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Original Article

Genetic Characteristics of *Echinococcus granulosus* from Fixed Paraffin-Embedded Tissue Samples in Human Isolates Based on the High-Resolution Melting Point Analysis in Sabzevar, Northeast Iran

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Abstract

Background: There are ten genotypes of *Echinococcus granulosus* with different intermediate and final hosts affecting the parasite's life cycle and its transmission to humans. Therefore, this study was conducted to determine the genotype of isolated hydatid cysts using the simple and fast high-resolution melting point analysis (HRM) method.

Methods: The paraffin tissue samples of patients who underwent surgery were obtained from the pathology sample bank of Vasei and Emdad Hospitals in Sabzevar, Iran during 2010-2020. The DNA content of the samples was extracted after collecting and determining the characteristics using the DNA extraction kit. PCR was performed on the samples and the presence of the hydatid cyst genome was confirmed using the special Master Kit. Mix PCR of Solis Biodyne Company and Real-Time device (Bio-Rad) were used, and the genetic identity of hydatid cysts were determined.

Results: Out of 33 paraffin samples, 21 samples contained hydatid cyst DNA, two of which were from the brain and 19 from the liver tissues; 12 samples did not contain hydatid cyst DNAs. All liver samples were from sheep species (G1), and the brain samples were from buffalo species (G3). Therefore, 9.53% of the *Echinococcus* species collected were buffalo (G3), and 90.47% were sheep (G1) strain.

Conclusion: Based on previous patterns, HRM methods can be used for easy and quick identification of *Echinococcus* strains. The G1 strain was the dominant strain causing hydatid cyst in different human organs, including the liver and brain.

Introduction

Cystic echinococcosis (CE) or hydatid cyst is a chronic helminthic zoonotic disease distributed globally (1,2). This disease is widespread in sheep breeding countries or rural areas where animals are slaughtered unhygienically (3,4) in which CE or hematic cyst disease are endemic. Earlier studies, conducted in Iran, showed that hydatid cysts are common in sheep (5 to 72%), camels (11.4 to 70%), cows (3.5 to 38%), and goats (1.7 to 20%). Such human infections were continuously reported from different regions of the country (5). The organism causing this disease is the dog tapeworm or *E. granulosus*, which exists cyclically among carnivores and several herbivorous mammals (including sheep, camels, horses, cows, pigs, other mono-poisons, and wild herbivores). Humans are also considered as accidental intermediate hosts for the parasite. CE has significant economic and medical effects on livestock and humans in many parts of the world, including Iran (6).

This strain has a wide genetic diversity, and ten genotypes of different intermediate hosts have been reported for *E. granulosus* (G1-G10) so far, each of which is more compatible with a specific host. G1 is compatible with sheep, G2 Tasmanian sheep, G3 buffalo, G4 horse, G5 cow, G6 camel, G7 pig, G8 to G10 wild cycle (deer), and G9 milk strain; in addition, some of these strains have been reported in human cases (7).

Considering the lack of studying the genotype of human hydatid cyst in Iran and the importance of this disease to its population, the genotype of the disease-causing parasite was examined in the present study. In order to determine the parasite strain and to identify the main and intermediate hosts for better management and controlling this disease, the present study was designed to determine the molecular characteristics of human hydatid cyst strains in Sabzevar, Iran.

Materials and Methods

Area of Sampling

This study was conducted in Sabzevar, a northeastern city in Razavi Khorasan Province, Iran (Fig. 1). Its population is around 244,000 people; the area is a desert-like region with hot and dry climate at a longitude of 57° 42' 59.99" E and a latitude of 36° 11' 60.00" N. Its height above sea level is 978 meters, and the average annual rainfall is 191 mm, with an average annual temperature of 17.6 °C (8).

Fig. 1: The location of Sabzevar city in the map of Iran

Sampling

This study was carried out retrospectively during 2010 to 2020 in two major hospitals and medical training centers affiliated to Sabzevar University of Medical Sciences, Iran. The sample randomly included individuals who underwent surgery with a definitive diagnosis of the hydatid cyst disease.

Paraffin tissue samples of the patients, which were obtained from the bank of pathology samples at the laboratory of both Vasei and Emdad Hospitals of Sabzevar, Iran, were used. After collecting and determining the characteristics of the samples, they were transferred to the molecular cell laboratory at the medical school. After deparaffinization, the tissue inside the paraffin blocks was cut with a scalpel blade and placed inside a sterile microtube. Afterwards, 1000 microliters of xylenol were added to the tissue to dissolve the remaining paraffin completely. The numbered microtubes were then placed on the hot plate for 24 hours. The tissues were removed from the microtube and placed in a sterile one.

