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Original Article

Genetic Identification of *Echinococcus granulosus* in Slaughtered Domestic Animals from Two Northeastern Iranian Cities Using HRM and Sequencing Techniques

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Abstract

Background: Hydatidosis is one of the common parasitic diseases common to both humans and animals with global spread. The disease is very important from both public health and economic perspectives. The parasite presents with highly diverse genetic features, including a different genotype which affect the life cycle of the parasite, its transmission routes, pathogenicity, immunogenicity, response to drugs, epidemiology and disease control. Therefore, we aimed to identify the genotype of hydatid cysts isolated from slaughtered animals in Sabzevar and Neyshabour slaughterhouses by sequencing and high-resolution melting curve analysis.

Methods: Out of 35 hydatid cyst samples, including 19 from sheep (15 liver and 4 lung samples) and 16 from cattle (9 liver and 7 lung samples) were included in this study. After the extraction of protoscoleces and DNA, a high-resolution melting curve analysis method was used to identify the genotype of the samples using pre-determined G1, G3, and G6 standard samples.

Results: Out of 35 hydatid cyst samples, 89.5% G1 and 10.5% G6 were detected in sheep samples, and 93.7% G1 and 6.3% G6 were detected in cattle samples. Results showed in the Sabzevar City, dominant genotype there was G1 and *COX1* gene considered a perfect biomarker in a molecular identification and phylogenetic study of this parasite.

Conclusion: G1 was the most prevalent in samples of hydatid cysts (both sheep and cattle) in Sabzevar, while in Neyshabour samples, G1 and G6 were the most dominant types.

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Introduction

Hydatidosis caused by *Echinococcus granulosus* is one of the most important common zoonotic diseases, and it is common in most tropical and subtropical temperate regions of the world (1, 2).

According to the classification criteria established by the WHO, human infections of "echinococcosis" are categorized among the neglected diseases, disorders, or conditions of global health significance for which the WHO advocates control (3). The term "echinococcosis" encompasses a group of parasitic diseases including cystic (CE), alveolar (AE), and neotropical (NE) echinococcosis, with an estimated infection rate of over one million individuals at any given time (4,5). Among the infections caused by *Echinococcus* spp., CE stands out as the most widespread globally, leading to significant morbidity and mortality rates in human populations (6).

Variation in species/genotypes of *E. granulosus* s.l. are reflected in morphological and biological characteristics of the parasite, and it can influence the life cycle pattern, host specificity, development rates, pathogenicity, treatment, transmission dynamics, epidemiology and finally control of CE (7)

High-Resolution Melting (HRM) analysis is a powerful, reliable, and cost-effective technique widely used in molecular genetics for identifying genotypes and detecting mutations, such as polymorphisms and single nucleotide polymorphisms (SNPs). The importance of HRM testing lies in its ability to provide rapid and accurate results without the need for additional post-PCR processing, such as gel electrophoresis or sequencing (8).

In the context of this study, HRM testing was critical for differentiating genotypes of *E. granulosus*, a parasite known for its high genetic diversity. Identifying genetic variations in *E. granulosus* is essential for understanding its epidemiology, pathogenicity, and transmission dynamics, which ultimately informs effective control and prevention strategies.

Additionally, HRM offers significant advantages over other molecular techniques due to its simplicity, cost-effectiveness, and ability to analyze multiple samples simultaneously. This makes HRM particularly suitable for large-scale genotyping studies and resource-limited settings. Its application in this study highlights its utility as a robust tool for advancing genetic research in parasitology (9, 10).

The HRM method was chosen for this study for the following reasons:

High accuracy in identifying genotypes and polymorphisms, simplicity and speed, Lower cost than other methods, Ability to compare multiple samples simultaneously, and widely used in parasitological studies.

To investigate these issues in this study, the present study was designed to investigate the genotype of hydatid cysts of domestic animals slaughtered in the slaughterhouses of Neyshabour and Sabzevar (two major cities in the northeast of Iran).

