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Genotypic characterization of *Echinococcus granulosus* isolates based on the *mitochondrial cytochrome c oxidase 1 (cox1)* gene in Northwest Iran

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Abstract. Hydatidosis is one of the most important zoonotic parasitic diseases caused by the larval stage of *Echinococcus granulosus* which causes great health and economic losses. The aim of this study was to use the sequencing method to evaluate genotypes of *E. granulosus* isolated from humans and bovines using *mitochondrial cytochrome c oxidase subunit 1* (*cox1*) gene. The samples were taken in the East Azerbaijan Province, Northwest Iran. Overall, 26 hydatid cyst samples (10 human and 16 cattle isolates) were collected. DNA extraction was taken from the protoscoleces of human and germinal layer of bovine samples. PCR was performed using the *mitochondrial cytochrome c oxidase subunit 1(cox1)* gene, and then it was sequenced. Sequences were analyzed for identification of their genotypes. All 16 bovine isolates were recognized as G1 genotypes (sheep strain) and G1B subtypes. Out of ten human host samples, seven isolates were G1B subtypes, and three samples were identified as G3 genotypes. The results of this study showed that G1 and especially G1B are the predominant genotype and subtype in humans and cattle in Northwest Iran.

INTRODUCTION

Hydatidosis is a zoonotic parasitic disease in humans and animals caused by the larval stage of the *Echinococcus granulosus* tapeworm, which continues to be a substantial cause of morbidity and mortality in various parts of the world (Umhang *et al.*, 2013). Dogs and other canines are definitive hosts and numerous herbivores and omnivores, including wildlife and domestic livestock are known as intermediate hosts (Latif *et al.*, 2010). The *E. granulosus* located in the jejunum of dogs and other canines and eggs are excreted in the feces and are then ingested by an intermediate host (cows, sheep, mice, caribou and humans), move to the duodenum and pass through the intestines to the circulatory system. They are then trapped in the liver and other organs developing into hydatid cysts (Sayek *et al.*, 1980). The liver and lungs are the most common organs to be infected (Ghabouli-Mehrabani *et al.*, 2014). The risk of this disease occurs in many regions and cases in the Middle East, Russia, Australia, New Zealand, America and Africa have been reported (Schantz, 1995). The highest prevalence of the hydatid cyst is in rural areas where people breed livestock (Ma *et al.*, 2008). In a study conducted in 13 provinces of Iran, the prevalence of *E. granulosus* in sheepdogs was reported at 27.2% (Parsa *et al.*, 2012). According to studies in Iran, the prevalence of hydatid cysts in sheep and cattle range from 5.1–74.4% and 3.5–38.3% respectively. Human hydatidosis were estimated about 0.6–1.2 cases per 100, 000

individuals in Iran (Rokni, 2009). A high grade of intra-specific variation has been distinguished within *E. granulosus* which demonstrates significant differences in life cycle patterns and host preferences. Considerable information is accessible about the epidemiology of diverse genetic strains of *E. granulosus* around the world (Thompson & Mcmanus, 2002). To date, 10 genotypes (G1-G10) within the species *E. granulosus* have been detected based on *mitochondrial DNA* analysis. This complex is divided into four species: *E. granulosus* sensu stricto (G1, G2 and G3 genotypes), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5) and *Echinococcus canadensis* (G6-G10). The genotypes include the common sheep strains (G1); Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7and G9), and deer strain (G8and G10). So far, *E. granulosus* sensu stricto and *E. canadensis* genotypes have been found in Iran (Youssefi *et al*., 2013). Prior molecular studies in Iran have been performed on the larval stages of *E. granulosus* isolated from humans or different livestock species including sheep, cattle, goats, buffalo and camels, revealing the existence of G1, G3 and G6 genotypes in the country (Mahami-Oskouei *et al*., Sharifiyazdi *et al*., 2011). As a result of the hyperendemicity of hydatidosis in Iran, and the plurality of intermediate hosts that carry the disease, it was deemed necessary to conduct the present study in the East Azerbaijan region. The results from our genotyping of *E. granulosus* in different regions can be used in studies that focus on prevention and control, epidemiology, vaccine design, drug sensitivity, life cycle analysis, transmission and disease progression (Ergin *et al*., 2010).