DNA extraction

The DNA extraction was done using Gent Bio's DNA extraction kit according to the manufacturer's protocol.

HRM assay

In order to identify *E granulosus* and determine the DNA sequence, a bio-Rad device with an HRM program was used. As control samples, the samples that were genotyped as well as the standard samples of certain genotypes such as G1, G3, and G6 were used to compare the analyses (9). The 300bp regions of the mitochondrial cox1 gene were commonly used to identify *Echinococcus* species (9). R/JB5 was amplified using primers (F/JB3 (5'-TTTTTGGGCATCCTGAGGTTTAT-3' and 5'-TAAAGAAAGAACATAATGAAAATG-3) (10).

In order to carry out the reaction in a volume of 20 microliters, first we prepared the samples together with the positive controls of *E. granulosus* species (G1, G3, G6) from Master Mix and primers and some sterile distilled water, and then we divided the volumes into microtubes. The predicted amount of DNA was added to the microtubes of samples and controls with the same concentration. After putting the lid on the microtubes, they were numbered and placed in the Real-Time device (Bio-Rad).

The thermal program was done for 10 seconds at 95 °C for denaturation, 15 seconds at 55 °C for annealing, and 10 seconds at 72 °C for final elongation. The cycle was repeated for 40 minutes for the replication of fragments.

In the current study, the genotype of hydatid cysts was investigated. The relationship between the genotype of hydatid cysts and the type of infected organ (e.g., liver, lung, kidney, and other organs) were also examined.

Ethical considerations:

The study was retrospectively conducted on tissue samples rather than human beings; the samples were obtained from the hospital bank of samples with the permission of the university research committee. Also, the study design was approved by the Ethics Committee at Sabzevar University of Medical Sciences, Iran (IR.MEDSAB. REC.1400.083).

Results

Overall, 33 samples were collected from two major hospitals in Sabzevar, Iran; two samples (from the liver tissue) were obtained from Vasei Hospital. Also, 31 samples were collected from Emdad Hospital, two belonging to the brain and 29 to the liver.

From these 33 paraffinated samples, DNA of hydatid cysts was extracted from 21 samples, while the DNA of hydatid cysts was not extracted from 12 samples. Two of the extracted samples were from brain and 19 from liver tissues. The G6 genotype were not identified in the studied samples, and 12 DNA samples were not extracted (Table 1).

Table 1: Mean Melting point (TM), Standard Deviation (SD), and percentage of each genotype calculated for each genotype of *Echinococcus granulosus*

No	*Frequency*	*Sample type*	*Genotype*	*Percent*	T_M	*SD*
1	19	Liver	G1	90.48	81.4	0.1
2	2	Brain	G3	9.52	81.9	0.07

PCR melting curve results showed that the average melting temperature for G1 and G3 were 40.81 and 90.81 °C, respectively. The melting and HRM curves are shown in the normal and separation graphs in Fig. 2 and 3.

Fig. 2 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 G3) in the temperature range of 80-84 °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 G3 genotypes were used as standards to determine the samples. As can be seen, different genotypes are close to the known standard sample.

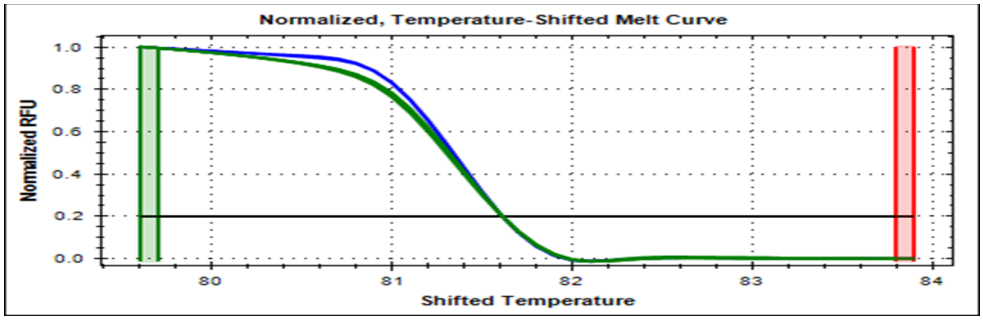

Fig. 2: The HRM analysis for samples in the normal graph

Fig. 3 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 and G3 genotypes have been used as standards to determine the samples. Hence, different genotypes are close to the known standard sample.

