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ORIGINAL ARTICLE

The phylogenetic similarity of hydatid cyst isolated from humans and sheep in Ilam Province southwest of Iran

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Abstract Hydatid cyst is a chronic zoonotic disease caused by the larval stage of the dog tapeworm, *Echinococcus granulosus*. To identify genotype of hydatid cysts of human and sheep jackal in Ilam Province (South West of Iran), the PCR-RFLP and DNA sequencing were used. A total of 10 human and 20 sheep protoscoleces hydatid cyst samples were collected from different hospitals and slaughterhouses. Then, the gene of cox1 of mitDNA of the parasite was amplified and PCR products were cut using *AluI* and HpaII restriction enzymes. Finally, a number of PCR products were bi-directionally sequenced. Based on the DNA sequencing and PCR-RFLP results, human and sheep samples indicated to pertain the genotypic similarities. Our data indicated that, the genotypes of larval stage of *E. granulosus* is similar in both intermediate hosts. According to the phylogenetic tree, there is at least one genotype of parasite, which belongs to *E. granulosous* sensu stricto (G1–G3) complex and overall isolates sequences of mtDNA indicated 100 % homology with references G1, G2, and G3 sequences in the GenBank database. G1 genotype was the dominant genotype of human and livestock.

Keywords *Echinococcus granulosus* · Human · Sheep · Ilam · Iran

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Introduction

Hydatid cyst disease is a chronic zoonotic infection caused by the larval stage of the tapeworm dog, *E. granulosus*. Because of the importance and the global spread of the disease in terms of medical, veterinary, and economic aspects, this disease is considered as one of the most important zoonotic cases (McManus et al. 2003; Craig et al. 2007; Brunetti et al. 2010). Usually, numerous species of domestic animals as incidental intermediate hosts and wildlife are infected with hydatid cyst. Numerous studies in Iran showed that hydatid cyst frequency has been reported as follows: sheep (5 to 72 %), camel (11.4 to 70 %), cattle (5.3 to 38 %), goat (1.7 to 20 %) (Dalimi et al. 2002; Rokni et al. 2009). Sheep is the most common and important intermediate host of parasite in a breeding cycle in Iran. Besides, human infections are also continuously reported from different regions of the country (Heidari et al. 2011; Fakhar et al. 2007). On the other hand, adult worm infection has been observed in all parts of the country in dogs, especially stray dogs and cattle (Ahmadi et al. 2011; Eslami et al. 2010). The average infection with this parasite worm in Iran's carnivores is 27.4 %. The average infection with this parasite in Ilam decreased in 2013 at a rate of 37 % than in 1997 (Abdi et al. 2013). Usually in areas where the disease is endemic, there is a relatively high diversity of *E.granulosus* in terms of biology, genetics, or strain (Panahi et al. 2015; Nakao M et al. 2007; Moks E et al. 2008; Eckert J et al. 1997). Several studies have demonstrated that, there are different strains of *Echinococcus* in these areas as a complex of different strains that may affect epidemiology and pathogenesis of hydatid cyst. Additionally, there are reasons that prove more virulence of some strains (Casulli et al. 2008; Utuk et al. 2008). So far, ten distinct genotypes