The aim of this study was to evaluate genotypes of *E. granulosus* isolated by sequencing method from human and bovine hosts using the *mitochondrial cytochrome c oxidase subunit 1 (cox1)* gene in Northwest Iran.

MATERIALS AND METHODS

Samples collection

Twenty- six cases of hydatid cysts (16 cattle and 10 human isolates) were collected. Cattle cysts were obtained from animals of province that were slaughtered in the Tabriz slaughter house. Human samples were obtained from surgical cases of hydatid cysts in the hospital. Each human or animal infected cyst was considered separately.

Samples preparation

In order to complete the microscopic examination of samples for the presence of a germinal layer and protoscoleces, the cyst fluid of all human samples was aspirated by a separate syringe under sterile conditions and then centrifuged. Protoscoleces and germinal capsules were removed and held in a 70% ethanol solution at -20ºC until DNA extraction (Kamenetzky *et al*., 2000).

DNA extraction

Before DNA extraction, the samples were washed with distilled water. To extract DNA, the protoscoleces and germinal layers of human cysts and 25-50 mg of the germinal layer of bovine samples (due to lack of protoscoleces) were used. DNA extraction was performed with a commercial kit *(AccuPrep® Genomic DNA Extraction kit)* according to the manufacturer's instructions.

PCR amplification

Polymerase Chain Reaction (PCR) was performed in a volume of 20 µl that included Taq DNA polymerase (1 U), from each dNTP (dATP, dCTP, dGTP, dTTP) 250 µM, Tris-HCl (pH 9.0)10 mM, KCl(30mM), MgCl2 (1.5mM), template DNA (50ng) 7µl and 10 pmol from each primer of Forward (5'- TTTTTT GGGCATCCTGAGGTTTAT-32) and Reverse (52TAAAGAAAGAACATAATGAAAATG-32) (Ergin *et al*., 2010). The thermal cycler was set for 94°C (5 min.) for initial denaturation and then denaturation at 94°C (30 s), annealing at 56°C (45 s), extension at 72°C (35 s) in 35 cycles, and the final extension

72°C (10 min.). The PCR product was electrophoresed on 1.5% agarose gel after staining with safe staining, then 440bp band of *cox1* gene was observed under UV light using transilluminator device. Gel purification was done using the kit (*AccuPrep® Gel Purification Kit Cat No: K-3035*) according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

The purified products of all samples were sent to the company and sequenced using the Genetic Analyzer 3130 ABI. After sequencing, the initial results of sequences were carefully compared and edited using Chromas and Sequencher software. The sequences were compared with available sequences in GenBank using the BLAST program. After multiple alignments by ClustalW, phylogenetic analysis and phylogeny tree drawing were done by the Maximum Composite Likelihood method using MEGA4 software. *Echinococcus Vogeli* sequence as an outgroup was used in the dendrogram.

Ethics statement

This study has been approved by the ethics committee of Tabriz University of Medical Sciences, Iran.

RESULTS

In microscopic examination of human cyst fluid, the specimens have both protoscoleces and germinal capsules. Due to the lack of protoscoleces in bovine samples, the germinal layer was isolated. DNA extraction of all samples was done successfully. PCR reaction was performed on the 26 samples using the *cox1* gene fragment and specific primers. The PCR product was electrophoresed using 1.5% agarose gel. We observed 440 bp bands of all samples under UV light and confirmed them. After sequencing, the sequences were compared with sequences available in GenBank using the Blast program (*http://www.ncbi.nlm.nih.gov./BLAST/BLASTdatabases.html*). All 16 bovine samples reported a G1 genotype (sheep strain) and G1B subtype. Out of ten human samples, seven samples were G1B subtype and three isolates recognized G3 genotype (buffalo strain). In this study, out of 26 samples, 23 (88.46%) samples were reported to be the G1B subtype and three (11.54%) samples were the G3 genotype (buffalo strain), which represents the predominance of the G1 genotype (sheep strain). Bovine C15 (G1 genotype), human H1 (G3 genotype) and human H4 (G1 genotype) sequences have been deposited in GenBank under accession numbers: KJ540226, KJ540230, KJ540228 respectively. In phylogenetic analyses, all sequences compared with other *Echinococcus granulosus* genotypes that were previously recorded (Figure 1). Representative GenBank accession numbers for the sequences obtained from this study and for the reference genotypes used in all analyses are shown in Table 1.

