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Original Article

Genotyping of *Echinococcus granulosus* Isolates from Human Clinical Samples Based on Sequencing of Mitochondrial Genes in Iran, Tehran

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Abstract

Background: The present study was aimed to investigate molecular diversity of *Echinococcus granulosus* isolates collected from human clinical samples using two mitochondrial genes *cox1* and *nad1* in Iran.

Methods: Forty seven human hydatid cysts were collected through surgery from two hospitals in Tehran during 2010-2012. To determine the fertility of protoscoleces, the cyst fluids were subjected to morphological microscopic examinations. Protoscoleces were removed from each cyst and their total genomic DNAs were extracted. PCR was performed to amplify fragments of 450 and 400 base pair (bp) for *cox1* and *nad1* genes, respectively. Genotype diversity and sequence variation of the strains were studied by bioinformatics software and in comparison with those mtDNA sequences already deposited in GenBank.

Results: Sixteen, (53.3%), 13 (43.3%), and 1 (3.3%) samples were related to lung, liver, and spleen, respectively. The remained 17 unfertile samples were excluded from the study. From the 29 isolates, 86.7% (n=26) and 10% (n=3) were related to G1, and G3 genotypes, respectively. The sole isolate with G6 genotype was obtained from lung sample. Analysis of concatenated sequences of *cox1*+*nad1* indicated the presence of 11 haplotypes among our strains that were related to genotypes G1 (n=9), G3 (n=1) and G6 (n=1).

Conclusion: In consistent to other reports from Iran, genotypes G1, G3, and G6 were observed in our human isolates. The rate of G3 genotype was however higher than other studies implying that human can be considered as a new appropriate host for G3 genotype. Further studies with more sample size from different geographic areas of Iran are needed for *E. granulosus* mapping.

Introduction

Human cystic echinococcosis (CE) is a chronic zoonotic infection caused by the larval stage of the canid tapeworm, *Echinococcus granulosus* (1). Both cystic and alveolar echinococcosis were reported from Middle East countries, which cystic echinococcosis was assumed as the predominant type (2-5). *E. granulosus* is one of the most important parasites in different parts of Iran (6). Although sheep, camels, cattle and goats are considered as commonly intermediate hosts of hydatid cysts in Iran, but sheep is the most common and important intermediate host found throughout the country (7).

Ten distinct genotypes of *E. granulosus* (G1–G10) were identified worldwide based on either morphological or molecular data (8-13). Several molecular studies on human isolates of *E. granulosus* have identified the genotypes G1, G3, and G6 in different regions of Iran (14, 15). Sheep, buffalo, and camels assume as main reservoirs of genotypes G1, G3, and G6 that are among common livestock in Iran. Numerous reports on the incidence of *E. granulosus* have indicated that G1 (common sheep strain) is the most common genotype in Iran (16, 17) and also most parts of the world (18-20). In many countries including Iran, camels have an important role in transmission cycle of G6 (common camel strain) of *E. granulosus* and have been reported principally from human (21, 24). Moreover, buffalo is the main intermediate host of genotype G3 in Iran (14, 18). The first molecular report in Iran for *E. granulosus* genotypes was done in 1998 by Zhang L *et al* (15). In this study a total of 16 isolates were collected from human and domestic animals and partial sequence variation of the mitochondrial cytochrome c oxidase I (*cox1*) and NADH dehydrogenase subunit I (*nad1*) genes were investigated. Accordingly, sheep and camel strains were presented as sole characterized genotypes (15). The G3 genotype was later described by other molecular

studies in Iran among both human and animal samples (6, 7, 13, 16). Analysis of evolutionary sequence variations could improve our knowledge about phylogenetic relationship of these strains in compare to other ones in different countries.

The present study was aimed to characterize genotype diversity and sequence variations of *E. granulosus* isolates from human hydatid cyst in different tissues based on PCR sequencing of two housekeeping genes *cox1* and *nad1*.

Materials and Methods

Suspected patients to hydatidosis from different geographic regions were referred to two hospitals, Children's Medical Center Hospital and Masih Daneshvari Hospital in Tehran, Iran. Totally, 47 samples of human hydatid cysts were collected via surgery during 2010-2012. Thirty three cases (70.2%) were isolated from males and 14 cases (29.8%) from females. Immediately after surgery, the cyst samples were collected and were rinsed in normal saline and transported to the Molecular Laboratory of Children's Medical Center Hospital for further analysis. The microscopic examination was performed on the cyst fluids to determine the fertility of protoscoleces. The protoscoleces were removed from each cyst, and rinsed three times with phosphate buffered saline (PBS). DNA of each sample was extracted by High Pure PCR Template preparation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. To ensure the accuracy of DNA extraction, concentration of the extracted DNA samples were analyzed by NanoDrop. The extracted DNAs were stored at -20°C until required for PCR.