(G1–G10) from this parasite have been described using molecular techniques based on the analysis of nuclear and mitochondrial genetic markers (Eckert J et al. 1997; Moro PL et al. 2009; Rosenzvit MC et al. 1999; Bowles J et al. 1993; Brody JR et al. 2004; Bowles J et al. 1992). Accordingly, the taxonomic status of parasite is proposed in four species as follows: *E. granulosus* sensu stricto (G1–G3), *E.equinus* (G4), *E. ortelpi* (G5), *E. canadensis* (G6–G10) (Nakao M et al. 2007; Moks E et al. 2008). Most human isolates are genotype G1 (sheep strain) and the most common genotype with the most geographical distribution in the world (Bowles et al. 1992; Villalobos et al. 2007). However, investigating operated cases of hydatid cysts implies the possibility of human infection with genotypes (Villalobos et al. 2007; Abdi et al. 2010; Ahmadi et al. 2006; Varcasia et al. 2006; Li et al. 2008; Rostaminejad et al. 2010). Due to the relatively high genetic diversity, different genotypes of *E. granulosus* may be associated with antigenic power, sensitivity to pharmacological agents, host specificity, life cycle, and mode of transmission and severity of virulence. All of the above can have a decisive role to design and develop vaccines, diagnostic tests and medical treatment of hydatid cyst disease (Moro et al. 2009; Rostaminejad et al. 2010). Therefore, due to epidemiological requirements and designing control strategies, it is necessary to determine the common genotypes of parasite in endemic areas exactly (Bowles et al. 1992; Altintas et al. 2013). Currently, for the identification of strains of *E. granulosus*, in addition to the morphological, biochemical, and biological characteristics, molecular methods are used, especially those depending on PCR-RFLP, according to genetic proximity of genes available in the nuclear DNA sequence, ribosomal RNA and mitochondrial DNA, specifically genes *nad-1* and *cox-1*. These areas are included as appropriate genetic regions in mitochondrial DNA that with appropriate quality and quantity are of importance to provide PCR product purification for molecular studies (Villalobos et al. 2007; Dousti et al. 2013; Parsa et al. 2011). The variants are classified according to their genotype that include genetic sequencing, mitochondrial or ribosomal gene fragment RFLP and comparative analysis of homologous sequences of DNA (Jamali et al. 2004). Although the characteristics of *Echinococcus* species of Iran have been studied in humans and livestock (intermediate host) by using morphological, biochemical, and molecular methods (Moks et al. 2008; Varcasia et al. 2006; Jamali et al. 2004; Zhang et al. 1998). But the study and identification of strains or genotypes of *E. granulosus* in humans (the operated patients) and animals with hydatid cysts (sheep) slaughtered with molecular methods especially PCR-RFLP in Ilam province with mitochondrial gene *cox-1* were conducted for the first time with this study. Animal husbandry is considered as one of the important and common business of the Ilam people, due to specific climatic conditions and large population of nomadic tribal.

Materials and methods

Parasite

In this study, 10 samples of human and 20 samples of sheep hydatid cyst isolates of liver and lung lesions were collected from patients undergoing surgery in different hospitals and abattoirs, respectively. In the laboratory, after disinfecting cyst area with lugol, the cyst contents were aspirated with a sterile syringe. Protoscolices isolated from the fertile hydatid cyst of the liver and lung were stored in Ethanol 80 % and held frozen at −20 °C until DNA extraction.

DNA extraction

Protoscolices were washed three times with distilled water for 10 min in 8000 rpm. Then repeated freezing and thawing as well as abrasion and crushing of samples were done in a Chinese mortar. Initial digestion of samples by adding lubricating buffer and proteinase K was done in 4 h at 56 °C. (Rahimi et al. 2007). For the extraction of genomic DNA of samples, DNA extraction kit (Geen all, Korea) was used. The concentration of extracted DNA was determined by spectrophotometer and extracted DNA were stored at −20 °C until use.

Molecular analysis

The sequences with the length of 450 bp were amplified from mitochondrial DNA (mtDNA) *cox*1 fragments, using the primers: *cox*1. F(JB3): 5′-TTTTTTGGGCATCCTGAGGT TTAT-3′ and *cox*1. R(JB4.5):

5′-TAAAGAAAGAACATAATGAAAATG-3′ (Moks et al. 2008; Rahimi et al. 2007; Utuka et al. 2008). PCR mixture with a final volume of 50 μl include 5 μl 10× buffer, 0.2 μl dNTPs, 2 ml 50 mM MgCl2, 25 pico moles of each primer, 1 unit of Taq DNA polymerase enzyme and 2 μl template DNA was prepared. Then the final volume of reactants reached to 50 μl with distilled water. The initial denaturation at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min.