The present cross-sectional study was conducted on hydatid cyst samples collected from Sabzevar and Neyshabour slaughterhouses in 2018-2019. According to the size of the target sample, a total of 55 liver and lung samples infected with hydatid cysts belonging to sheep and cattle, in which the diagnosis of hydatid cyst was clinically confirmed by the slaughterhouse veterinarians, were placed, collected, and taken to the parasitology laboratory at Sabzevar University of Medical Sciences, Iran.

Out of the 55 samples, 20 were excluded from the study due to being sterile and having no trace of protoscoleces, while 35 samples included 19 sheep samples (15 liver and 4 lung samples) and 16 cattle samples (9 liver and 7 lung samples). In the laboratory, after disinfecting the surface of the cysts with 70% eth-

anol, they were centrifuged at 3000 rpm for 5 minutes; the removed supernatant was then washed with normal saline 3 times. At the end, the liquid was poured away, and by preparing a wet slide from the sediment and examining it microscopically, the resulting sediments were confirmed to be protoscoleces; they were then stored in 70% alcohol at -20°C until molecular methods were performed in separate tubes with special codes.

From December 2018 up to September 2019, livestock hydatid cysts were collected from cattle and sheep slaughtered in Sabzevar and Neyshabour slaughterhouses. The fertile cysts containing clear and colorless liquid were selected for molecular analysis. The sterile normal saline was used to wash hydatid cysts three times; they were stored in 70% ethanol at -20°C until molecular processes were applied

DNA Extraction

Genomic DNA was extracted using a genomic DNA extraction kit (Ge Net Bio, South Korea) according to the manufacturer's instructions with minor modification. The concentration of the extracted DNA was determined by NanoDrop (Thermo Scientific, Rockford, IL, USA); also, we stored the DNA at -20°C to reach the molecular analysis application.

Mitochondrial PCR Amplification

In order to amplify a 446 bp DNA fragment of the mitochondrial subunit 1 of the *cytochrome c oxidase 1* gene (*COX1*), PCR was performed, as delineated by Bowles et al., with some modifications (11). The primer sequences utilized were 5′ TTTTTTGGGCATCCTGAGGTTTAT 3′ (For-ward) and 5′ TAAAGAAAGAACATAATGAAAATG 3′ (Reverse). The primer length was 300 bp.

The PCR reaction was carried out in a total volume of 20 µl consisting of 2 µl PCR buffer (10×), 2 µl NTPs, 1 µl $MgCl_2$, 1 µl of each primer, 1 unit Taq DNA polymerase, 11 µl distilled water, and 1 µl DNA. The steps in the PCR protocol are described below. First, the thermal cycler for the COX1 primer was set at 95 °C (for 3 min) for initial denaturation and denaturation at 95 °C (for 30 sec), annealing at 54 °C (for 30 sec), and extension at 73 °C (for 30 sec) in 35 cycles; the final extension step was set at 73 °C (for 5 min). Also, the PCR reaction products were isolated by electrophoresis on a 2% agarose gel mixed with safe stain and observed on a UV transilluminator.

Therefore, first, sterile distilled water was prepared for the number of samples under study as well as positive controls of *E. granulosus* species (G1, G3, and G6 from the Master Mix and primers) and divided into microtubes. Finally, the predicted amount of DNA with the same concentration from each sample was added to the microtubes of samples and controls. After placing the lids on the microtubes, they were numbered and placed in Realtime device (Bio-Rad).

We have used sequenced and identified *Echinococcus* species samples from the *Echinococcus* Research Center of Kerman University of Medical Sciences as positive controls.

After extraction, the purity of DNA was checked and confirmed with a Nanodrop device at a wavelength of 260/280.

We used SPSS 20 (IBM Corp., Armonk, NY, USA) statistical software and Fisher's exact test to investigate the relationship between hydatid cyst genotype and the type of infected organ (liver, lung, kidney, etc.).

