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SPECIAL ARTICLE

Echinococcosis: based on molecular studies in Iran

Mohammad Rostami Nejad[1], Ehsan Nazemalhosseini Mojarad[1], Mohsen Norouzina[1], Majid Fasihi Harandi[2]

[1] *Research Institute for Gastroenterology and Liver Disease, Shahid Beheshti University, M.C., Tehran, Iran*

[2] *Department of Parasitology, Kerman Medical University, Kerman, Iran*

ABSTRACT

Iran is an important endemic focus area of cystic hydatid disease where several intermediate host species are commonly infected with *Echinococcus granulosus*. Annually, most livers are shipped out, stored away, or destroyed due to contamination among livestock. For instance, in Aleshtar (Lorestan province), liver and lung losses due to hydatid cysts during 2002-2003 were approximately 864,360,000 Rials and 35,530,000 Rials for sheep and cow, respectively. In other words, given the risk of human contamination with this parasite, the costs associated with diagnosis, treatment, and surgery are important. Epidemiology studies have been performed in different part of Iran, but there is no international estimate of infection in livestock. Molecular studies have indicated the presence of G1 and G6 genotypes in different parts of Iran. We discuss these molecular studies in this article.

Keywords: *Echinococcus granulosus*, Hydatid cyst, Molecular studies, Iran.

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INTRODUCTION

Echinococcusis/ hydatidosis is one of the most prevalent zoonotic diseases in Iran, especially in rural areas where the slaughtering of offal is practiced on farms (1-3).

Several reports have indicated that hydatid cysts (HCs) are commonly found in sheep, camels, cattle and goats throughout Iran (1).

Hydatidoisis is a disease that is not readily apparent to the farmers but of considerable economic and public health importance. Hydatidosis occurs throughout the world and causes considerable economic losses and public health problems in many countries (4, 5).

Microscopicmorphology, biochemical and most importantly molecular genetic techniques have been used to characterize group strains within *E. granulosus*. Using mainly mitochondrial DNA (mrDNA) sequences, nine distinct genotypes (G1-G9) have been identified for *E. granulosus* to date (6). These include the important sheep strain (G1), two bovid strains (G3, G5), a horse strain (G4), the camelid strain (G6), a pig strain (G7) and one strain of cervid (G8) (4). A ninth genotype (G9) has also recently been described in humans and pigs in Poland (7).

Echinococcosis is endemic in Iran and is maintained in three distinct cycles, a livestock/ dog domestic cycle, a desert cycle between dogs and camel and a cylvatic cycle between wild carnivores and wild ruminants (1).

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Reprint or Correspondence: Mohammad Rostami Nejad, PhDs. Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University, M.C., Iran

E-mail: m.rostamii@gmail.com

Sheep are the most common and important intermediate host of *E.granulosus* in Iran followed by cattle, goats and camels. The prevalence of infection and fertility rates of the cyst in sheep is high (1).

Many investigators have reported the prevalence of HCs in intermediate hosts in different part of Iran (1, 2). For example the prevalence of HCs in sheep, cattle, and goats in Aleshtar were 26.7%, 21.2%, and 12.9%, respectively (8). In one study, the fertility and viability rates of HCs were 83.7-88% in sheep, 13-60% in cattle and 60-75% in goats (9). Human cases of HCs are also reported from different medical centers in different parts of the country (10-18). In a study carried out in Khorram-Abad, the prevalence of HCs in the liver and lung were 61.5 % and 20.5 %, respectively (19). Few molecular studies have identified the prevalence of hydatid cysts in different parts with various techniques and shown two distinct genotypes [camel strain (G1) and sheep strain (g6)] in Iran (1, 2, 20). However, the sources of infection in humans and the role of intermediate host reservoirs remain to be determined (20).

