

Genetic characterization of human-derived hydatid cysts of *Echinococcus granulosus* in Lorestan Province, Western Iran

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Abstract. The aim of the present investigation was genotyping of hydatid cysts of cystic echinococcosis (CE) patients residing in Lorestan province by sequencing and analyzing of the partial mitochondrial cytochrome c oxidase subunit 1 (cox1). A total of 26 hydatid cysts (6 hydatid cysts) from 6 patients suffering from hepatic CE and 20 formalin-fixed paraffin-embedded (FFPE) tissue samples from 20 human patients with histologically confirmed CE. DNA was extracted, and genotyping was performed by sequencing and analyzing mitochondrial cox1 gene. All the samples were analyzed using mitochondrial cox1 primers. In sequencing analysis, the alignment of the determined sequence with those of known genotypes of *E. granulosus* demonstrated that the isolates belonged to genotype G1 (sheep strain). Representative nucleotide sequence obtained in this study was deposited in the GenBank database under the accession number LC068913.1. The phylogenetic analysis showed two clusters. One of the clusters includes G3 genotype (HM5630221) and the other cluster represents all strains related to G1 and G3 genotypes which contain genotype defined in this study as well. The obtained findings demonstrated that G1 genotype is predominant strain of human CE in Lorestan province, Iran, which indicating the sheep-dog cycle in this area. Further studies with more sample size should be conducted to be sure of the sheep strain G1 which is predominant strain in this province.

INTRODUCTION

Cystic echinococcosis (CE) disease caused by *Echinococcus granulosus* is well-known major cause of economic and community health problem in several countries around the world such as Iran (WHOs, 1996). Typically, human infection may happen after ingestion of infective eggs, which are passed in the feces of definitive host during direct expose or environmental contamination (Eckert & Deplazes, 2004, Brunetti *et al.*, 2010, Mahmoudvand *et al.*, 2014, 2016). Current studies have reported that clinical symptoms of CE generally depend on the

cysts location, size, and the number of cysts (Junghanss *et al.*, 2008). According to previous molecular epidemiological studies 10 different genotypes of *E. granulosus* (G1–G10) including: *E. granulosus sensu stricto* (G1, G2, G3), *Echinococcus equines* (G4), *E. ortleppi* (G5), *E. intermedius* (G6, G7, G8, G10) have been demonstrated (Thompson, 2008). In Iran, three genotypes including G1, G3, and G6 have been identified in humans and animals in different regions of Iran (Nejad *et al.*, 2012, Sharbatkhori *et al.*, 2009). In a study conducted by Schneider *et al.* (2010), it has been shown that there is an association between the type of genotypes

and the size of hydatid cysts, where the infected individuals with G7 genotype had smaller liver cysts than those infected with G1 genotype (Schneider *et al.*, 2010). Several investigations on the prevalence of *E. granulosus* have reported that G1 genotype (common sheep strain) is the principal genotype in Iran and also in most countries (Thompson, 2008, Nejad *et al.*, 2012, Sharbatkhori *et al.*, 2011). Based on current studies the mitochondrial DNA (mtDNA) is more influential than nuclear DNA within *E. granulosus* in creating phylogenetic communications between intimately related species due to its fast sequence progress (Nejad *et al.*, 2012, Sharbatkhori *et al.*, 2011). Moreover, studies on mitochondrial genomes have discovered its capability to detoxify difficult subjects in taxonomy of *Echinococcus* (Nejad *et al.*, 2010).

Regarding the genotyping of *E. granulosus* in Lorestan Province, western Iran, Parsa *et al.* (2012) have reported three genotypes (G1, G2 and G3) from 71 stray dogs by means of DNA sequencing of the partial mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase 1 (*nad1*). However, little is known regarding *human E. granulosus* isolates in this province. Therefore, the aim of this investigation was genotyping of hydatid cysts of CE patients residing in Lorestan province by sequencing and analyzing of the partial mitochondrial *cox1*, to guess on likely transmission patterns of this parasite by homology analysis, and to identify the phylogeny of genotypes of *E. granulosus* by creating neighbor-joining trees.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Committee on the Ethics of Animal and Human Experiments of the Lorestan University of Medical Science, Khorramabad, Iran (Permit Number: 89/6). For human isolates, all adult subjects gave their written informed consent for surgery.