Next, the PCR products were sequenced by Bioneer Corporation (South Korea). Also, we performed nucleotide sequence analysis with BLAST (http://www.ncbi.nlm.nih.gov), but alignments were undertaken by the use of such software packages as ClustalX and BioEdit. Later, the *COX1* sequences of the representative isolates were sent to GenBank. Also, a phylogenetic tree was rendered through Molecular Evolutionary Genetics Analysis (MEGA7). The sequences we obtained in the present study and those from the reference

sequences of *E. granulosus* genotypes (G1-G10) existing in the GenBank were compared so that the dendrogram was drawn. Consequently, *Taenia saginata* was regarded as the outgroup in the model.

Accordingly, we obtained the evolutionary history using the maximum likelihood (ML) approach and drawing on the Kimura 2-parameter model. Automatically, the Primary tree(s) were obtained after the neighbor-joining (NJ) method was applied to a matrix of pairwise distances estimated utilizing the maximum composite likelihood method and subsequently selecting the topology with a higher log likelihood value. Finally, the representative tree was appropriately scaled for drawing.

Results

We successfully performed the PCR amplification of the *COX1* region of rDNA on 46 animal DNA samples (13 sheep and 36 cattle samples) and found that the amplicons produced were approximately similar to those of the sheep strain (Fig.1).

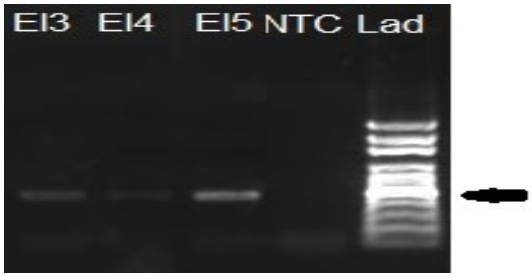

Fig. 1: Agarose gel electrophoresis of *COX1*-PCR (446 bp) products of E. granulosus isolates from livestock Lanes 1, 2, 3 (E13, E14, E15) *COX1* gene, Lane 4(NTC): negative control lane 5: ladder 100bp

The PCR samples included hydatid cysts (38 liver and 11 lung samples). Upon the negative control in each PCR run, we noted no amplification. In total, 27 representative amplicons of *COX1* DNA from the obtained isolates were submitted to sequencing. The partial consensus sequences of the *E. granulosus COX1* rDNA region of the isolates were achieved; these were compared with the reference sequence genotype G1 (Accession No. MG 322623.1) and others stored in the GenBank. After aligning the obtained sequences with the reference sequence, 99.9% homology was noted, and the isolates were found to be corresponding with *E. granulosus* genotype G1 (Fig. 2).

Phylogenetic analysis

Using *COX1* sequencing, we generated the Phylogenetic trees (Fig. 3). Also, the alignment process was done using ClustalW; then the aligned sequences were manually refined in BioEdi software (version 7.2.5); also, maximum likelihood (ML) trees were inferred by MEGA 7 software. Finally, we assessed the nodal support through bootstrapping with 1000 replicates.

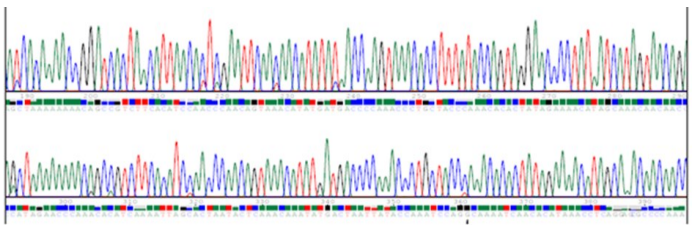

Fig. 2: Partial sequence alignment of the *COX1* rDNA region of *E. granulosus* isolates was compared with reference strains (G1 genotype: GenBank Accession No. MG 322623.1)