PCR-RFLP and DNA sequencing

Restriction fragment length polymorphism method was carried out by Bowles et al. and Hop et al. protocol with slight modifications (Bowles J et al. 1992; Hop M et al. 1997). This method was done on the PCR product

mtDNA *cox*1 region of samples from sheep and human by *AluI* and *HpaII* restriction enzymes. Electrophoresis was done on agarose gel 1.2 % (*w*/*v*) in SB 1× buffer and stained with 0.5 μg/ml safe stain under voltage 80 for 50 min and were analyzed (Bowles et al. 1992, 1993). To study DNA sequencing, a number of PCR product was randomly bi-directionally sequenced using PCR primers by the Seqtech Company in the USA. The phylogenetic tree was built with the neighbor-joining (NJ) algorithm using molecular evolutionary genetics analysis (MEGA) software (version 6.0).

Results

A fragment of about 450 bp was amplified from all isolates using *cox*1 PCR, and no amplification was observed in the negative control (Fig. 1).

All PCR products were digested by 2-base digestion restriction endonucleases enzymes. The endonuclease digestion of the PCR products resulted in the RFLP patterns that were the same for all isolates as the pattern. From the restriction patterns of the *cox*1 RFLP products by *AluI* and HpaII enzymes, two bands with sizes of 190, 260, and 170, 280 bp were obtained, respectively (Figs. 2 and 3).

According to the phylogenetic tree, there is at least one genotype of parasite, which belongs to *E. granulosous* sensu stricto (G1–G3) complex and overall isolates sequences of mtDNA indicated 100 % homology with references G1, G2, and G3 sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/). Consistently approved the same genotype and all of the isolates were identified as *E.granulosus* sensu stricto (G1–G3) complex and G1 genotype (sheep strain) is the dominant genotype of human and sheep in Ilam province (Fig. 4). The result obtained from sequence revealed that 100 % homology with GenBank reference sequence for G1 (M84661), G2 (M84662), and G3 (M84663) genotypes (Fig. 4).

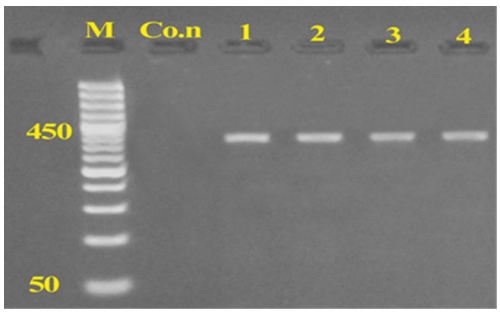

Fig. 1 PCR amplified mtDNA-*cox1* (450 bp) of protoscolices and adult's worm on agar gel. *M* marker with 50 bp molecular weight, *Co.n* control negative, *1* human sample, *2* sheep sample, *3* dog sample, *4* jackal sample

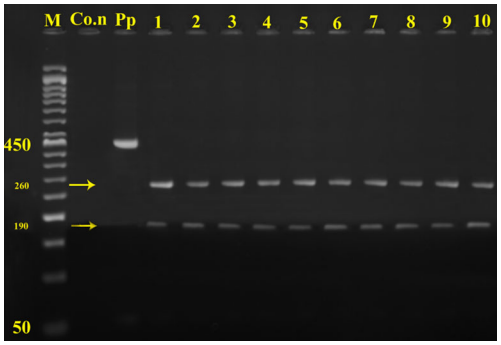

Fig. 2 Digestion pattern of 450 bp PCR products of mtDNA *cox-1* fragment with *Alu*I enzyme on agar gel. *M* marker with 50 bp molecular weight, *Co.n* negative control, *Pp* PCR products without enzyme, *1* human sample, *2* sheep sample, *3* dog sample, *4* jackal sample

Discussion

In the present study for digesting, *E. granulosus* isolates sheep and human DNA endonuclease digestion; two restriction enzymes of Alu1 and HpaII with different sequences of nucleotides were used. The results showed the same pattern of *E. granulosus* DNA fragments. DNA patterns of *E. granulosus* after digestion with two restriction enzymes showed that all samples had a similar RFLP pattern and the single bands of DNA are at the size 450 bp which indicates the same pattern of the genotype of DNA sequence (homolog) of *E. granulosus* or intraspecific similarity of parasite in all isolates. The sequencing of mtDNA obtained from our samples was characterized as *E. granulosous* sensu stricto, which is the same as what has been previously reported in Iran. It can be concluded that the hydatid cyst isolates in our study area, Ilam, is basically similar to that of other endemic areas of Iran. Numerous studies around the world indicated the infectivity of all genotypes except genotype G4 and G10 in humans (McManus et al. 2003; Casulli et al. 2008; Utuk et al. 2008; Moro et al. 2009; Rosenzvit et al. 1999; Bowles et al. 1993) and G1 genotype is the most common cause of human infection in the world (Bowles et al. 1992; Hop et al. 1997;