Collection of hydatid cyst samples

A total of 26 hydatid cysts including (i) 6 hydatid cysts from 6 patients suffering from hepatic CE the size of the cysts was recorded, that ranged from 7.2 cm to 10.9 cm and their endocysts were preserved in 70% ethanol at 4°C; (ii) and 20 FFPE tissue samples from 20 human patients histologically approved CE (observation of laminated layers and/or hooklets and/or protoscoleces) were collected from the collection in the Pathology Departments Lorestan University of Medical Sciences, Khorramabad, Iran between 2013 and 2014.

Extraction of genome DNA

In order to extract the genomic DNA (gDNA) of human tissue samples, 10 µm thick sections were obtained from tissue blocks, and extra paraffin was trimmed.

To prevent probable contamination between tissues blocks, disposable razor blades were prepared and all the tools were sterile. To remove paraffin, 1 ml xylene was added to sections for 10 min at 37°C then centrifuged at 1500 g for 5 min, and the supernatant was discarded. This process was repeated once.

For rehydration, sections were followed in 100%, 90%, 80% and 70% ethanol (Schneider *et al.*, 2010). After that, the 70% ethanol was eliminated and added lysis buffer. The tissues were digested overnight at 56°C within 400 µl of the lysis buffer with the addition of 40 µl Proteinase-K (20 mg/ml) (Sigma, USA). Genomic DNA extraction of each sample was done by High Pure PCR Template preparation kit (Bioneer, South Korea) according to the manufacturer's instructions. Extracted DNAs were stored at -20°C until further use in PCR. To make sure the accuracy of DNA extraction, the concentration of the extracted DNA samples were evaluated by NanoDrop.

Mitochondrial PCR amplification

In the present study, the mitochondrial *cox1* gene was amplified using specific primers JB3/JB4.5 according to the method explained by Bowles *et al.* (1992) (Bowles *et al.*, 1992). PCR amplification was done in a 25 µl final

volume containing 1 X PCR buffer, 2.5 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 μM of each primer and 1.5 μM Ampli-Taq Polymerase. Two primers, JB3 (forward), 5'-TTT TTT GGG CAT CCT GAG GTT TAT-3' and JB4.5 (reverse), 5'-TAA AGA AAG AAC ATA ATG AAA ATG-3', were used to amplify a 450 bp fragment of *cox1* gene.

The PCR conditions were one initial denaturation 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension for 45s at 72°C. Final extension was carried out at 72°C for 10 min. The PCR product was electrophoresed on 1.5% agarose gel. The non-template water as negative control was used in all PCR amplifications.

Sequencing analysis

DNA sequence analysis was performed by the Illumina Genome Analysis System, employing the same primers used in the primary PCR. The electropherogram of each sequence was checked by eye, and the sequences were compared with each other using the software BioEdit. Sequencing in both directions was used as assessment of the sequencing data accuracy.

Nucleotide sequences obtained in the current study were subjected to BLAST searches (<http://www.ncbi.nlm.nih.gov/blast>), and then aligned and analyzed with each other and also with *E. granulosus* reference sequences which were downloaded from GenBank using Clustal X 1.83.

Phylogenetic analysis

To understand phylogeny of *E. granulosus* genotypes, a neighboring-joining tree was constructed at *cox1* using the Molecular Evolutionary Genetics Analysis (MEGA 6) software based on the evolutionary distances calculated by Kimura-2-parameter model. The reliability of the tree was assessed using the bootstrap analysis with 1000 replicates.

The tree was drawn using the sequence obtained in the present study and some reference sequences available for the *E. granulosus* (G1 and G3 genotypes) in GenBank. *Taenia saginata* (accession

no. NC009938) and *E. multilocularis* (accession no. KT318128.1) were applied in the model as the out groups.