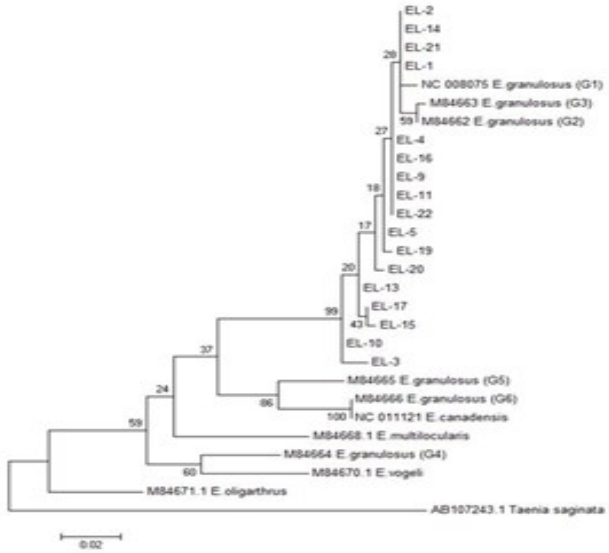

Fig. 3: Molecular phylogenetic analysis by maximum likelihood method

HRM

Out of 20 samples obtained from Sabzevar slaughterhouse, (13 liver samples and 7 lung samples) 65%, 35%, respectively; also, out of 15 samples obtained from Neyshabour Slaughterhouse (8 liver samples and 7 lung samples) related to 21% and 14%, respectively, were reported.

Fig. 4 shows the normalized melting analysis curves between the examined and standard samples. The normalized view of the graph is concerned with *E. granulosus* samples (G1 and G6) in the temperature range of 80-82 °C. The HRM analysis of genes is determined by using standard samples determined in advance. On the other hand, melting analysis curves of G1 and G6 genotypes were used as standards to determine the samples. As can be seen, different genotypes are close to the known standard sample.

Fig. 5 shows the melting analysis curves separating the examined and standard samples. The HRM analysis of genes is determined by using pre-determined standard samples. Melting analysis curves of G1, G3, and G6 genotypes have been used as standards to determine the samples. Hence, different genotypes are close to the known standard sample.

Table 1 shows the frequency of genotypes G1 and G6 in cow and sheep samples.

Table 2 shows the Mean Melting point (TM) and standard deviation (SD) calculated for each genotype of *E. granulosus*

Table 1: Frequency of Genotypes *E. granulosus*

Genotype Sample type	G1 N(%)	G3 N(%)	G6 N(%)
Sheep	17(89.5)	0	2(10.5)
Cow	15(93.7)	0	1(6.3)