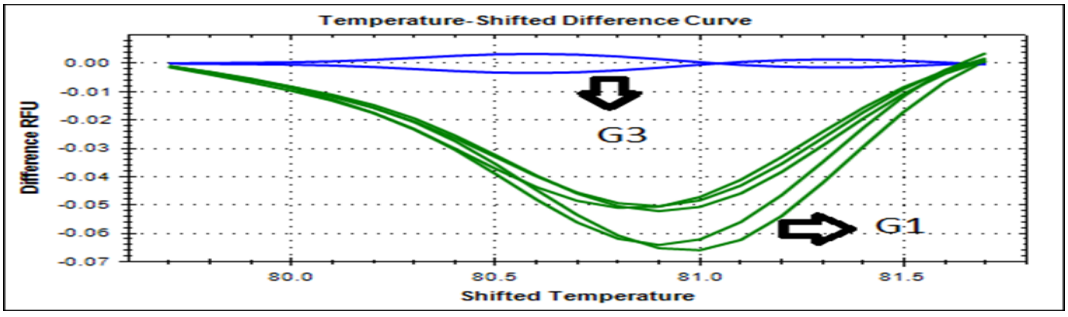

Fig. 3: The HRM analysis for samples in the discriminant graph

Color separation was used to identify the species in the charts. Therefore, the green color was used in the G1 separation graph, and blue was used in the G3 separation graph.

Discussion

The present study was conducted to determine the genotype of isolated hydatid cysts using the HRM method in Sabzevar, a northeastern city in Iran. The study results primarily indicated that the genetic identity of hydatid cysts in a region could be determined with simple methods such as HRM methods. The dominant species in this region were G1 or the sheep species. In various studies conducted by researchers in different regions of Iran, other genotypes such as G1, G3 or G6 were also found (11-36) (Table 2).

Table 2: Published information concerning *Echinococcus granulosus* isolated in different regions of Iran

No.	*Frequency*	*Source*	*Area*	*Strains*	*Methods*	*Gene*	*References*
1	50	Human (liver, lung, Spleen)	Khorasan Razavi	G1-G3 Senso strico	RFLP	ITS1	11
2	22	Dog, Jackal (adult worm)	Ilam	G1-G3	RFLP, Sequencing	nad1	12
3	49	Domestic Animal	Central Province	G1, G2, G3	Sequencing	cox1	13
4	47	Human (liver, lung, Spleen)	Tehran	G1, G3	Sequencing	cox1, nad1	14
5	60	Human (liver)	Mashhad	G1, G6	RFLP, Sequencing	ITS1, cox1, nad1	15
6	55	Human, Domestic animal	Ardabil	G1, G3	Sequencing	cox1, nad1	16
7	43	Human	Busher	G1	Sequencing	cox1	17
8	9	Dog, Jackal (adult worm)	Ardabil	G1	Sequencing	cox1, nad1	18
9	72	Human (liver)	Hamedan	G1, G3	Sequencing	Cox1, nad1	19
10	20	Human (liver)	Iran	G1, G3	Sequencing	nad5	20
11	122	Domestic Animal	North Khorasan	G1, G3, G6	Sequencing	cox1	21
12	120	Domestic Animal, Human	Tabriz	G1	Sequencing	cox1	22
13	15	Dog, Jackal (adult worm)	Mazanderan	G1, G3	Sequencing	cox1	23
14	38	Dog (adult worm)	Khorasan Razavi	G1	RFLP	ITS1	24
15	19	Human (liver, lung)	Yazd	G1, G6	Sequencing	cox1	25
16	8	Human (liver, lung)	Birjand	G1, G6	Sequencing	cox1, nad1	26
17	62	Domestic Animal, Human	Mazanderan	G1, G2, G3, G1-G3	Sequencing, RFLP	cox1, ITS1	27
18	23	Human	Esfahan	G1-G3	Sequencing	cox1, nad1	28
19	4	Human	Tehran	G1-G3	Sequencing, RFLP	cox1, nad1, ITS1	29
20	55	Human	Azerbaijan	G1	RFLP	ITS1	30
21	29	Human	Tehran	G1, G3, G6	Sequencing	cox1, nad1	31
22	30	Human	Golestan	G1	RFLP	ITS1	32
23	11	Human	Ardabil	G1, G3	Sequencing	cox1, nad1	33
24	17	Human	Fars	G1, G6	Sequencing	nad1	34
25	1	Human	Kerman	G6	Sequencing	cox1, nad1	35
26	17	Domestic Animal	Sabzevar	G1	Sequencing	Cox1	36

