

Original Article

## Genotypic characteristics of hydatid cysts isolated from humans in East Azerbaijan Province (2011-2013)

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### Abstract

**Introduction:** Cystic echinococcosis (CE) is one of the important helminthic diseases of human and animals, which causes by *Echinococcus granulosus*. Canids are its definite and grazers especially sheep, and cattle, and also wild herbivores are its intermediate hosts. Human can also be accidentally infected by a parasite. This study aimed to investigate genotypes of the hydatid cysts isolated from hydatidosis patients in order to confine the source of the infection, 2013.

**Methods:** In this cross-sectional study 55 paraffin blocks of identified hydatid cysts have been undergone genotyping using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The ITS1 region of rDNA has been amplified using BD1 forward and 4s reverse primers. PCR products have been digested using HpaII and RsaI restriction endonucleases. RFLP products studied using gel electrophoresis. Data were analyzed using SPSS for Windows using the chi-square test.

**Results:** About 29 (52.72%), 16 (29.1%), 3 (5.45%), 3 (5.45%), 1 (1.81%), 1 (1.81%), 1 (1.81%) and 1 (1.81%) out of 55 hydatid cysts were located in lung, liver, spleen, kidney, heart, pancreas, brain, and femore, respectively. The frequency of hydatidosis observed higher in patients from rural areas ( $P = 0.013$ ; odds ratio = 0.599; 95% confidence interval: 0.28, 1.27). Based on RFLP results, the entire studied hydatid cysts identified as sheep strain (G1).

**Conclusion:** According to the results of the present observation, it can be concluded that the majority of cases of human hydatidosis in East Azerbaijan Province are caused by sheep strain (G1) of *E. granulosus*, which indicates the sheep-dog cycle in the studied area.

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### Introduction

*Echinococcus granulosus* is a parasitic tapeworm infects canids as definite hosts and causes hydatidosis mostly in herbivores as intermediate hosts.<sup>1,2</sup> Humans can be accidently infected by ingesting its eggs, which results in human cystic echinococcosis (CE) or hydatidosis.<sup>2</sup> CE is an important and sometimes life-threatening disease, which mostly affects lungs and liver, but other organs can be also affected.<sup>2,3</sup>

Despite the fact that the prevalence of parasitic diseases has been decreased over the past decades,<sup>4,6</sup> hydatidosis is remaining as a health problem in Iran. CE is an important zoonotic disease, which is distributed throughout the country. It is estimated that it is responsible for almost 1% of surgical operations in Iran.<sup>7</sup> Different genotypes of *E. granulosus* have been identified from a variety of hosts worldwide. Until now, 10 genotypes (G1-G10) has been described.<sup>8,9</sup> The G1 and G2

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strain are called sheep strains, G3 and G5 bovid strains, G4 horse and G6 camel strain, G7 pig strain, and G8 and G10 cervid strains. The genotypes are different in some criteria such as pathogenicity, host specificity, pattern of life-cycle, transmission dynamics and developmental rates, human infectivity and response to chemotherapeutic drugs.<sup>7</sup> The transmission patterns in each region are related to diversity of the reservoirs of the parasite. In Iran, the presence of G1, G3, and G6 strains have been reported, but the sheep strain (G1) is the most prevalent genotype.<sup>7</sup> Polymerase chain reaction (PCR) based methods such as PCR-restriction fragment length polymorphism (RFLP) has been vastly used for genotyping of *E. granulosus*.<sup>10-14</sup> This study aimed to investigate genotypes of the hydatid cysts isolated from hydatidosis patients in order to confine the source of the infection in East Azerbaijan, Iran using PCR-RFLP technique.

## Methods

To study the transmission patterns of *E. granulosus*, genotypic analysis was performed on hydatid cysts obtained from 55 paraffin blocks of CE patients that have been surgically operated in Imam Reza Hospital, Tabriz, North West of Iran. PCR method was done Using BD1 (forward) and 4s (reverse) primers. PCR products were digested using HpaII and RsaI restriction endonuclease.

In this cross-sectional study totally 55 paraffin blocks of hydatid cysts isolated from hydatidosis patients that are surgically operated during 2011-2013 in Imam Reza Hospital, Tabriz, have been collected. Demographic variables of patients such as sex, age, the affected organ and residential status have been also gathered from their hospital documents and interviewing.