DISCUSSION

The prevalence of hydatid cysts in slaughtered animals in Iran has been reported to be from 1.5 to 70% (Rokni, 2009). With respect to hyperendemicity in East Azerbaijan Province, rare research has been conducted to determine the genotype of hydatid cysts; so far, no study has been done on their nucleotide sequencing. In genotyping survey of hydatid cyst isolates using the sequencing method with *cox1* gene in the present study, the predominant genotype in human and bovine samples were reported to be the G1 genotype (sheep strain). The results of this study indicate that cattle could also be suitable hosts for the sheep strain. In conducted studies, in order to determine the genotype of bovine hydatid cyst samples using the sequencing method with the *cox1* gene in Pakistan, China, Turkey, Tunisia, Algeria, Spain and Peru, G1 is reported as the predominant genotype that is consistent with this study (Bardonnet *et al.*, 2003; Daniel Mwambete *et al.*, 2004; M'rad *et al.*, 2005; Ma *et al.*, 2008; Utuk *et al.*, 2008; Latif *et al.*, 2010;

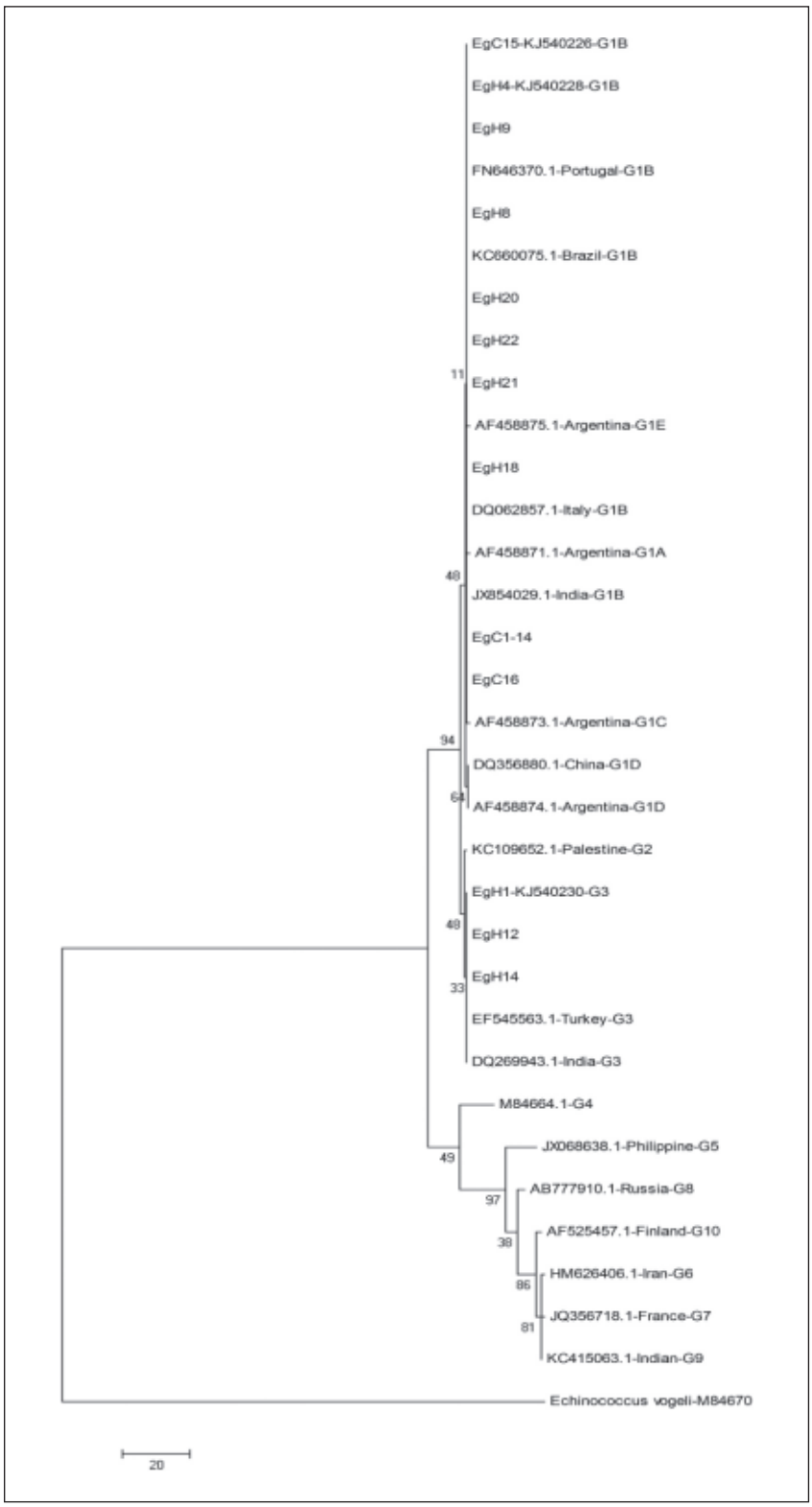

Figure 1. Phylogeny and genetic relationships of *Echinococcus granulosus* human and bovine isolates from Northwest Iran and reference sequences for G1-G10 genotypes of *E. granulosus* as well as *Echinococcus vogeli* as outgroup

Table 1. *Echinococcus granulosus* haplotypes from Northwest Iran and origin of sequences used for *cox1* sequence

