Original Article

Molecular Characterization of *Echinococcus granulosus* Sensu Lato from Livestock in North Khorasan Province, Iran

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**Abstract**

**Background:** *Echinococcus granulosus* is one the most important zoonotic disease which is endemic in worldwide. Molecular method has allowed discrimination of different genotypes (G1-G10), providing new approach in development of prevention and control program of hydatid cyst. This study was conducted to identify the genotypes of *E. granulosus* from domestic animals in nine districts of North Khorasan Province using the mitochondrial cox1 gene sequence.

**Methods:** Overall, 122 hydatid cyst were collected during 2016-2017 from sheep (n=43) and cattle (n=79). DNA was extracted from protoscoleces and germinal layers and amplified by PCR. Phylogenetic analysis was also performed by analyzing the complete nucleotide sequences of mitochondrial cytochrome C oxidase subunit 1 (cox1) of *E. granulosus* genotypes from various locations.

**Results:** Sequencing of the amplified products revealed the presence of G1 as dominant genotype, G3 and *Echinococcus canadenesis* in one isolate each. Altogether, 9 haplotypes were detected based on cox1 gene. Haplotype 3 was the common variant that found in 58 including 42 cattle and 16 sheep.

**Conclusion:** This study provided knowledge on the identity of *E. granulosus* cysts collected from sheep and cattle in North Khorasan Province. Furthermore, these results showed the potentials of sheep as a main source of infection to humans, contributing the transmission and maintain of hydatid cyst in this region.
Introduction

Cystic echinococcosis (CE), also known as cystic hydatid disease, is a severe zoonotic disease caused by the larval stages of Taeniid cestodes of Echinococcus granulosus sensu lato (s.l.). Echinococcus spp. require two mammalian hosts to perpetuate their life cycle. Dog and other canids act as definitive hosts for adult worms, and ungulates serve as intermediate hosts for the cystic larva. Human is accidental intermediate host that become infected through ingestion of parasite eggs excreted by the feces of the infected dogs (1, 2). Despite the major control and prevention programs in reducing hydatid disease, this disease remains as a serious human and animal health concern (3).

CE is a cosmopolitan diseases that is endemic in many rural and pastoral areas of Asia (4-6). This disease is known to be endemic in many parts of Iran (7). Furthermore, CE has been reported with different prevalence (5% to 49%) in Iranian dogs (8-10). Human hydatidosis is responsible for about 1% of the surgical operation in Iranian hospitals (7, 11) and the incidence rate of this disease is reported to be 0.6-1.2 cases per 100000 inhabitants (7, 12), indicating high prevalence of CE in Iran.

Sheep-dog cycle is mainly present in Iran. Sheep and camel serve as the most important intermediate hosts (88% and 70%, respectively) (7, 13).

Recent molecular phylogenetic analysis using mitochondrial genetic data have revealed 10 different genotypes for E. granulosus that differ in infectivity, host range and genetic characteristic (14). The following reconstruction based mainly on mitochondrial data of E. granulosus s.l. suggests four major species as follows: E. granulosus sensu stricto (s.s) (G1-G3), E. equinus (G4), E. ortleppi (G5) and E. canadensis (G6-G10) (15). "Camel and cattle strain cycles of E. granulosus require the shorter intervals for chemotherapy of dogs with respect to the shorter pre-patent period of these strains" (16). Therefore, knowledge of Echinococcus species involved in a region have benefits for the development of prevention and control programs and epidemiological studies (17).

An extensive body of evidence has indicated the high prevalence of CE in livestock and human in Iran (18). The annual economic loss incurred as a result of hydatid cyst-related condemnation of offal was estimated over U.S$219,349 in North Khorasan, where this study was conducted (19). Furthermore, surgical survey has been found evidence for the presence of human hydatidosis (20), considering the importance of molecular studying for elucidating the parasite epidemiology.

This study was conducted to extend the knowledge on molecular characterization of the larval stage of E. granulosus collected from sheep and cattle originating from North Khorasan Province, Iran.