Presence and sequence diversity of two mitochondrial genes *cox1* and *nad1* were investigated by PCR, separately. PCR was performed in a 25 µl final volume containing 1 X PCR

buffer, 2.5 mM MgCl₂, 0.2mM 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' (10), were used to amplify a 450 bp fragment of *cox1* gene under the following conditions: initial denaturation 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing at 50°C for 45s and elongation for 45s at 72°C. Final extension was performed at 72°C for 7 min. Forward primer MS1: 5'-CGTAGGTATGTT GGTITGTITGGT-3') and reverse primer MS2: 5'-CCATAATCAAATGGCGTACGAT -3' were used to amplify a 400 bp fragment of the second locus, *nad1* gene (25). The amplification was carried out with 5 min initial denaturation at 94°C, and 30 cycles of 30s denaturation at 94°C, 45s annealing at 50°C and 30 s elongation at 72°C. Final extension was performed at 72°C for 5 min. In the next step, PCR products of *nad1* and *cox1* genes were purified using a commercial purification kit (Bioneer, South Korea) and sent to Takapouzist Company (Tehran, Iran) for sequencing. Consensus characteristic nucleotide sequences of each genotype from relevant mtDNA in GenBank database (Accession numbers DQ062857 and AJ237632 for G1, M84663 and AJ237634 for G3, and M84666 and AJ237637 for G6 for *cox1* and *nad1*, respectively) were used for genotype characterization. Multiple alignments of concatameric sequences of *cox1* and *nad1* genes were created in compare to their related reference sequences. Identity and similarity of the sequences were determined using the BioEdit software version 7.2.

Phylogenetic analysis was performed using Maximum Likelihood method based on the Tamura-Nei model. Nucleotide sequence of *T. saginata* (GenBank accession number NC 009938) was used as outgroup.

Results

Out of the 47 collected hydatid cysts, DNA extracts of 30 samples were used for molecular analysis. Extracts of the remained unfertile 17 samples showed low quality for amplification and sequencing experiments, which were excluded from the study. The obtained samples were related to cysts of lung (53.3%), liver (43.3%), and spleen (3.3%). All of the DNA samples successfully present amplicons of the *cox1* and *nad1* genes as was shown for the control samples (450 and 400 bp respectively).

Three genotypes G1, G3 and G6 were identified among our DNA samples based on *cox1* and *nad1* sequences data. Genotype 1 was the most common available genotype in the studied population. Out of the 30 studied samples, 26 samples (86.7%) belonged to genotype G1. These samples belonged to lung (57.7%), liver (38.5%), and spleen (3.3%). Three isolates belonged to the genotype G3 (10%), all related to samples from liver (Strains HT13, HT22, HT55), and one isolate belonged to genotype G6, that obtained from the lung. Analysis of the strains with genotype G1 indicated common nucleotide sequences in position 66 (C) and 257 (T). Presence of mutation at position 56 (a definitive mutation for genotype G2) was also detected among 10 strains with G1 genotype. Point mutations in other positions are summarized in table 1. Among all the strains with G3 genotype, mutation at position 40 (A to G) was observed in compare to reference strain M84663. However, no sequence diversity was observed in the identified strain with G6 genotype compared with camel strain M84663.

In the case of *nad1* gene, the analysed sequences showed common point mutations at positions 43 (A and G) and 429 (A and G) for all the strains with G1 and G3 genotypes, respectively. Presence of point mutation in position 282 (C to T; a definitive point mutation for strains with G2 genotype) was observed among all but one strain with G1 genotype

based on their *cox1* nucleotide sequence. The only obtained strain with G6 genotype (Strain HM42, from Isfahan Province) displayed 100% identity with *nad1* gene of *E. granulosus* camel strain (Accession no. Aj237637). Other point mutations for *nad1* gene are summarized in table 1. Comparison of concatameric sequence data presented a total of 11 haplotypes among our isolates. Nine of these haplotypes

belonged to the strains with G1 genotype (Table 1).

The phylogenetic analysis of concatenated sequences of *cox1*+*nad1* showed three clusters A, B and C. Cluster A represents all strains related to G1 genotype, and cluster B and C characterize strains related to G3 and G6 genotypes, respectively. The largest cluster included 26 concatenated sequences that were grouped as G1 genotype (Fig. 1).