Molecular analysis Techniques for Echinococcus isolates

Nuclear and mitochondrial genomes have been investigated for genetic variation in *E. granulosus*. The nuclear ribosomal RNA gene (rDNA) repeat unit has different regions evolving at varying rates and has been used extensively to study variation and phylogeny in *E. granulosus* (21, 22) at a number of different taxonomic levels. Mitochondrial DNA (mtDNA) is useful for the discrimination of closely related organisms because of its relatively rapid rate of evolution. Furthermore, as mtDNA is haploid, allele haplotypes can be determined unambiguously. Mitochondrial DNA has the additional advantage of being maternally inherited. Furthermore, it does not recombine, thus simplifying analysis (23).

RFLP analysis

Recent studies of molecular genetic variation in *E. granulosus* were comprised of restriction fragment length polymorphism (RFLP) analysis in Iran (1, 2, 20, 24). The technique allowed several distinct strains of *E. granulosus* to be distinguished, and extensive study showed that the RFLP patterns were stable within a particular strain. The conventional RFLP procedure was simplified, without loss of resolution or accuracy, by linking RFLP analysis with polymerase chain reaction (PCR) targeting of the nuclear rDNA ITS1 region (25). Characteristic PCR-amplified ITS1 and PCR-ITS1 RFLP banding patterns were produced when samples within the *Echinococcus* species were analyzed. The approach was rapid and although its usefulness has been questioned (22), it has proven to be applicable and reliable in the hands of a number of researchers for the identification of newly collected isolates and for the investigation of *E. granulosus* transmission patterns where strains occur sympatrically (23, 26-31).

Nucleotide sequences

Nucleotide sequence comparison of defined DNA segments between organisms provides the most direct and sensitive means of detecting genetic variation. PCR has made sequence comparison a feasible approach for genetic variation studies. The advent of PCR has provided a highly sensitive approach that is now widely used for *E. granulosus* identification purposes, including discrimination of eggs (23).

Mitochondrial sequences, particularly fragments of the mitochondrial protein-coding genes, cox1 [(cytochrome c oxidase subunit 1, COX1, 366 bp (32)], nad1 [(NADH dehydrogenase I, ND1, 471 bp (25)], and atp6 [(ATP synthetize subunit 6, ATP6, 706 bp (33)] have proven to be invaluable for *E. granulosus* strain identification and genetic variation. The consensus view of the strain pattern within *E.*

granulosus, based on a variety of other criteria (35), was broadly upheld when the DNA sequences of the different genotypes were compared. Furthermore, remarkable intra-genotypic strain homogeneity was found at the DNA sequence level. Recent studies have shown that the ATP6, COX1, and NAD1 genes are good molecular markers for investigating genetic variation in a number of isolates of *E. granulosus* from a range of hosts (i.e., cattle, sheep, and goat) in Iran (33). With specific PCR, those *Echinococcus* genotypes, that are considered to have the greatest public health impact, can be easily and rapidly identified by a specific PCR that is faster than PCR-RFLP.

Here, the various molecular approaches that have been used for accurate identification of *E. granulosus* are briefly reviewed. Their use in epidemiological surveys in different hosts and various parts of Iran are also reviewed.

Indication of the presence of two distinct strains of *E. granulosus* in Iran by mitochondrial DNA markers

In a preliminary study, 16 isolates of *E. granulosus* collected from Iranian patients at surgery and domestic animals (sheep, goats, cattle, and camels) at slaughterhouses in Tehran and central and southern Iran (2) were analyzed for DNA nucleotide and predicted amino acid sequence variation within regions of the mitochondrial cytochrome c oxidase I (COX1) and NADH dehydrogenase subunit I (NAD1) genes. A polymerase chain reaction–restriction fragment length polymorphism method, based on the DNA sequence variation in the NDI gene, was subsequently used to rapidly survey the *E. granulosus* isolates. The isolates were categorized into two distinct and uniform genotype groupings. The analysis clearly indicated that the camel/dog strain (G6 genotype) of *E.granulosus* and the cosmopolitan, common sheep strain (G1 genotype) occur in Iran. The G1 genotype was found present in all four human isolates examined. It was more prevalent in domestic animals than the camel-restricted G6 genotype. In these areas the majority of *E. granulosus* infected livestock animals can potentially act as reservoirs of human infection.