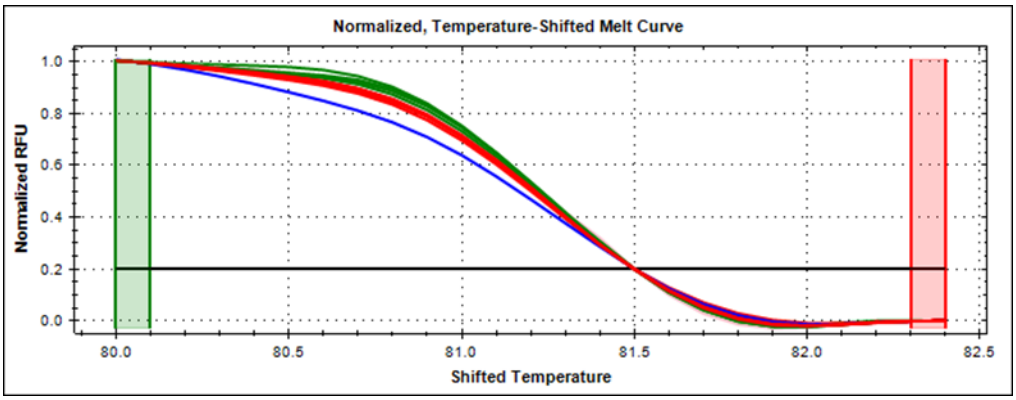

Fig. 4: HRM analysis for samples in normal graph

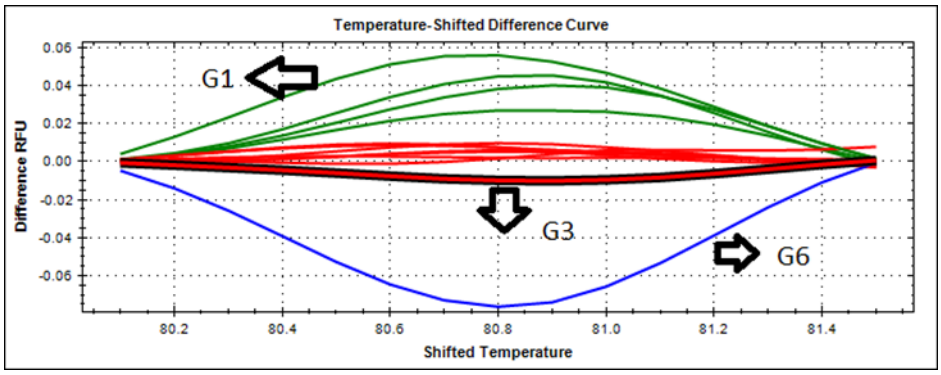

Fig. 5: HRM analysis for samples in the discriminant graph

Table 2: Mean Melting point (TM), Standard Deviation (SD) calculated for each genotype of *Echinococcus granulosus*

No	Genotype	TM	SD
1	G1	81.10	0.13
2	G3	81.50	0.12
3	G6	81.90	0.14

Discussion

The results of this study primarily indicated that the genetic identity of hydatid cysts in a region could be determined with simple methods such as HRM. The dominant species in this region were G1 and, secondly, G6 (camel species). In various studies conducted by researchers in different regions of Iran, other genotypes such as G1, G2, G3, and G6 were also found (12-29) (Table 3).

Each of the genotypes G1 to G10 has a different main reservoir, life cycle, and pathogenicity; for example, G1 is the most common and pathogenic genotype for humans. Knowing the dominant genotype shows which animals play a key role in the transmission cycle. Also, it is possible to determine whether transmission occurs mainly through the dog-sheep cycle or, for example, the dog-camel cycle. This is important for designing a disease control and prevention program (15,26,30).

Arbabi and his colleagues conducted a similar study on molecular identification and genotyping of *E. granulosus* strains isolated from camels and dogs in Isfahan; they identi-

fied mitochondrial genes *COX1 and NAD1*. Unlike our study, where we did not find the G3 genotype, their study showed the presence of sheep (G1), cow (G3), and camel (G6) genotypes in this region; the predominant genotype in both hosts was the common sheep strain (G1) (30).

In Ilam, a similarity between the size of DNA bands of *E. granulosus* in dog isolates and sequencing, was reported which indicated the presence of similar genotypes of the parasite in the region, where there was at least one genotype of the parasite that belonged to *the E. granulosus* complex (G1-G3) (31).

Jafar Zadeh and his colleagues conducted another study on determining the genotype of *E. granulosus* from paraffin tissues of hydatid cysts operated in North Khorasan. PCR-RELP was performed on the *ITS1* gene using Rsa I and Hep II restriction enzymes. In their study, a phylogenetic study was conducted using the mitochondrial *COX1* gene. The results obtained with the enzyme cut pattern of the *ITS1* gene as well as the *COX1* gene synonym and phylogenetic tree drawing showed that all genotypes were G1 or sheep strain but unlike our study, they did not obtain the G6 genotype (32).

Abedi et al conducted a study on determining the genotype of *E. granulosus* isolated from livestock using mitochondrial *COX1* in the Central Province. Unlike our study, they found the G2 genotype in addition to G1.In their study, the main genotypes were identified as G1 (61%) and G3 (37%). Also, one of the samples showed a genotype similar to G2 genotype; they showed that there was a significant difference between the genotypes of the samples, and that the main genotypes of *E. granulosus* in the urban regions of the Central province were G1 and G3(33).

Rostami et al. used the HRM technique to differentiate the G1, G3, and G6 genotypes of *E. granulosus* (Sensu lato), where 280 *E. granulosus* samples were obtained from sheep, cattle, and camels. The percentages of animals infected with different strains of sheep, cattle, and camels were reported as 93, 94, and 25 percent (G1), 4, 7, and 4 percent (G3); and 0, 2, and 71 percent (G6). The HRM results were completely consistent with the results obtained from sequencing (as a reference method) and their hook measurement. Unlike our study, where we did not find the G3 and G2 genotypes in this study, the G2 genotype was reported for the first time in humans in Iran. Due to the easy transportation of livestock from one city to another, it is possible to observe each of the genotypes mentioned in various studies within the country in any city. This research showed that molecular evaluation with the help of the HRM method can be used as a molecular epidemiological screening tool (34).