Table1: *Echinococcus granulosus* haplotypes, accession numbers and nucleotide substitution detected in this study using concatenated sequence (*cox1* + *nad1*)

Representative geno- types&haplotypes ^a	Isolation source			ACCESSION NUMBERS (<i>coxI</i>)	ACCESSION NUMBERS (<i>nadI</i>)	Nucleotide substitu- tion (<i>coxI</i>) ^b	Nucleotide substitution (<i>nadI</i>) ^b
	Lung Spleen	Liver					
G1							
Haplotype 1 (n=9)	3	5	1	KF 612381	KF 612357	ND	282 C→T
Haplotype 2 (n=7)					KF 612356	56 C→T	282 C→T
Haplotype 3 (n=3)	6	1	0	KF 612390	KF 612360	ND	342 and 345 T→C
Haplotype 4 (n=2)	1	2	0	KF 612380	KF 612358	ND	375 T→C
Haplotype 5 (n=1)	1	1	0	KF 612376	KF 612349	252 T→C	282 C→T
Haplotype 6 (n=1)	1	0	0	KF 612395	KF 612350	252 T→C	ND
Haplotype 7 (n=1)	1	0	0	KF 612396	KF 612355	56 C→T, 123 T→C	282 C→T
Haplotype 8 (n=1)	1	0	0	KF 612394	KF 612343		150 T→C,282 C→T
Haplotype 9 (n=1)	0	1	0	KF 612386	KF 612351	56 C→T	170 G→A, 282 C→T
	1	0	0	KF 612393		56 C→T, 38 T→C	
G3							
Haplotype 10 (n=3)	0	3	0	KF612397	KF612369	40 A→G	ND
G6							
Haplotype 11 (n=1)	1	0	0	KF612400	KF612372	ND	ND

^aHaplotypes 1-11 defined based on concatenated nucleotide sequences of *cox1* + *nad1*.

^bND: No sequence diversity was detected in compare to definitive reference mutations presented by Bowek *et al.* for *cox1* and *nad1* (10).