In a study by Sadri et al., the characteristics of hydatid cysts isolated during 2001-2002 from slaughtered animals in the industrial slaughterhouse of Yasouj, Iran was determined by PCR-RFLP method. Their research aimed to determine the molecular characteristics of hydatid cyst strains and their spread in the liver and lungs of animals slaughtered in the industrial slaughterhouse of Yasouj, Iran (37). In thir study, 93 animal hydatid cysts (31 sheep samples, 56 goat samples, and six cow samples) were collected from Yasouj industrial slaughterhouse; genomic DNA was extracted from the corresponding protoscolex by the standard phenol-chloroform method. They used PCR to amplify the rDNA-ITS1 fragment of each sample with EGR and EGF primers. The PCR products were first examined by electrophoresis and then cut with Alu I and Rsa I enzymes. The RFLP-PCR products of the said fragments were electrophoresed and investigated. The PCR method obtained the size of rDNA-ITS1 fragments of all liver and lung samples with similar bands and size of 1000bp. The pattern created using the RFLP test with AluI and RsaI enzymes showed the G1 genotype, the same sheep genotype of *E. granulosus*. The Yasouj study revealed that strain G1 was the dominant strain causing hydatid cyst disease in different organs of livestock, including the liver and lungs (37).

In the present study, a specific primer of the cox1 gene was used to identify different species of *E. granulosus*. Our results as presented in the graphs of the test samples' control samples (standard), showed the characteristics of the double-stranded DNA product based on its melting behavior. In addition, based on HRM PCR analysis, 19 samples of G1 genotype and two samples of G3 were identified from the total of 21 samples examined, where the G1 genotype was detected only in 19 sheep samples (90.48%), and G3 genotype was detected only in 2 buffalo samples (9.52%). However, the G6 genotype was not detected in our samples. Therefore, the predominant genotype found in Sabzevar, Iran was the G1 genotype. Therefore, our findings are consistent with the above-mentioned study conducted in Yasouj, Iran (37), even though the study methods were different.

In another study, 46 hydatid cyst samples, 18 obtained from female and 28 from male cases, were subjected to molecular analysis (38). Then, the samples were identified using PCR and sequencing methods, where all the samples showed G1 or sheep genotype in humans (38). Still another study conducted in southwest Iran examined 334 hydatid cyst samples collected from the liver and lungs of 141 sheep, 104 calves, 84 goats, and 5 human samples (39). DNA extraction and PCR of the ITS1 fragment were done. Then RFLP was performed and fragments of the ITS1 gene were sequenced. Their results showed that the G1 genotype was present in all human, bovine, goat and sheep samples but the camel strain (G6) was not present among the samples. In line with the present study, their molecular findings also indicated that the dominant genotype in southwestern Iran was the sheep strain (G1), which is common among human populations, cattle, sheep, and goats (39).

In Kania region of Turkey during 2018-2019, after DNA extraction and sequencing of 83 cysts (57 bovine and 26 human cysts), 82 cysts with Sensu strict genotype (G1-G3) were identified together with several human cysts as G4 (equinus) (40). Moreover, Shafiei et al., examined the relationship between parasite genotype and hydatid cyst location, in which they found that some liver cysts had a G1 genotype. In contrast, six cysts related to the peritoneum, kidney and brain were G2, but one of the pelvic cysts was identified as G3 (41).

Moreover, Hedayati and colleagues collected 47 paraffin blocks related to patients with hydatid cysts in Mazandaran, the coastal province to the Caspian Sea in the north of Iran. After deparaffinization, PCR, and sequencing, it was determined that 66.6% of the samples had G1 genotype while 33.3% had G1 geno-

type, which were related to G2-G3 genotypes (42).

Conclusion

All liver samples were G1, and all brain samples were G3. Therefore, 9.52% of the *Echinococcus* species collected were G3 (buffalo species), while 90.48% were G1 (sheep species). The predominant strain of the *E. granulosus* parasite in Sabzevar, a city located to northeast of Iran, was the same as other reports from other parts of Iran. It is worth mentioning that this parasite is the main host in sheep and dogs, and the intermediate hosts are the cattle.

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Conflict of interest

The authors declare that there is no conflict of interest.

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