices of *E. granulosus* G1 isolates (Tajima's D; -0.27403 and Fu's Fs; -0.193), indicating a substantial divergence from neutrality (Table 2). To illustrate the expansion and the divergence time for unequal population sizes of *Echinococcus,* the indices of Tau: 1.911, Raggedness r (0.0948), and R2 statistic (0.1158) are shown in Fig. 3. The multiple sequence alignments of the nucleotides and amino acids of G1 and G3 genotypes are shown in Fig. 4 and 5. The non-synonymous substitutions of *E. granulosus* G1 occurred at codons 2,3, 85 (MAZ02), 71 (MAZ11), 74 (MAZ13), and 85 (MAZ12), besides two amino acid substitutions were identified at codons 65 and 66 in *E. granulosus* G3 (Fig. 5).
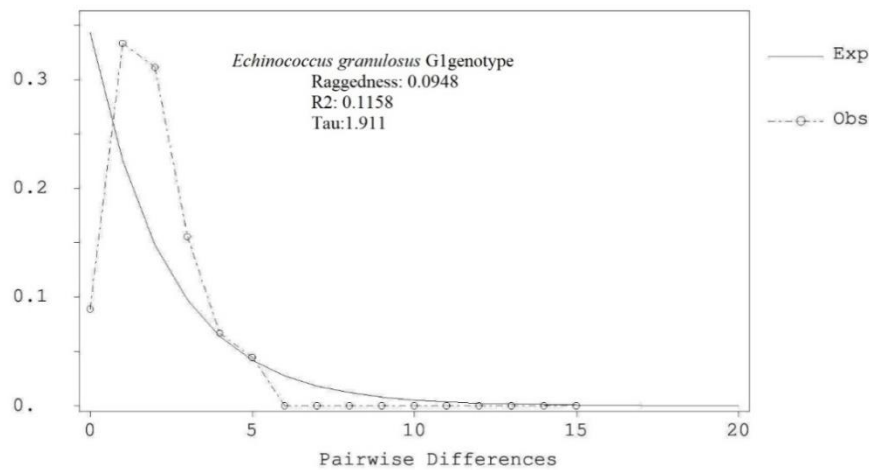

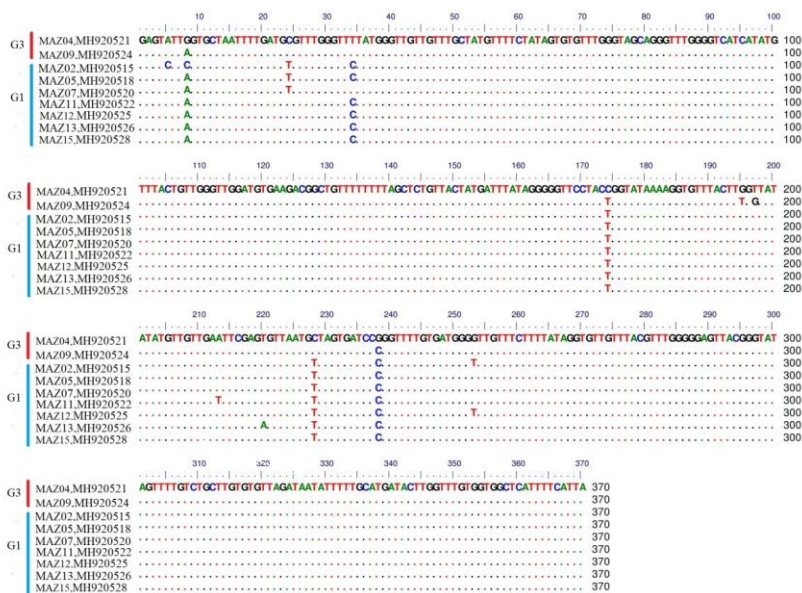**Fig. 3:** Expected and observed mismatch distribution for *E. granulosus* G1 genotype inferred by *cox1* gene

Fig. 4: Nucleotide sequence alignment of *cox1* gene based on identified haplotypes of *E. granulosus* G1 and G3 genotypes

Fig. 5: Amino acid sequence alignment of *cox1* gene based on identified haplotypes of *E. granulosus* G1 and G3 genotypes