Molecular and morphological characterization of *E. granulosus* of human and animal origin in Iran

Two hundred thirteen *E. granulosus* isolates were collected from humans and other animals from different geographical areas of Iran and characterized using both DNA (PCR-RFLP of ITS1) and morphological criteria (20). The sheep and camel strains/genotypes were again shown to occur in Iran. As shown previously, the sheep strain was shown to be the most common genotype of *E. granulosus* affecting sheep, cattle, goats and occasionally camels. The majority of camels were infected with the camel genotype as were 3 of 33 human cases. This is the first time that cases of HCs in humans have been identified in an area where a transmission cycle for the camel genotype exists.

In addition, the camel genotype was found to cause infection in both sheep and cattle. Results also demonstrated that both sheep and camel strains can be readily differentiated on the basis of hook morphology alone.

Characterization of *Echinococcus granulosus* isolates from human, sheep and camel in Iran

In this study, *E. granulosus* isolates collected from human, sheep, and camel samples in Iran were characterized on the basis of rostellar hook morphology of protoscoleces as well as PCR-RFLP (1). Morphological study on human and animal isolates showed the presence of two distinct strains of the parasite, one in sheep and the other one in camels. Rostellar hooks from sheep

isolates were significantly different from those of camel origin; meanwhile, human isolates were found to be similar to those isolated from sheep. Molecular analysis of the ITS1 region of ribosomal DNA derived from human, sheep and camel isolates were in agreement with the morphological findings. Based on the PCR-RFLP method, the sheep and human isolates appeared to have the same genotype, and the camel isolates appeared to have a different genotype.

A new primer pair in the ITS1 region for molecular studies on *E. granulosus*

Using the grinder method, a pure and high concentration of DNA was extracted from 10 human HCs collected from Isfahan (central Iran) hospitals and processed for PCR reaction. Using DNASIS, the primers were designed in the internal transcribed spacer 1 (ITS1) region, following analysis of 30 *E. granulosus* nucleotide sequences extracted from the gene bank (24). As a result, this new and specific *E. granulosus* primer, which amplified DNA thoroughly, can be applied for molecular studies on Echinococcusis.

Echinococcus granulosus strain differentiation in Iran based on sequence heterogeneity in the mitochondrial 12S rRNA gene using new specific primer pairs

Infected organs (liver and lungs) of 30 animals (cattle, sheep and goats) naturally infected with *E. granulosus* were collected from the abattoirs of northern and western Iran during June-Oct 2007 (33). Protoscoleces were removed from each fertile cyst and DNA was extracted. New and genotype-specific primers of the mitochondrial 12s rRNA gene were designed for two existing genotypes (G1 and G6) of *E. granulosus* known to occur in Iran and were applied in PCR reactions. The new primers specifically amplified the G1 and G6 genotypes of *E. granulosus* with specific bands of 259 and 676 bp respectively. The G1 primer pairs did not amplify the G6 genotype and vice versa. The G1 genotype was identified in all fertile cyst samples. The 259 and 676 bp fragments of the G1 and G6 reference isolates were sequenced and compared with the G1 and G6 sequences deposited in GenBank. Our sequences were the same as those reported previously for these strains.

Echinococcus granulosus genotypes in livestock of Iran indicate high frequency of G1 genotype in camels

In this study, 112 *E. granulosus* isolates from sheep, goats, cattle and camels were genotyped by PCR amplification in the rDNA-ITS1 region followed by RFLP with the enzyme RsaI (34). The possibility of intra-genotype variation was also investigated using randomly amplified polymorphic DNA (RAPD) analysis. The predominant genotype belonged to the G1 genotype and a few camel isolates (6 of 18; 33.3%) belonged to the G6 genotype. Overall G1 and G6 genotypes were identified in 94.6% (106 of 112) and 5.3% (6 of 112) of all isolates, respectively. No differences were observed among isolates from different hosts and between the infected organs based on each individual primer. The result of this study is compatible with other studies from Iran. This study suggested the studying of intra-genotypic variation of *E. granulosus* using other molecular methods.