RESULTS

Totally 26 isolates from human patients with CE operated on between 2013 and 2014 were tested for the molecular analysis. All hydatid cysts from 6 patients suffering from hepatic CE were confirmed to be fertile with protoscoleces. All these samples were analyzed using mitochondrial *cox1* primers. For all of the isolates, a fragment of about 450 bp was successfully PCR-amplified within the *cox1* gene (Figure 1). In sequencing analysis, the alignment of the determined sequence with those of known genotypes of *E. granulosus* demonstrated the isolate belonged to genotype G1 (sheep strain). Representative nucleotide sequence obtained in this study was deposited in the GenBank database under the accession number LC068913.1.

The phylogenetic analysis demonstrated that the G1 and G3 genotypes were placed in two separate clusters. The genotype defined in the present study was located in the G1 cluster (Figure 2).

DISCUSSION

Cystic echinococcosis caused by the *E. granulosus* parasite is one of the main neglected tropical diseases newly categorized by WHO (1996) (World Health Organization, 1996). At present, the several studies were conducted on the frequency and molecular identification of different parasites in Lorestan province but there is no data about genotyping of human hydatid cyst (Badparva *et al.*, 2014, Kheirandish *et al.*, 2011, Kheirandish *et al.*, 2013, Kheirandish *et al.*, 2014). Various investigations have been carried out on the genetic characterization of both human and animal *Echinococcus* isolates by means of some resources including germinal layers, protoscoleces, and adult worms (Dinkel *et*

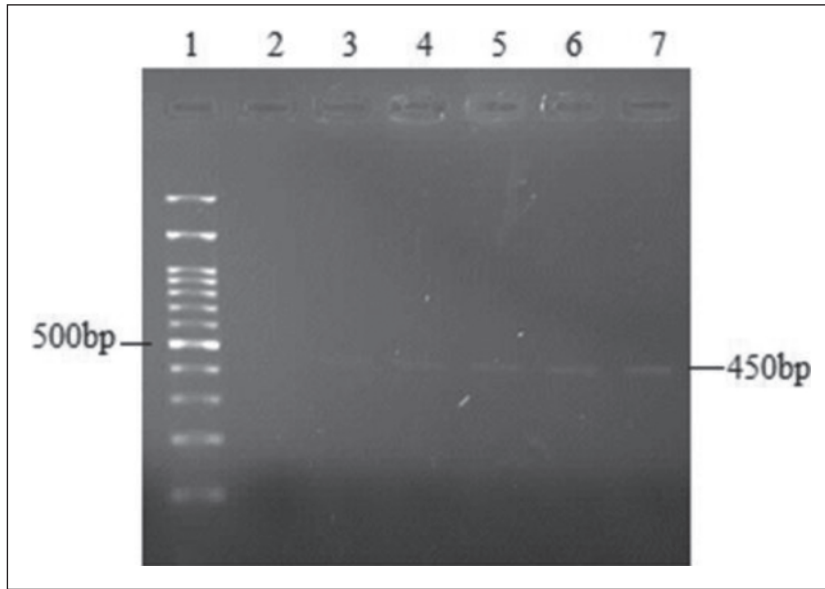


Figure 1. Agarose gel electrophoresis of PCR products of *E. granulosus* to show DNA extracted from FFPETs and fresh protoscoleces. Lane 1: 50bp DNA ladder marker; Lane 2: Negative controls; Lane 3-5: *E. granulosus* isolates DNA extracted from FFPETs; Lane 6, 7: *E. granulosus* isolates DNA extracted from fresh protoscoleces.