Materials and Methods

Collection of hydatid cysts

Overall, 122 hydatid cysts were collected during 2016-2017 from slaughtered animals (sheep and cattle) during post-mortem inspection from various locations within North Khorasan Province, Iran. Collected cysts from lung and liver were placed in sterile saline solution and transported to the laboratory in ice box. To evaluate the cysts fertility, cyst contents were aseptically aspirated, centrifuged at 1500 gr for 10 min, and examined for the presence of protoscoleces. Protoscoleces were collected from fertile cysts, whereas germinal layers were collected from infertile cysts. Collected protoscoleces and germinal layers were washed several times in sterile saline and saved in -20 until DNA extraction.

DNA extraction and Polymerase chain reaction (PCR)

Genomic DNA (gDNA) was extracted individually from the larval tissues of E. granulosus using a DNeasy blood and tissue kit (Qiagen,
Germany) according to the manufacturer’s instructions and used as a template for polymerase chain reaction (PCR). Partial fragment of a mitochondrial gene for cytochrome c oxidase subunit 1 (cox1) was subjected to amplify by PCR using specific primers as described previously (21). PCR reaction was conducted in a 50 µl final volume containing 50-100 ng of gDNA, 200 µM of each dNTP, 3 mM of MgCl2, 10 pmol of each primer, and 1.5 U of Taq DNA polymerase. The DNA fragment of cox1 was amplified under following cycling condition, initial denaturation step of 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 45 sec, annealing at 50 °C for 45 sec and extension at 72 °C for 45 sec; followed by a final extension at 72 °C for 10 min. The resulting amplicons from each PCR were analyzed through 1.5% agarose gel electrophoresis and were visualized by ethidium bromide staining under UV.

**DNA sequence analysis**

Amplified products were commercially purified and sequenced using the forward primer employed for PCR (Bioneer, South Korea). The quality of the sequences was evaluated and edited by BioEdit software 7.0.5 (22) and then compared to those available in the GenBank database using BLAST sequence algorithms to determine the genotype of Echinococcus isolates (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

All 122 nucleotide sequences obtained in the present study were deposited in GenBank under accession numbers KR733081-KR733083-88, KR920697, KR920700-701, KT200218-20, KT200222-23, KT254111-19, KT254121-25, KT320877-88, KU360296-325, KU603673-79, KU603681-707, KU603709-726, and KU603728-729 for cox1 sequences. Nucleotide data including reference sequences and haplotypes sequences from this study were aligned with the Clustal W (23) algorithm using BioEdit version 7.0.5 (22). The HKY + gamma + T model was selected as the best fit model using j model test 0.1.1 software (24). The selected model based on the Akaike Information Criterion was applied to construct phylogenetic relationships between the haplotypes using the Maximum likelihood tree as implemented in PAUP 4.0b10 (25). Reliability of internal branches was evaluated using non-parametric bootstrapping with 1000 replicates. *Taenia saginata* was included as outgroup.

**Sequence homology**

Haplotype segregation in obtained sequences in the present study was performed by DnaSP software version 5 (26). Multiple alignments of sequence information using Clustal W estimated the extent of variation in detected genotype by pairwise comparison of haplotype sequences with each other and reference sequences. To determine the synonymous and non-synonymous substitution, the nucleotide sequences translated into the corresponding amino acids using CLC genomics software version 9 (CLC bio, Aarhus, Denmark).

**Ethical aspects**

All samples were collected post-mortem in the slaughterhouse and caused no suffering to the animals.

**Results**

The examination of organ distribution of CE indicated pulmonary and hepatic cysts in both animals. In cattle, lung was more likely to be infected than liver, 64.55%, and 35.44% respectively. While liver and lung cysts were equal in sheep (22 vs 21). The fertility rates of hydatid cysts were 78% and 12.22% in sheep and cattle, respectively.
Table 1: Accession number and geographical locations of *Echinococcus* cox1 sequences used in the present phylogenetic analysis