Phylogeny tree and haplotype network

To authenticate the taxonomic status of *E. granulosus* G1 and G3 genotypes, a phylogeny tree was generated by analyzed sequences of *Cox1* gene (Fig. 6). The phylogeny tree represented the human hydatid cyst isolates placed in two distinct clades as G1 and G3 genotypes of *E. granulosus*. *Taenia multiceps* was addressed as an out-group (AN: JQ710587). A haplotype network was provided to demonstrate a genealogical relationship among the haplotypes of G1 and G3 genotypes (Fig. 7). The haplotype network indicated star-like characters including MAZ11 and MAZ15 as the dominant haplotypes. The haplotypes MAZ04 and MAZ09 of G3 genotype were placed at a distinct clade compared with G1 haplotypes.
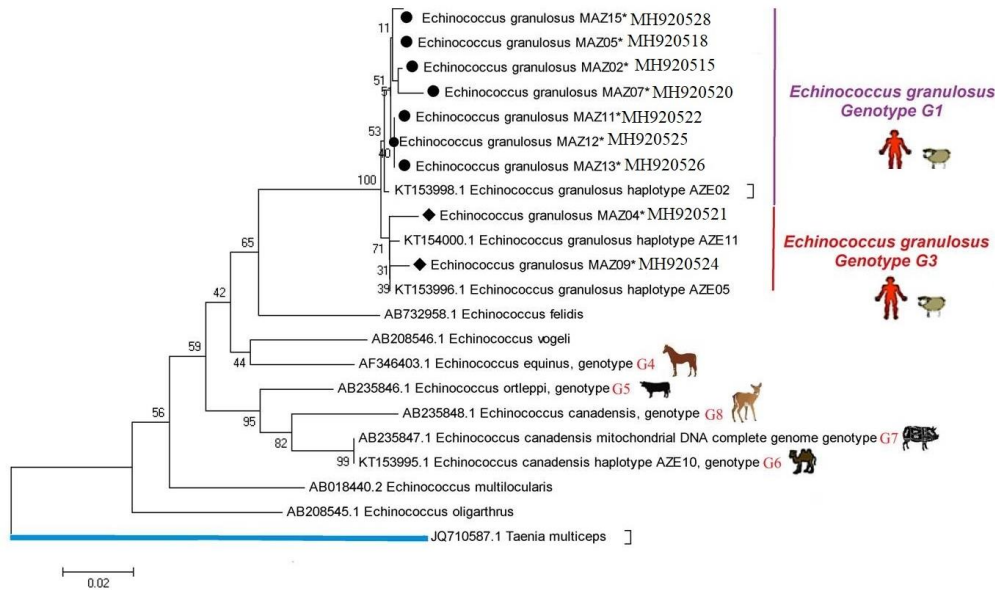

Fig. 6: Phylogenetic analysis of human *E. granulosus* isolates using *cox1* gene based on maximum likelihood algorithm with kimura 2-parameter model. *Taeniamulticeps* (Accession no., JQ710587) was used as outgroup. The sequences relevant to this study marked by asterisk (*)
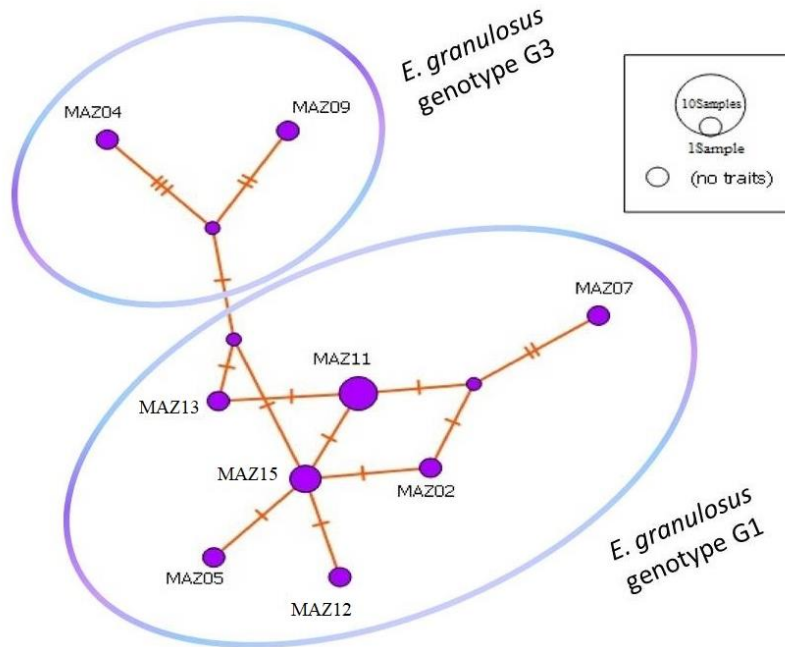
Fig. 7: Haplotype network in *E. granulosus* sensu stricto based on mitochondrial sequences of *cox1* from Northern Iran (constructed by PopART software)