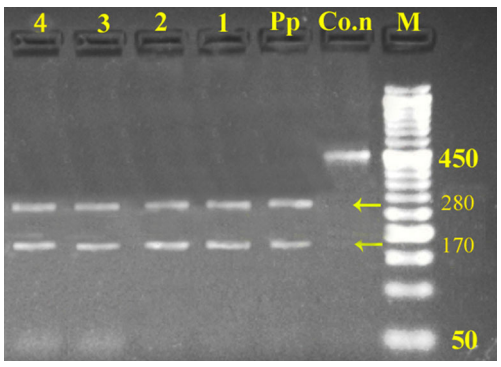

Fig. 3 Digestion pattern of 450 bp PCR products of mtDNA-*cox1* fragment with *Hpa*II enzyme on agar gel. *M* marker with 50 bp molecular weight, *Co.n* negative control, *Pp* PCR products without enzyme, *1* human sample, *2* sheep sample, *3* dog sample, *4* jackal sample

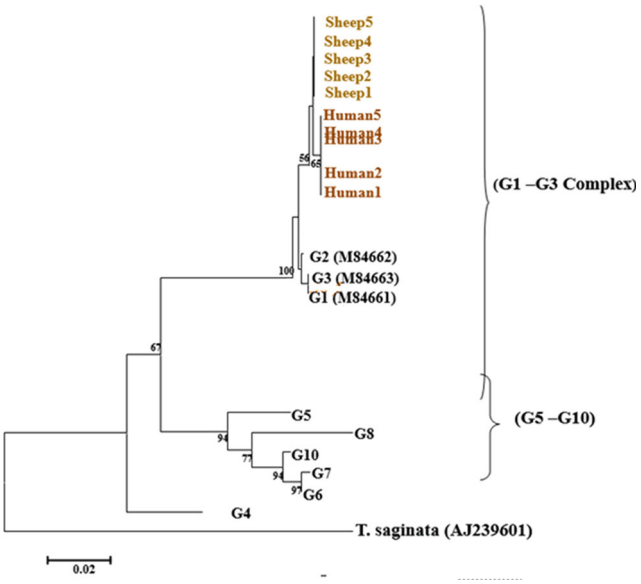

Fig. 4 Phylogenetic relationships among *E. granulosus* isolated from human and sheep based on mtDNA *cox1* gene sequence. The evolutionary history was inferred using the neighbor-joining method, supported by 1000 bootstrap replicates