| Genotype | Host      | Accession number | Country               |
|----------|-----------|------------------|-----------------------|
| G1       | Cattle    | HM636639         | Lybia                 |
| G1       | Sheep     | HQ717149         | Turkey                |
| G1       | Sheep     | DQ856467         | Greece                |
| G1       | Human     | JX854034         | India                 |
| G1       | Sheep     | HM563001         | Iran (Kerman)         |
| G1       | Goat      | HM563010         | Iran (Kerman)         |
| G1       | Dog       | JN604097         | Iran (Lorestan)       |
| G1       | Sheep     | JF775380         | Turkey                |
| G2       | Sheep     | M84662           | Tasmania              |
| G2       | Dog       | JN604103         | Iran (Lorestan)       |
| G2       | Goat      | KJ162562         | Iran (Kashan)         |
| G3       | Sheep     | DQ856466         | Greece                |
| G3       | India     | JX854028         | India                 |
| G3       | Sheep     | HM563016         | Iran (Kerman)         |
| G3       | Buffalo   | M84663           | India                 |
| G3       | Dog       | JN604104         | Iran (Lorestan)       |
| G4       | Horse     | M84664           | Spain                 |
| G5       | Camel     | AB921055         | Egypt                 |
| G5       | Human     | JX854035         | India                 |
| G5       | Cattle    | AB235846         | Argentina             |
| G5       | Cattle    | M84665           | Holland               |
| G6       | Camel     | NC011121         | Kazakhstan            |
| G6       | Camel     | AB921058         | Egypt                 |
| G6       | Camel     | AB921084         | Egypt                 |
| G6       | Camel     | HM563018         | Iran (Kerman)         |
| G6       | Camel     | M84666           | kenya                 |
| G6       | Human     | KC415063         | India                 |
| G6       | Camel     | HM856354         | Iran                  |
| G7       | Human     | KJ556997         | China                 |
| G7       | Pig       | M84667           | Poland                |
| G8       | Moose     | AB235848         | USA                   |
| G10      | Reindeer  | AF525457         | Filand                |
| G10      | Human     | KJ663947         | China                 |
| G10      | Moose     | AB777911         | Russia                |
| Taenia saginata | Human | AB465246 | South Korea |

Pulmonary cysts had higher fertility than liver cysts in both sheep and cattle. The highest rate of fertility was determined in pulmonary cysts of sheep (80.95%), and the lowest in cattle’s liver (10.3%).

**Molecular analysis**

All genomic DNA samples derived from individual *E. granulosus* cysts were subjected to PCR of cox1 gene. Successful PCR amplification of cox1 gene yielded amplification product of 446 base pair. Single bands on agarose gel indicated the specificity of the PCR protocol employed. The obtained consensus haplotype sequences of cox1 were 304 bp. Alignments of the obtained sequences derived from sheep isolates indicated the existence of G1 genotype (sheep strain) in 42 of 43 isolates and *E. canadensis* in one isolate. Totally, 78 of 79 cattle were infected with G1 (*E. granulosus*...
sensu stricto), and the remaining one with G3 (buffalo strain).

**Phylogenetic analysis**

Phylogenetic analysis of cox1 sequences revealed four main clades including the previously well-known G1-G3 complex, G4, G5 and G6-G10 complex. G5 (*E. ortleppi*) formed a sister phylogenetic group with G6-G10 complex. G4 was distinct from other *E. granulosus* genotypes (G1-G3, G5, and G6-G10). Totally from 9 haplotypes detected in cox1 sequences, 8 haplotypes grouped with reference sequences from G1-G3 complex, particularly G1. Haplotype 9 clustered with G6 and G7 genotypes, separating from G10 genotypes. Intra-group genetic variation observed in all main groups. Maximum likelihood analysis of the 9 haplotypes along with reference sequences was shown in Fig. 1. Furthermore, the integration of the phylogenetic tree with geographical information from reference sequences used in this study was represented in Fig. 2.

**Sequence polymorphism in COX1 gene**

The alignment of the cox1 sequences indicated 9 different haplotypes (including 7 G1s, one G1-3, and one *E. canadensis*). Among all 9 haplotypes, haplotype 3 was the common variant, found in 58 isolates including 42 cattle and 16 sheep. Haplotype 1 was the second current variant with 42 isolates including 17 sheep and 25 cattle. The other haplotypes (7 haplotypes) observed in 22 isolates (Table 2).