There were several limitations to the HRM method, including:

1. Sensitivity to DNA Quality: HRM analysis is highly sensitive to the quality and integrity of the DNA used. Degraded or contaminated DNA can lead to inconsistent melting profiles, which may affect the accuracy of genotype identification. Therefore, the quality of the extracted DNA must be carefully monitored to ensure reliable results.

2. Resolution Limitations: While HRM is an excellent tool for detecting small genetic variations, it may have limitations in distinguishing large genomic changes such as insertions, deletions, or large structural variations. This can affect the accuracy of results in detecting certain mutations or genotypes.

3. Interpretation of Melting Profiles: The interpretation of melting curves in HRM requires careful analysis, and subtle differences in melting temperature (Tm) may be difficult to distinguish in certain cases. The presence of overlapping curves or slight variations in Tm can complicate genotype calling, especially when dealing with closely related genotypes or variants with minimal genetic differences.

Table 3: Published information concerning *E.granulosus* isolated in different regions of Iran

No.	Number of cases	Source	Area	Strains	Methods	Gene	Reference
1	60	Human(liver)	Mashhad	G1,G6	RFLP, Sequencing	*ITS1,COX1, NAD1*	12
2	47	Human(liver,lung,Spleen)	Tehran	G1,G3	Sequencing	*COX1,NAD1*	13
3	49	Domestic Animal	Central Province	G1,G2,G3	Sequencing	*COX1*	14
4	22	Dog,Jackal(adult worm)	Ilam	G1-G3	RFLP, Sequencing	*NAD1*	15
5	50	Human(liver,lung,Spleen)	Khorasan Razavi	G1-G3 Senso strico	RFLP	*ITS1*	16
6	55	Human,Domestic animal	Ardabil	G1,G3	Sequencing	*COX1,NAD1*	17
7	43	Human	Busher	G1	Sequencing	*COX1*	18
8	9	Dog,Jackal(adult worm)	Ardabil	G1	Sequencing	*COX1,NAD1*	19
9	72	Human(liver)	Hamedan	G1,G3	Sequencing	*COX1,NAD1*	20
10	122	Domestic Animal	North Khorasan	G1,G3,G6	Sequencing	*COX1*	21
11	15	Dog,Jackal(adult worm)	Mazanderan	G1,G3	Sequencing	*COX1*	22
12	38	Dog(adult worm)	Khorasan Razavi	G1	RFLP	*ITS1*	23
13	8	Human(liver,lung)	Birjand	G1,G6	Sequencing	*COX1,NAD1*	24
14	23	Human	Esfahan	G1-G3	Sequencing	*COX1,NAD1*	25
15	55	Human	Azerbaijan	G1	RFLP	*ITS1*	26
16	30	Human	Golestan	G1	RFLP	*ITS1*	27
17	17	Human	Fars	G1,G6	Sequencing	*NAD1*	28
18	8	Dog	East Azerbaijan, Ardabil	G1	Sequencing	*COX1,NAD1*	29

4. Device and Protocol Variability: The performance of HRM can vary depending on the specific instrument and protocol used. Differences in equipment calibration, reagent quality, or experimental conditions can affect the reproducibility of the results. To mitigate this, standardization and validation of the method are essential for consistent outcomes.

5. Limited to Known Variants: HRM is best suited for detecting known genetic variations. Novel or rare mutations that are not represented in the reference database may not be easily detected using HRM, highlighting the need for complementary methods such as sequencing to confirm and identify new genetic variants (8,9).

It is recommended that standard genotyped samples be available at a reference center so that researchers can use these samples to determine the genetic identity of samples from their own region using the HRM method.

Ethics statement

The present research project was scientifically reviewed and approved by the Ethics Committee at Sabzevar University of Medical Sciences, Iran (Ethics Code: IR.MEDSAB.REC.1398.104).

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Conflict of interest

No conflict of interest.

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