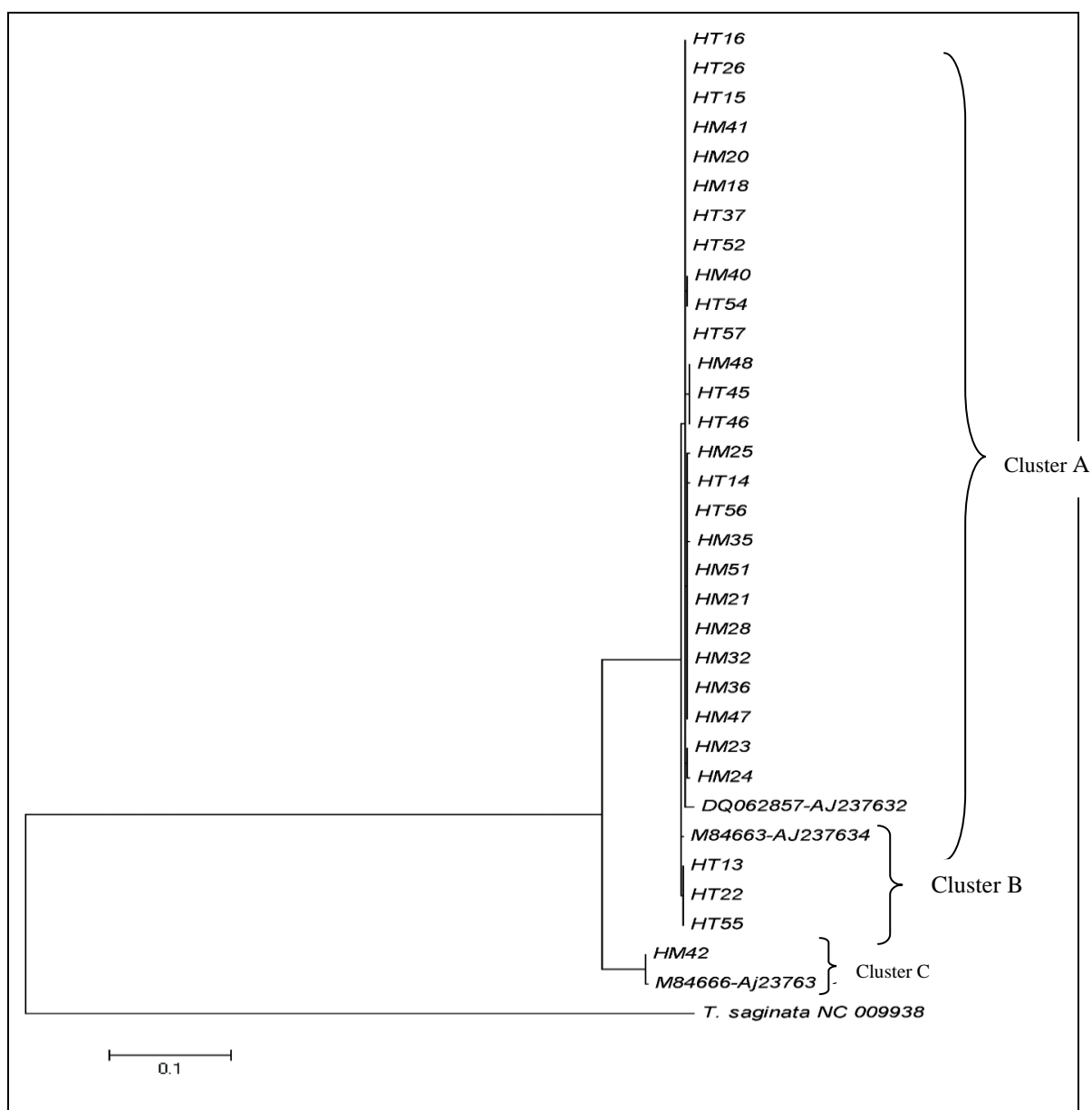


Fig. 1: Phylogenetic tree of Iranian *E. granulosus* isolates from human hydatidosis using the Maximum Likelihood method based on the Tamura-Nei model. The scale bar indicates the number of substitutions per site for each isolate. Partial DNA sequences of concatenated mitochondrial *cox1+nad1* genes were used as input data. Isolate HM42 represent genotype G6, isolates HT13, HT22, HT55 represent G3 genotype, and the other ones represent G1 genotype. Accession numbers of M84666-AJ237637, M84663-AJ23763, and DQ062857-AJ237632 represent reference sequences of *Echinococcus granulosus* genotypes G6, G3, and G1, respectively. *Taeniasaginata* NC 009938 was used as out group sequence data

Discussion

E. granulosus is known to be widespread in whole country, with “sheep–dog” and “camel–dog” strains described in the literature as

infecting humans (21-24). There are many reports on the molecular identification of *E. granulosus* genotypes in domestic animals (6-8, 13-17). Three *E. granulosus* genotypes, including genotype G1 in human, sheep, cattle, cam-

el and goat, genotype G3 in buffalo, sheep, cattle and human and genotype G6 in sheep, camel and human, were reported from different studies (6-8, 13-17, 21-26). Various mitochondrial and nuclear genes have previously been applied effectively in differentiation of these genotypes. Rostami Nejad et al. investigated genotype diversity of three mitochondrial genes *cox1*, *nad1* and *atp6* and also partial sequences of the 12S rRNA gene in their isolates and confirmed the presence of G1 and G6 genotypes in different intermediate hosts, including cattle, camels, sheep, buffalo and goats in different geographic areas in Iran (17). Fasihi Harandi et al. (16) and Ahmadi & Dalimi (5) genotyped their isolated using PCR-RFLP of the internal transcribed spacer (ITS1) region. They demonstrated that sheep and camel strains are similarly detected in cattle and also humans as well. According to the results of our study, G1 genotype was the predominant genotype of *E. granulosus* in infected patients that is in accordance with other reports from Iran and other countries. In the preliminary study in 1998 by Zhang et al., the G1 genotype was found in all four human isolates examined (15). In a study in Golestan province in Iran, PCR products and RFLP patterns of ITS1 gene of *E. granulosus* showed presence of G1 genotype in all 30 human samples (27). Our findings also confirmed by two studies of Dousti et al. in Ilam province and Khademvatan et al. in Khuzestan provinces, which all of their human isolates belonged to G1 genotype (28, 29). On the other hand, there are few reports for existence of human infection with strains belonged to G3 genotype. In a study in Ardabil province, two human isolates of G3 genotype were described for the first time in Iran (26). The presence of *E. granulosus* genotype G6 (camel genotype) has been reported previously in domestic animals and humans in different countries (21, 30, 31). In Shahnazi et al. study using RFLP patterns of ITS1 gene, 6 out of 31 human samples (19.3%) were infected with the camel genotype (24). Diversity of these results could

be explained by type of the studied samples, difference in geographic area, and exploited method for genotyping. Results of our study showed presence of various point mutations in both *cox1* and *nad1* genes that are region specific in compare to other strains in the world. Many of the studied strains didn't show described mutations by Bowel et al. for differentiation of strains with genotypes G1-G3 at positions 56 (for *cox1* gene) and 282 (for *nad1* gene).

Conclusion

The results of this study showed presence of the three common genotypes G1, G3, and G6 among our isolates. Higher frequency of strains with G3 genotype compared with other reports in Iran is of great concern that suggest human as new appropriate host for G3 genotype. The described sequence variations in *cox1* and *nad1* genes among the human isolates proposed incompatibility of point mutations 56 (C to T) and 282 (C to T) in differentiation of G1-G3 genotypes in Iran. Further studies with more sample size from different geographic areas of Iran are needed to evaluate these results for genetic mapping of *E. granulosus*.

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