Genetic categorization of *Echinococcus granulosus* from humans and herbivorous hosts in Iran using an integrated mutation scanning-phylogenetic approach

Cystic material from 148 *E. granulosus* isolates from humans, sheep, goats, cattle, and camels utilizing DNA regions designated pcox1 and pnad1 for mt genes (Cox1 and NAD1 genes) were analysed by PCR-based SSCP (35).

E. granulosus revealed five (pc1–pc5) and nine (pn1–pn9) electrophoretic profiles, respectively. The result of this study have shown that the majority of cyst isolates (142 of 148; 95.9%) were assigned to the G1–G3 complex of *E. granulosus* (or *E. granulosus* sensu stricto), whereas some *E. granulosus* cysts (6 of 19; 31.6%) from camels were assigned to the G6–G10 complex (or *E. canadensis*). The present study reinforces the advantages of the mutation scanning-sequencing-phylogenetic approach to explore variation in multiple mitochondrial loci within and among *Echinococcus* populations, which provides a platform for future, detailed studies of the molecular epidemiology of *E. granulosus* in Iran and other countries.

New mitochondrial primer pairs in 12S rRNA, atp6 and nad1genes for genetic variation studies on *Echinococcus granulosus* in Iran

Thirty isolates of *E. granulosus* were collected from cattle, sheep and goats from different geographical areas and genotypes 1 and 6 were determined using new and specific primers in the mitochondrial 12SrRNA gene with specific bands of 259 and 676 bp (36). Eighteen isolates were genotyped and selected for investigation and comparison of their atp6 and nad1 genes sequence. Primers for the atp6 and nad1 genes were designed and selectively amplified and produced bands of 708 and 1157bp, respectively. The G1 genotype was present in the investigated isolates. The result of this study showed that the specificity of designed primers was 100% when blasted in NCBI and compared with other primers.

Conclusion

There is considerable evidence demonstrating the importance of determining genotypic variation in *E. granulosus* in Iran (23, 37). This is particularly significant in endemic regions such as Iran, where more than one species of intermediate host is present.

The range of DNA techniques and valuable information on the molecular categorization of the different genotypes for molecular epidemiology studies of cystic Echinococcusis is now available. Importantly, in many cases, molecular techniques have validated the genetic basis of important morphological differences that can now be used with confidence as a reliable and simple way of identifying and differentiating between strains and species of *Echinococcus* (e.g., 20).

In this context, the recent study of Fasihi Harandi et al. (20) is important and worthy of particular comment as they highlight the complexity and genomic organization differences that exist in *E. granulosus*.

Based on studies of Zhang et al., Ahmadi and Dalimi, Fsihi Harandi et al. and Rahimi et al., molecular techniques were used to confirm the presence and reveal the host preferences of sheep (G1 genotype) and camel (G6 genotype) strains in Iran (1, 2, 20, 24)

They used the approach to identify distinct G1 and G6 genotypes within Iranian *E. granulosus* isolates. Sequencing of the nad1 and cox1 genes and ITS1-PCR coupled to RFLP confirmed these observations.

Rostami Nejad et al. developed a specific and sensitive PCR system for rapid diagnosis of *E.granulosus* genotype1 and genotype6 (33, 36) in Iran. The Eg-PCR-RFLP and protocols could thus be used as additional methods to discriminate the recognized *E. granulosus* genotypes and Eg-specific PCR that is

especially useful for resolving the issue of the G6/G7 genotypes and human infection in Iran.

This molecular technique confirms the results of an earlier study conducted using a restricted number of isolates collected from various areas of Iran. Any attempt that eliminates dogs in the life cycle of this parasite will reduce the incidence of hydatid disease in humans and animals (36, 37).

Finally, it should be emphasized that in addition to proving the value of investigating genetic variation in *Echinococcus*, the majority of *E. granulosus* infected livestock can potentially act as reservoirs of human infection. The role of ranchers in transport of sick or unsafely livestock to slaughterhouses should be considered, as this has important implications for hydatid control and public health impact (38).

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