Nikmanesh et al. 2014; Yakhchali et al. 2011). The disease is common in domestic animals and human infection has been reported from different regions (Rokni et al. 2009; Sharbatkhori et al. 2011; Rostaminejad et al. 2012). Moreover, cox-1 RFLP patterns were obtained from sheep and human samples indicated the active presence of *E. granulosus* sensu stricto (G1–G3) complex in Ilam province. In a study by Utuka. et al. in Turkey on isolates of *E. granulosus* with enzymatic digestion of *cox*-1 and *ITS*1 genes, prevalent strain in the region was reported as G1 genotype (sheep strain) (Nikmanesh et al. 2014). In the study, which was conducted by Scott et al., in Poland in 1997, for the first time a separate G9 genotypes was identified from *E. granulosus* (Bowles et al. 1993). In a study by Ahmadi and Dalimi, Zahng et al. and Thompson et al., isolates with two life cycles of dog—sheep and dog-camel—as the active cycle of the parasite, have been confirmed with morphological and molecular techniques in human and animal (Dalimi et al. 2002; Bowles et al. 1992; Zhang et al. 1998). In a study by Yakhchali et al., with PCR-RFLP method, molecular findings based on the nucleotide sequence of the *nad*-1 gene showed that all the samples originating from ruminants and dog had similar RFLP patterns that belonged to G1 genotype (sheep strain) (Gholami et al. 2012). Based on the results of a medical study by direct sequencing of nad1 and cox1 PCR product, G1 genotype was the dominant strain found in sheep, cattle, goat and human, and G3 genotypes (buffalo strain) not only was reported in one sheep isolate and one cattle isolate but for the first time it was also reported in two human isolates in Iran (Khademvatan et al. 2013). Based on studies in Iran, G1 genotype was the most common genotype in human isolates (Rokni et al. 2009; Moro et al. 2009; Ahmadi et al. 2006; Sadri et al. 2012). Shahnazi et al. study in Isfahan showed that the G1 genotype is the most dominant genotype, which was found in human, cattle and sheep isolates, and a small number of G6 genotype (camel strain) were also found in humans, camels, and cattle which shows that, camel strain can be an important source of infection for humans (Varcasia et al. 2006). Hanilo et al. in 2012 with the PCR-RFLP method using *ITS1* gene on human and animal isolates of *E. granulosus* in Zanjan showed that the predominant strain of *E. granulosus* in animals and humans is genotype G1 or the dominant strain in sheep (Sadri et al. 2012). Shrbatkhori et al., in 2010, by sequencing mitochondrial *nad-1* and *cox-1* genes, reported camel isolates belonged to the two genotypes G1 and G6 (Pezeshki et al. 2013). In the study on isolated animal piece ITS1 gene by PCR-RFLP in Yasouj, G1 strains, the predominant strain causing hydatid cyst disease were reported (Adwan et al. 2013). In a study of Dousti et al., in Ilam conducted using ITS1 gene on human and animal isolates, G1 and G3 genotypes were identified in the region that are consistent with the results of this study (Varcasia et al. 2007). In studying PCR-RFLP by Moqaddas et al., conducted in five different geographical regions of East of Iran using two genes cox-1 and ITS1 on 50 camel hydatid cyst samples, 54 % of samples were reported as G1 genotype and 46 % of them were reported as G6 genotype (Bart et al. 2006; Busi et al. 2007; Vural et al. 2008). In the present study in the same areas on mtDNA of *cox*1 gene of adult worms, the findings were analogous to that of other researchers (Bowels et al. 1993; Bhattacharya et al. 2008; Villalobos et al. 2007). Based on the results of cox1 and nad1 gene sequencing of the adult worms isolated from dogs in Lorestan, all of the samples belong to the G1–G3 complex and G1 genotype (sheep strain) of *E. granulosus* sensu stricto were dominant genotype (Parsa et al. 2011). These findings in Lorestan near to our results in Ilam province. The results showed that the predominant strain of the parasite in Ilam province, like the rest regions of the country is G1 genotype or the same common sheep strain belonged to *Echinococcus granulosus* sensu stricto (G1–G3 complex), which is in its life cycle, dogs are the definitive host and livestock is intermediate host and is the cause of human infections in the region that play crucial role in the control and prevention of this disease. Genetic similarity between the size of fragments of DNA bands of *E. granulosus* human and sheep isolates by PCR-RFLP method indicates that the similar genotype of parasite is present in the Ilam province. According to the phylogenetic tree, there is at least one genotype of parasite, which belongs to *E. granulosous* sensu stricto (G1–G3) complex and G1 genotype (sheep strain) is a dominant genotype of human, livestock in Ilam province. Overall isolates sequences of mtDNA indicated 100 % homology with references G1, G2, and G3 sequences in the GenBank database.

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Authors' contributions Sample collection and molecular studies were conducted by Morteza Shamsi under supervision of Prof Abdolhossein Dalimi. Dr. Afra Khosravi and Dr. Fatemeh Ghafarifar were advisors of this research.

Compliance with ethical standards

Funding This study was financially supported by the Tarbiat Modares University, Tehran, Iran.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Tarbiat Modares University Ethical Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statement of animal rights All procedures performed in studies involving animals were in accordance with the ethical standards of the Tarbiat Modares University Ethical Committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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