Deparaffinization of the paraffin embedded samples was performed according to the method described by Schneider et al.<sup>15</sup> The brief procedure of deparaffinization is as follows. The paraffin blocks were cut into 6-10  $\mu\text{m}$  sections with a quick rotation of the microtome wheel. The tissue sections incubated with xylol in 37 °C for 10 min. After the incubation had ended, they

centrifuged at 15000g for 5 min and then the supernatants were discarded. The sedimented materials kept in 70% ethanol and then subjected DNA extraction.

DNA extraction has been performed using phenol-chloroform technique using CTAB as summarized below. Hydatid cysts samples underwent enzymatic digestion by proteinase K and sodium dodecyl sulfate for 2 days at 50 °C. Then 700  $\mu\text{l}$  isoamyl alcohol and chloroform mixture added to the digested parasite particles and centrifuged at 4000 rpm. The supernatants transferred to new microtubes and 1:1 volume alcohol 2-propanol added to the mixture then kept in -20 °C for 30 min. After freezing time, samples centrifuged at 1400 rpm and the supernatant discarded, then 1 cc 70% ethanol added and centrifuged at 14000 rpm. The supernatants discarded and microtubes in placed on the absorbent paper in the opposite side and dried in room temperature. At the end 300  $\mu\text{l}$  Tris-ethylenediaminetetraacetic (EDTA) acid buffer added to the microtubes and kept in 4 °C.<sup>16</sup>

PCR has been performed using BD1-F5'-GTCGTAACAAGGTTTCCGTA-3' and 4S-R3'-TCTAGATGCGTTCGAA(G/A)TGTCGATG-3' primers (CinnaGen, Iran) for amplification of ITS1 region of parasite rDNA.<sup>17</sup> PCR amplification performed in 50  $\mu\text{l}$  volume containing 5  $\mu\text{l}$   $\times 10$  PCR buffer (500 mmol KCl and 200 mmol Tris-HCl), 1  $\mu\text{l}$  deoxynucleotide triphosphate mix (2 mmol), 0.5  $\mu\text{l}$  BD1 primer (100  $\mu\text{mol}/\mu\text{l}$ ), 0.5  $\mu\text{l}$  4S primer (100  $\mu\text{mol}/\mu\text{l}$ ), 0.3  $\mu\text{l}$  Taq polymerase 5 u/ $\mu\text{l}$ , 5  $\mu\text{l}$  template DNA, 35.7  $\mu\text{l}$  deionized water (DNase and RNase free) and 2  $\mu\text{l}$  MgCl<sub>2</sub> (50 mmol). PCR thermal cycling condition were as follow: 5 min of pre-denaturation at 95 °C, then 30 cycles of 60 s denaturation, 60 s annealing, and 72 s extension at 95 °C, 55 °C, and 72 °C, respectively.

PCR products were underwent electrophoresis in 1% Agarose with 80 V for 90 min using Loading buffer (Jena Bioscience, Germany), 100 bp DNA ladder (Jena Bioscience, Germany). The gel stained by ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ) and studied

under UV light using transilluminator device (Figure 1).<sup>18,19</sup> The PCR products of ITS1 (900 bp) region were subjected to DNA purification for RFLP using High Pure PCR Product Purification kit according to the manufacturers' instruction (Bioneer, Korea).

The purified PCR products subjected to enzymatic digestion by HpaII and RsaI restriction endonucleases following. Digestion with RsaI enzyme performed in 20 µl volume containing 11.5 µl sterile double distilled water, 2 µl RsaI ×10 buffer, 1.5 µl RsaI enzyme (10 u/µl) and 5 µl purified PCR product. The mixture has been incubated at 37 °C for 2 h. Also, enzymatic digestion with HpaII restriction endonucleases carried out in 20 µl volume containing 11.5 µl sterile double distilled water, 2 µl HpaII × 10 buffer, 1.5 µl HpaII enzyme (10 u/µl) and 5 µl purified PCR product. The mixture has been incubated at 37 °C for 2 h.<sup>16</sup> After the enzymatic digestion the products stained by ethidium bromide (0.5 µg/ml) and studied under UV light using transilluminator device. Data were analyzed using SPSS for Windows (version 16, SPSS Inc., Chicago, IL, USA) software using the chi-square test.