The alignment of the cox1 sequence indicated intra-genotype sequence variation within G1 and G6 genotypes (Fig.3). Haplotype 1 showed complete identity (100%) to G1 reference sequence (HM563001). Haplotype 2 had a single nucleotide substitution of C to T at position 105 as compared to reference sequence HM563001, but this substitution was synonymous. Synonymous substitution was also observed in haplotype 6 with a transition of C to T at position 13. Haplotype 3 showed a nucleotide change of C to T at position 3, leading to non-synonymous substitution of Alanine to Valine. Two variable non-synonymous substitutions were observed in haplotype 4, one substitution (C to T) at position 3 led to substitution of Alanine to Valine and the other (A to G) at position 134, causing transition of Isoleucine to Valine. Comparison of G1 reference sequence (HM563001) and haplotype 5 showed two differences.

Fig. 1: Phylogenetic relationships among obtained haplotypes in this study and reference sequences retracted from NCBI. The phylogenetic tree was constructed on COX1 sequences using the Maximum likelihood algorithm as implemented in PAUP 4.0b10. *Taenia saginata* served as outgroup. The scale bar represents distance
**Fig. 2:** Phylogeography of *E. granulosus* species. GenGIS software was used to represent a clear view of the relationship between geography and genomic diversity. Each of the four genotypes within *E. granulosus* is assigned a unique color (G1-G3: orange, G4: black, G5: blue and G6-G10: yellow). A sequence of *Taenia saginata* as the correspondent outgroup sequence is identical by green color.

**Table 2:** Accession number for the partial cox 1 sequences derived from this study

| Haplotype (Genotype) | Host origin (number) | Accession numbers |
|----------------------|----------------------|-------------------|
| Haplotype 1 (G1)     | Sheep (17), Cattle (25) | KU603689 KU603713 KU603012 KU603718 KU603717 KU603716 |
|                      |                      | KU603703 KU603711 KU603678 KU603712 KU6036101 KU603723 |
|                      |                      | KTD208779 KTD254121 KTD254117 KTD208811 KTD208821 KTD254111 |
|                      |                      | KU602098 KU603020 KU602096 KTD254114 KTD254125 KU603679 |
|                      |                      | KU603673 KTD20220 KTD254116 KU603675 KU603608 KU603685 |
|                      |                      | KU603719 KU603707 KU603726 KTD208888 KU603725 KU603722 |
|                      |                      | KR920697 KU603715 KU603721 KTD208834 KTD208844 KTD20887 |
| Haplotype 2 (G1)     | Sheep (1), cattle (4) | KU603016 KU603709 KU603693 KU603728 KTD20877 |
| Haplotype 3 (G1)     | Sheep (16), Cattle (42) | KU603097 KU603034 KU603681 KR733081 KU603023 KU603700 |
|                      |                      | KU603006 KU603000 KU603019 KU603683 KU603024 KU603677 |
|                      |                      | KU603003 KU603701 KU603022 KR733088 KTD254123 KU603692 |
|                      |                      | KU603004 KU603699 KU603007 KU6030314 KU603010 KU603684 |
|                      |                      | KU603691 KU603688 KU603611 KU603682 KU603714 KU603729 |
|                      |                      | KTD20880 KTD20878 KU603710 KU603686 KU603690 KU603698 |
|                      |                      | KTD254122 KR920700 KU603020 KTD254119 KU603697 KU603621 |
|                      |                      | KTD254124 KU603706 KTD254113 KU603705 KU603035 KU603613 |
|                      |                      | KU603674 KU603025 KU603702 KTD200219 KU603687 KTD254118 |
|                      |                      | KU603090 KU603015 KTD200218 KTD20223 |
| Haplotype 4 (G1)     | Sheep (1), cattle (2) | KTD200222 KTD254112 KTD254115 |
| Haplotype 5 (G3)     | Cattle (1)           | KU373086 |
| Haplotype 6 (G1)     | Sheep (4), cattle (3) | KU603099 KU603676 KU603695 KU603696 KU603618 KU920701 |
|                      |                      | KTD20885 |
| Haplotype 7 (G1)     | Sheep (1), cattle (2) | KTD20886 KU603720 KU603724 |
| Haplotype 8 (G1)     | Sheep (2)            | KU603694 KU360317 |
| Haplotype 9 (G6)     | Sheep (1)            | KU373084 |