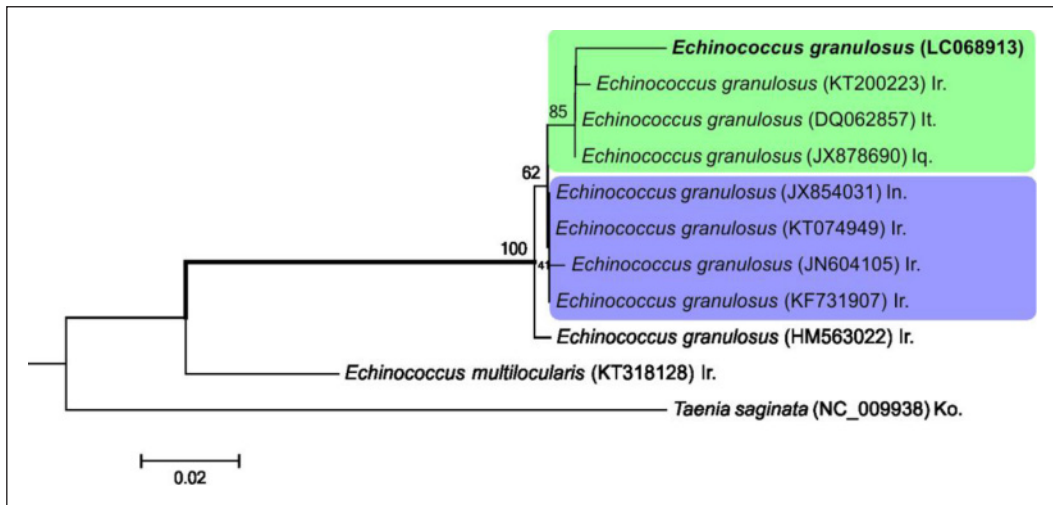


Figure 2. Molecular phylogenetic tree of Iranian *E. granulosus* isolate from human hydatidosis. Isolate LC068913.1 represent genotype G1. Accession numbers of KT200223, DQ062857, JX878690 represent reference sequences of *E. granulosus* genotypes G1. Accession numbers of JX854031, KT074949, JN604105, KF731907 and HM563022 represent reference sequences of *E. granulosus* genotypes G3. *Taenia saginata* (accession no. NC009938) and *E. multilocularis* (accession no. KT318128.1) were applied in the model as the out groups.

al., 2004). Generally, gaiting of human cystic contents is not accessible, besides in hospitals situated in highly endemic regions for *E. granulosus*; however, FFPE tissue samples are effortless to transport and store in the laboratory (Schneider *et al.*, 2010). Reviews have shown that mitochondrial genes such as *cox1* and *nad1* genes are proper molecular markers for studying genetic variation in many isolates of *E. granulosus* from various hosts including cattle, sheep, and goat in Iran (Nejad *et al.*, 2012, Sharbatkhori *et al.*, 2011). Moreover, mitochondrial genomes have the capability to eliminate some limitations in *Echinococcus* taxonomy (Nejad *et al.*, 2010).

In line with our findings, previous molecular epidemiological studies have revealed that G1 genotype is responsible for more than 80% of human CE worldwide (Thompson, 2008). Khademvatan *et al.* (2013) in Khuzestan province and Dousti *et al.* (2013) in Ilam province have revealed that all of their human isolates belonged to G1 genotype. Similarly, Vahedi *et al.* (2014) have reported that among the human hydatid cysts isolated in East Azerbaijan Province, North West of Iran recognized as sheep strain (G1) using PCR-RFLP.

In contrast, Nikmanesh *et al.* (2014) have reported from the 30 human hydatid cyst isolates, 26 (86.7%), 3 (10%), and 1 (3.3%) were belonged to G1, G3, and G6 genotypes, respectively. Rostami *et al.* (2015) have demonstrated that from 200 FFPE tissue samples of human isolates in three provinces of Iran including Alborz, Tehran, and Kerman provinces, 54.4, 0.8, 1, and 40.8% of the samples were determined as the G1, G2, G3, and G6 genotypes, respectively. In the other study, Parsa *et al.* (2012) showed that G1 strain is principal in the *E. granulosus* isolates of 71 stray dogs, from Lorestan province by DNA sequencing of the partial mitochondrial *cox1* and *nad1* genes. The obtained results corroborated the importance of sheep as a source of human hydatidosis. Therefore, an affluent control plan against human CE cannot be established unless training the farmers and in susceptible individuals about the importance and life cycle of the *E. granulosus* in this region.

Moreover, controlled slaughtering of livestock, mainly sheep, in abattoirs can be also useful in control strategies. The obtained findings demonstrated that G1 genotype is predominant strain of human CE in Lorestan province, Iran; which indicated the epidemiological significance of sheep in retaining the transmission cycle of *E. granulosus* certify more attention.

Declaration of Interest

The authors report no conflicts of interest.

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