**Results**

**Demographics**

About 24 (43.63%) and 31 (56.37%) out of 55 hydatidosis patients were male and female, respectively. The mean age of the patients

was 35 years old (13-87 years old). About 29 (52.72%), 16 (29.10%), 3 (5.45%), 3 (5.45%), 1 (1.81%), 1 (1.81%), 1 (1.81%) and 1 (1.81%) out of 55 hydatid cysts were located in lung, liver, spleen, kidney, heart, pancreas, brain, and femore, respectively. Lung was the most affected (52.72%) and heart, bone, pancreas and brain were the less affected organs (1.81%). Frequency of hydatidosis observed higher in patients from rural areas (P = 0.013; odds ratio = 0.599; 95% confidence interval: 0.28, 1.27) (Table 1).

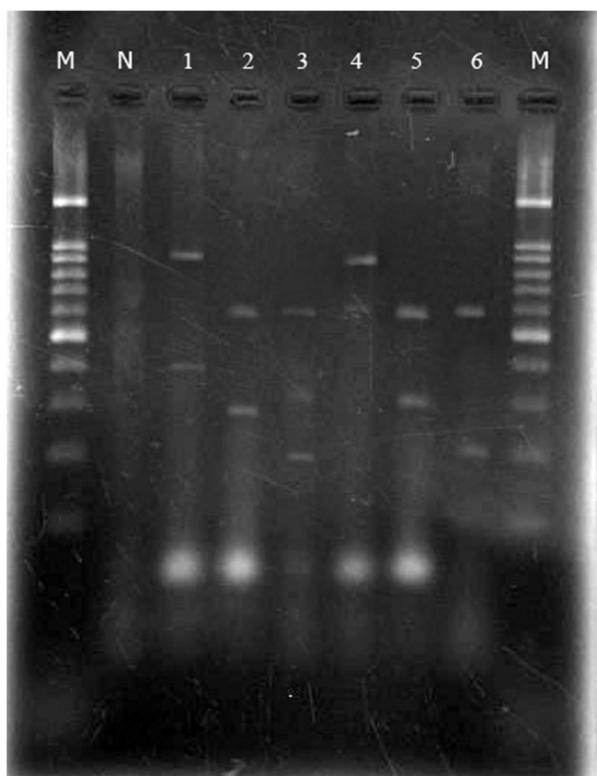
PCR: All samples except one were harboring 900 bps ITS1 region, which is characteristics of hydatid cysts.<sup>16</sup> Different amplification has been observed in one sample, 900 bps and 400 bps. The 400 bps region has been sent to nucleotide sequencing. The results illustrated a high homology of the sequence with region in *Peronospora plantaginis*, but not *E. granulosus* (Figure 1).

RFLP: Digestion of purified ITS1 region by restriction endonucleases showed one distinct pattern for each enzyme. Electrophoresis of all digestion products of RsaI restriction endonucleases showed two different bands, 300 and 600 bps. Furthermore, digestion with HpaII restriction endonuclease resulted in two distinct bands, 200 and 600 bps (Figure 1). Both are identical of sheep strain (G1) of *E. granulosus*.<sup>16</sup> It is concluded that all studied human hydatid cysts were G1 strain of *E. granulosus*.

**Table 1. Odds ratio estimated for hydatidosis among demographic variables**

Variable	Involved organ								Total	OR	95%CI	P
	Lung	Liver	Kidney	Spleen	Heart	Pancreas	Brain	Femore				
Sex												
Male	13	7	1	2	0	0	1	0	24	0.599	0.28, 1.27	0.182
Female	16	9	2	1	1	1	0	1	31	1.000		
Residence												
Urban	11	5	2	1	1	0	1	0	21	0.381	0.17, 0.82	0.013
Rural	18	11	1	2	0	1	0	1	34	1.000		
Age group (year)												
< 20	4	2	0	0	0	0	0	0	6			
20-30	8	6	0	0	1	0	0	0	15			
30-40	7	4	0	1	0	0	0	1	13			
40-50	4	2	2	0	0	0	1	0	9			
50-60	3	1	1	0	0	1	0	0	6	-	-	-
60-70	2	0	0	1	0	0	0	0	3			
70-80	1	1	0	0	0	0	0	0	2			
> 80	0	0	0	1	0	0	0	0	1			