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)
One substitution at the 204 positions (T to C), leading to substitution of Valine to Alanine and the other at the 13 positions, causing non-synonymous substitution. On the other hand, this haplotype generated a sequence with 100% identity to G3 reference sequence (M84663). Haplotype 7 had the most sequence variation with one synonymous (C to T at position 13) and two non-synonymous substitutions. Nucleotide substitution (G to A) at position 295 changed Aspartate to Asparagine. Substitution of Alanine to Valine was generated by transition of C to T at position 3. Haplotype 8 had also both non-synonymous and synonymous substitution at position 3 and 13, respectively. Haplotype 9 showed 99% identity to G6 and G7 reference sequences (M84666) with a single transition (C to T) at position 204, leading non-synonymous substitution (Alanine to Valine).

Fig. 3: ClustalW alignments of partial cox1 amino acid sequences. Accession numbers HM563001, M84662, M84663, M84666, and M84667 represent G1, G2, G3, G6 and G7 reference sequences, respectively

Discussion

In the present study, three genotypes were identified to infect cattle and sheep: G1, G3 and E. canadensis. G1 was found as the most common genotype in North Khorasan Province, consistent with the earlier study from Iran (27, 28). The presence of G1 was found in all thirty liver and lung samples from cattle, sheep, and goats of abattoirs in northern and western Iran using DNA sequences of the mitochondrial 12S rRNA gene (29). A predominance of G1 with a small number of G3 using cox1 gene was showed in five different provinces of Iran (30). In contrast, a study on 19 camel hydatid cysts collected from central Iran revealed the majority of G3 genotype in isolates (31). The dominant of G1 over the other genotypes was also reported from other countries: such as China (32), Turkey (33) and Southern Brazil (34). The occurrence of both sheep strain (G1) and buffalo strain (G3) have been demonstrated in different intermediated host in Iran (35, 36) and other countries (34, 37). For example, the presence of G3 genotype was found in 3 cattle and 2 sheep along with the majority of G1 in both animals (107 isolates) (38). A similar finding was reported in Italy on 80 cattle and water buffalos (78.75% G1 vs. 12.5% G3) (39). In contrast, G3 and G6 were the dominant genotypes in India (40, 41) and Egypt (42, 43), respectively. Considering that sheep strain (G1) was the most frequent genotype, it seems to sheep-dog cycle was responsible for establishment and maintenance of Echinococcus life cycle in North Khorasan Province where this study was conducted. However, G3 and G6 genotypes are known human pathogens and should be considered as a significant public health concern.

In the present study 9 haplotypes were identified based on the alignment of Cox1 sequences. In comparison with our result, haplotype segregation of previous studies from other provinces in Iran showed a higher diversity of E. granulosus sensu stricto (G1-G3). For example studies in Ardabil (44), Lorestan (8) and Zanjan province described 13 haplotypes (35). This difference may be related to the length of the gene analyzed, province of study and sample size. The outcomes of haplotype
segregation could be affected by the length of the gene analyzed (45).

In this study, the topology of *Echinococcus* clade from this tree was consistent with previous studies (46-48). The present phylogeny based on maximum likelihood supported the validity of the G1-G3 complex to distinct from other genotypes and withhold that G2 was a distinct genotype. Moreover, the tree showed a monophyly of *E. ortleppi* and *E. canadensis* and supported the nation that *E. canadensis* are closely related to each other. The tree topology suggested that G10 and G8 were paraphyletic and G10 was sister to G6 and G7. Our results provided supportive evidence for the revision of genotype G4 into *E. equinus* (17).

Concluding remarks, the *Echinococcus* genotypes identified in this study, G1, G3, and G6, are known human pathogen, exerting significant public health concern. Molecular analysis showed the presence of G1 (sheep strain) as the prominent genotype of *Echinococcus* in sheep and cattle in North Khorasan Province of Iran. Considering the presence of poor rural communities where people and livestock are in close contact to dog, prevention and control program should be imposed on sheep – dog cycle. Although this study has provided a glimpse of the genotypes of *E. granulosus* in North Khorasan Province, a large study is needed to investigate the isolate from different hosts and from multiple geographic areas to better understand the transmission and epidemiology of different genotypes in Iran.

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Conflict of interest

The authors declare that there is no conflict of interests.

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