Discussion

Findings of this study showed that the human CE with *E. granulosus* G1 and G3 genotypes containing various genetic traits are explicitly being circulated in bordering the Caspian Sea, Northern Iran. Presently, CE/alveolar echinococcosis as an ignored public health concern threat a wide range of at-risk populations, particularly those who settle in hyperendemic areas. Despite the initiate of preventive strategies by Iran's health policymakers, there is an increasing incidence of human hydatidosis in some areas of Iran (e.g., Tabriz city, 10 cystectomy CE cases/1 month) (24-26). Currently, the G1, G2, G3, and G6 strain have been reported from human hydatidosis isolates in Iran (13). Identification and genotyping of *Echinococcus* spp., in human CE isolates are potentially critical to determine probable drug-resistance codons, genetic diversity traits, and transmission patterns of this parasite (27).

In concurrence with earlier studies in Iran, the G1 genotype was the most dominant strain associated with human hydatidosis. We also detected a considerable haplotype variability of *E. granulosus* G1 (Hd; 0.830) and G3 (Hd; 1.00) genotypes in human clinical isolates obtained from bordering the Caspian Sea, Northern Iran. The occurrence of significant diversity (novel haplotypes) of G1/G3 genotypes indicates that the sheep and buffalo hosts may potentially contribute in maintenance and transmission of probable emergent *Echinococcus* sp., to candies, and will also lead to establishment of resistant alleles to treatment in the region. Furthermore, genetic diversity in *Echinococcus* parasite may frequently take place after high gene flow, in which migration increases the effectual population size in various geographical regions, where the diversity characters are likely dominant (28). In addition, occurring codons 65 and 66 of G3 genotype should be taken into consideration in intensification of *E. granulosus* G3 pathogenicity or development of plausible drug-resistance alleles (21,29). According to the above-mentioned descriptions, our earlier study showed that the gene migration (gene flow) of *cox1* in support of *E. granulosus*s. G1 and G3 strains are unambiguously

shared between Northern-Central, Northern-Southeastern, and Northern-Western pair populations in Iran (30-32). Previous study *E. granulosus* isolates from definitive hosts (Dogs and Golden jackals) in Mazandaran northern Iran, using partial cox1 sequence, 66.7% and 33.3% of isolates belonged to G1 and G3 genotypes, respectively (33). Another study of *E. granulosus* isolates from livestock and humans in Golestan Province, northern Iran indicated G1 (78.3%), G2 (2.7%), G3 (15%) and G6 (4%) genotypes among the 74 CE isolates (34).

The presence of negative neutrality indices (-0.27403 to -0.193) in support of *E. granulosus* G1 genotype implies to population expansion after the bottleneck incident and purifying selection (32,33). The findings of Tau value (1.911) and unimodal mismatch distribution test revealed that the *E. granulosus* G1 genotype have newly experienced a population expansion due to mitochondrial *cox1* gene migration of parasite in bordering the Caspian Sea, Northern Iran.

In present study, the majority of *E. granulosus* G3 genotype was only limited to human liver hydatidosis, on the one hand, a letter from Iran shown that, *E. canadensis* G6 isolates (n=8) has a positive tropism to shape the human cerebral CE (34). Although, in the current study, no cases of Central Nervous System CE were obtained, however more samples are needed to confirm the potential tropism of genotypes of *E. granulosus* sensu latoto organ specificity of humans.

Conclusion

In this study, for the first time, G3 genotype (buffalostrain) was identified from two human hydatidosis isolates in Mazandaran Province which indicating the water buffalo can play in the transmission of *Echinococcus* G3 to canids. Present results strengthen our knowledge concerning taxonomic status, transmission patterns of *Echinococcus* parasite to human and heterogeneity aspects of this parasite in Northern Iran which will also assist in the expansion of strategies for controlling of intermediate hosts and monitor of clinical isolates in the region.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

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