E. granulosus Haplotype	Host	Accession number	Reference
C15	Cattle	KJ540226	This study
C16	Cattle	–	This study
C1-14	Cattle	–	This study
H4	Human	KJ540228	This study
H1	Human	KJ540230	This study
H8	Human	–	This study
H9	Human	–	This study
H12	Human	–	This study
H14	Human	–	This study
H18	Human	–	This study
H20	Human	–	This study
H21	Human	–	This study
H22	Human	–	This study
G1A	Livestock-human	(AF458871)	(Kamenetzky *et al.*, 2002)
G1B	Sheep-cattle	(FN646370)	(Beato *et al.*, 2010)
G1B	Pig	(KC660075)	(Monteiro *et al.*, 2014)
G1B	Sheep	(DQ062857)	(Varcasia *et al.*, 2006)
G1B	Human	(JX854029)	(Sharma *et al.*, 2013)
G1C	Livestock-human	(AF458873)	(Kamenetzky *et al.*, 2002)
G1D	Homo sapiens	(DQ356880)	(Bart *et al.*, 2006)
G1D	Livestock-human	(AF458874)	(Kamenetzky *et al.*, 2002)
G1E	Livestock-human	(AF458875)	(Kamenetzky *et al.*, 2002)
G2	Sheep	(KC109652)	(Adwan *et al.*, 2013)
G3	Sheep	(EF545563)	(Vural *et al.*, 2008)
G3	Sheep	(DQ269943)	(Bhattacharya *et al.* 2007)
G4	Horse	(M84664)	(Bowles & McManus, 1992)
G5	Spotted deer	(JX068638)	(Boufana *et al.*, 2012)
G6	Camel	(HM626406)	(Sharifiyazdi *et al.*, 2011)
G7	Pig	(JQ356718)	(Umhang *et al.*, 2013)
G8	Alces alces	(AB777910)	(Konyaev *et al.*, 2013)
G9	Homo sapiens	(KC415063)	(Sharma *et al.*, 2013)
G10	Reindeer	(AF525457)	(Lavikainen *et al.*, 2003)
Out group *E. vogeli*	Rodent	(M84670)	(Bowles & McManus, 1992)