OR: Odds ratio; CI: Confidence interval



**Figure 1. Gel electrophoresis of enzymatic digestion of 900 bp polymerase chain reaction (PCR) products of ITS1 region; M: 100 bp DNA ladder; N: Negative control; 2, 5: Products of enzymatic digestion by *RsaI* enzyme (300 and 600 bp); 3, 6: Products of enzymatic digestion by *HpaII* enzyme (200 and 600 bp); 1: PCR product of ITS1 region (900 bps and 400 bps); 4: PCR product of ITS1 region (900 bp)**

## Discussion

In this study, totally 55 human hydatid cysts have been undergone PCR-RFLP in order to identify their genotypes and source of human infection in East Azerbaijan Province, North West of Iran, during 2011-2013. According to the results of this cross sectional study, sheep strain (G1) is the main source of human hydatidosis in the area. Also, statistical analysis of the demographic variables of the patients revealed that the residents of rural areas, which sheep are the main livestock, are at higher risk of the infection than the others from urban areas. Also, the lung is the most affected organ in the patients and liver takes the second place.

Vahedi and Vahedi, in a study, evaluated the demographic variables and human hydatidosis during 10 years period on 318 patients in East Azerbaijan, Iran. They reported that females were predominant

among hydatidosis patients in the area, which our results are supporting their finding. In the present study, hydatidosis was much prevalent among female patients, but the difference was not significant. In their study, like ours, the age group, 20-30 years old possesses the highest rate of the infection. Also, lung and in the second place liver were the most infected organs.<sup>20</sup> The present study is supporting their finding in most of the aspects, which was expected.

All the human cases studied in this study were infected by G1 strain of *E. granulosus*. This finding is emphasis on the importance of sheep as a source of canine and subsequently human infections. Other studies have also been supporting the idea that G1 strain is predominant in the East Azerbaijan Province, which some are as follows.

Jamali et al. studied the genotypes of human, cattle and sheep hydatid cysts in Tabriz District. They reported all the strains as G1, which is the same as our finding. They concluded that a single strain of *E. granulosus* is the predominant genotype in Tabriz District.<sup>16</sup>

Jafari et al. reported a different genotype of *E. granulosus* in Mazandaran Province, Iran, in paraffin blocks of hydatid cyst samples. Like the present study, they found the region about 400 bps and described them as a new genotype,<sup>4</sup> but in our study, after nucleotide sequencing of the 400 bp PCR product of ITS1 region, a high homology with *P. plantaginis*, a kind of plant, has been observed.

Hanifian et al. studied a similar study in the neighboring Province, West Azerbaijan, but on the isolates from hydatid cyst of sheep and cattle. They amplified the ITS1 region and patterns of fragments of endonuclease digestion of the region showed that a single strain (G1-G3 complex) is also dominant in the West Azerbaijan.<sup>21</sup>

Ahmadi and Dalimi studied the genotypic characteristics of hydatid cysts isolated of human, camel and sheep from different parts of Iran. Their results illustrated that the RFLP pattern of hydatid cysts isolates from human and sheep are the same, but camel isolates were different genotype.<sup>22</sup> Harandi et al.



reported that the G1 strain of *E. granulosus* is the most predominant strain infecting goat, cattle, sheep and camel in Iran. They reported the camel strain from three humans.<sup>23</sup>

Most of the studies reviewed above have emphasized on the importance of G1 strain of *E. granulosus* in human and animal hydatidosis in Iran. The present study also showed that in East Azerbaijan Province a single strain of *E. granulosus*, the sheep strain, is responsible for human hydatidosis. In this area sheep are the most dominant livestock and consequently the most dominant genotype of *E. granulosus* is the G1 strain. These facts, beside the molecular studies, introduce sheep-dog cycle of *E. granulosus* as a human source of hydatidosis.

A successful control program against human hydatidosis cannot be fulfilled unless educating the farmers and in charged people about the importance and life cycle of the parasite in this area. Controlled slaughtering of livestock, especially sheep, in abattoirs

may be also effective.

### Limitations

The sample size of the study and budget were the most important limitations of the study.

### Conclusion

This study shows that the single strain of *E. granulosus*, the G1 strain, is responsible for human hydatidosis in East Azerbaijan Province, which indicates the sheep-dog cycle in this area.

### Conflict of Interests

Authors have no conflict of interest.

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