Sánchez *et al*., 2010). Also in the studies in Iran, all bovine samples were the G1 genotype that is consistent with the current study (Zhang *et al*., 1998). In conducted studies in Peru, Italy and Argentina, G1-G3, G1 and G3, and G1, G2, G5 genotypes are reported, respectively. (Kamenetzky *et al*., 2002; Busi *et al*., 2007; Moro *et al*., 2009). In a study in Isfahan (central Iran), 35% of the samples were the G6 genotype, which obtained different results in comparison with our study (Shahnazi *et al*., 2011). In the Ardabil province, genotypes of the hydatid cyst were reported as follows: out of nine human cysts, seven cases were G1 and two cases were the G3 genotype. From 19 bovine hydatid cysts, 18 samples were G1 and one sample was the G3 genotype (Pezeshki *et al*., 2013). There are very few reports of the G3 genotype from humans in the world, and only a few countries, such as Italy and Brazil have reported them for the first time (Busi *et al*., 2007; De La Rue *et al*., 2011). The results of our study are quite similar with this study, especially due to the existence of G3 genotype in human that could be due to the proximity of Ardabil with East Azerbaijan province in terms of the life cycle of the parasite. This indicates that the G3 genotype could be one of the etiologic factors in the region. Thus, in order to identify the transmission cycle, the study of G3 genotype reservoirs as intermediate hosts in this region is necessary. Also, these results are not consistent with some studies conducted in Iran and the world (Zhang *et al*., 1998; Daniel Mwambete *et al*., 2004; M'rad *et al*., 2005; Busi *et al*., 2007; Ma *et al*., 2008; Utuk *et al*., 2008; Moro *et al*., 2009; Sánchez *et al*., 2010; Latif *et al*., 2010; Shahnazi *et al*., 2011). Due to the various distributions of *E. granulosus* intermediate hosts in different parts of the world, there is definite probability for different genotypes in different regions. On the other hand, the predominant or high prevalence of a genotype in an area shows the significance and role of its intermediate hosts in the life cycle of the parasite. Other than sequencing, there are different molecular methods for genotyping of *E. granulosus*, including RFLP, SSCP and semi nested-PCR. However, these methods were not able to differentiate genotypes correctly, such as strains of *E. granulosus* sensu stricto and *E. canadensis* (Spotin *et al*., 2015; Jabbar *et al*., 2011; Simsek *et al*., 2011; Varcasia *et al*., 2007). In a study in Greece that examined 20 sheep and goat cysts using semi nested-PCR and sequencing, all sheep samples were G1, and goat samples were G6/G7 as sequenced by the semi nested-PCR method. However, 18 samples of sheep were G1, and two samples were the G3 genotype, as identified by sequencing. All goat samples were reported to be the G7 genotype (Varcasia *et al*., 2007). In a study in Mongolia, the genotype of 50 samples of human hydatid cyst using SSCP was reported as 34 samples of the G1-G3 genotype (*E. granulosus* sensu stricto) and 16 samples of the G6-G10 genotype (*E. canadensis*) (Jabbar *et al*., 2011). Also, in a survey in Turkey, all bovine and sheep samples were reported as the G1-G3 genotypes using the SSCP method (Simsek *et al*., 2011). It can be concluded that the sequencing method is preferable over other methods for genotyping and phylogenetic analysis. Different molecular studies were performed to identify genotypes of *E. granulosus* based on mitochondrial DNA genes such as *cox1* and *nad1* and *nuclear DNA* genes. According to these investigations, mitochondrial DNA is more accurate for the genotypic analysis of *E. granulosus* than nuclear DNA (Euzeby, 1991). Results of this study showed that G1, and especially G1B are the predominant genotype and subtype in humans and cattle in Northwest Iran.

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