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ORIGINAL ARTICLE

Genotyping and phylogenetic analysis of hydatid cysts isolated from livestock in Bushehr province, Iran

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Abstract Hydatid cyst is one of the parasitic zoonoses caused by infection with the larval stage of *Echinococcus granulosus* tapeworm. The spread of this parasite is global and is of great importance in terms of public health. To date, ten different species of this parasite have been identified that differ in characteristics such as life cycle, epidemiology and pathogenesis. The purpose of this study was to determine the genotype and phylogenetic relationship of hydatid cysts isolated from livestock of Bushehr province, Iran. About 62 samples of hepatic and pulmonary hydatid cysts were collected from slaughtered animals. DNA extracted by phenol–chloroform method was amplified by PCR using primers specific for the cox1 gene. The PCR products of 50 samples were sequenced and analyzed using BioEdit software and compared with sequences in the GenBank. The phylogenetic tree was drawn using Neighbor Joining tree-NJ method, and its reliability was evaluated. Sequencing results showed that out of 50 sequenced samples, 43 samples had the genotype of *Echinococcus granulosus* and 7 samples had the genotype of *Taenia hydatigena*. By drawing a phylogenetic tree, all 43 hydatid cyst samples belonged to G1 strain. The predominance of G1 strain of hydatid cyst in livestock of Bushehr province shows the main role of this genotype in establishing the life cycle of parasite in this region and if the genotype of the parasite in dogs and humans is determined, then these findings can be used to disrupt the life cycle of the parasite and reduce the human infections.

Keywords Genotype · Hydatid cyst · *Echinococcus granulosus* · Bushehr province

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Introduction

Hydatid disease or hydatidosis is one of the most important parasitic zoonoses caused by infection with the larval stage of *Echinococcus granulosus* tapeworm (Thompson 2017). In the life cycle of this parasite, Canidae, especially herding dogs and street dogs, plays the role of the final hosts and infect the pastures and vegetable fields by repelling the eggs. Humans and herbivores, especially livestock, can host the larval form (hydatid cyst) of this parasite as intermediate hosts (McManus et al. 2003). Carnivores infected with the adult form of the parasite usually do not have many symptoms and problems, but the establishment of parasite larvae in various organs of intermediate hosts, including humans, causes various clinical and even severe and lethal symptoms (Eckert and Deplazes 2004).

This disease is common in most tropical, subtropical and temperate regions of the world and is one of the health problems of many developing countries and various studies

indicate a high prevalence of this disease in the Middle East (Sadjjadi 2006; Deplazes et al. 2017). Numerous studies in Iran have shown a significant variable prevalence in various livestock such as sheep (5–72%), camels (11.4–70%), cattle (3.5–38%) and goats (1.7–20%) (Ahmadi 2005; Rokni 2009). In addition to health problems and various clinical complications in infected individuals, hydatid cyst also imposes significant economic losses on ranchers in infected communities (Harandi et al. 2012). Infection of animals with hydatid cysts, in addition to a significant reduction in livestock products such as meat and dairy products, causes the unusable viscera of slaughtered animals in slaughterhouses and subsequently causes significant losses (Tavakoli et al. 2008).

Various studies have shown that *Echinococcus granulosus* has ten different genotypes or intra-species strains, and in recent classifications often based on the mitochondrial DNA (mtDNA) sequence of the parasite, they are named into four main groups of sensu stricto (genotypes G1 to G3), equinus (G4), ortleppi (G5) and canadensis (G6 to G10). The sensu stricto group consists of sheep strain (G1), Tasmanian sheep strain (G2), buffalo strain (G3), and the G1 strain has the largest human and animal range around the world. There is another genotype of this parasite called *Echinococcus felidis* (*E. felidis*) which has been isolated from South African lions and is classified in a separate group (Nakao et al. 2007; Hüttner et al. 2008).

Due to the intraspecies differences and genetic diversity of this parasite, it is possible that different genotypes act differently in terms of antigenic potency, drug sensitivity, host specificity, life cycle and transmission routes and pathogenicity (Bowles et al. 1992; Thompson and McManus 2001; Thompson 2017). Studies have shown that some strains of *Echinococcus granulosus* are more pathogenic to humans than others and it has also been shown that some of these strains tend to specific organs of the intermediate host body. These genetic findings can play a role in the design and development of vaccines, diagnostic tests, therapeutic treatments, epidemiology, and disease control (Thompson 1995; Ahmadi et al. 2006; Barazesh et al. 2020). On the other hand, various studies have shown that there is strain diversity among species according to the geographical distribution of parasite (Shamsi et al. 2017). Therefore, accurate determination of the dominant parasite genotypes in each region can be applied in designing health programs for preventive and control measures (McManus 2002; McManus and Thompson 2003).

Iran is located in the hyperendemic region of this disease, so it is necessary to have genetic and epidemiological information about this disease in all regions of the country. Bushehr province is located in southwestern Iran and northwest of the Persian Gulf, which is considered a high-risk area for hydatidosis due to its tropical climate and large population of seasonal and nomadic nomads. Therefore, the existence of information about *Echinococcus granulosus* genotype in this region as a basis for health and prevention programs is absolutely necessary.

Materials and methods

Study location

This study was conducted in Bushehr province (Fig. 1), where is located on the northwestern margin of the Persian Gulf and southwest of Iran, with the population of about one million people and hot and humid climate most of the year. The geographical coordinates of this region is between 27° and 19′ to 30° and 16′ north latitude and 50° and 1′ to 52° and 59′ longitude, and the mean annual temperature is 25.7 °C. The weather in the province is warm for 7 months during the year, moderate-cold for 2 months and mild-warm for 3 months.

Sample collection

In this study, 62 samples were collected from organs (liver and lung) infected with hydatid cyst from infected animals in slaughterhouses of Genaveh and Deylam cities in the north, Khormoj and Choghadak cities in the center and Kangan in the south of Bushehr province randomly over a period of 9 months from the early January to September 2019, and were transferred to the parasitology laboratory located at Bushehr University of Medical Sciences. Hydatid cyst fluid (HCF) was aspirated separately and the protoscoleces were washed and fixed by 70% ethanol and kept at − 20 °C until testing.

DNA extraction

First, 200 µl of lysis buffer containing Tris–HCl, EDTA, NaCl, SDS and proteinase K was added to the protoscolex suspension collected from pulmonary and hepatic cysts, and incubated at 55 °C overnight until completely lysed. After adding Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) solution and centrifuging at 12000 rpm for 10 min, the supernatant was discarded and isoamyl alcohol and sodium acetate solution (0.3 M) was added and incubated overnight at − 20 °C. The next day, centrifugation was performed after the temperature of the tubes had reached room temperature, and the precipitate was washed with 70% ethanol. DNA samples were dissolved in 100 µl of water (PCR grade), and their quantity and quality were evaluated by NanoDrop device.

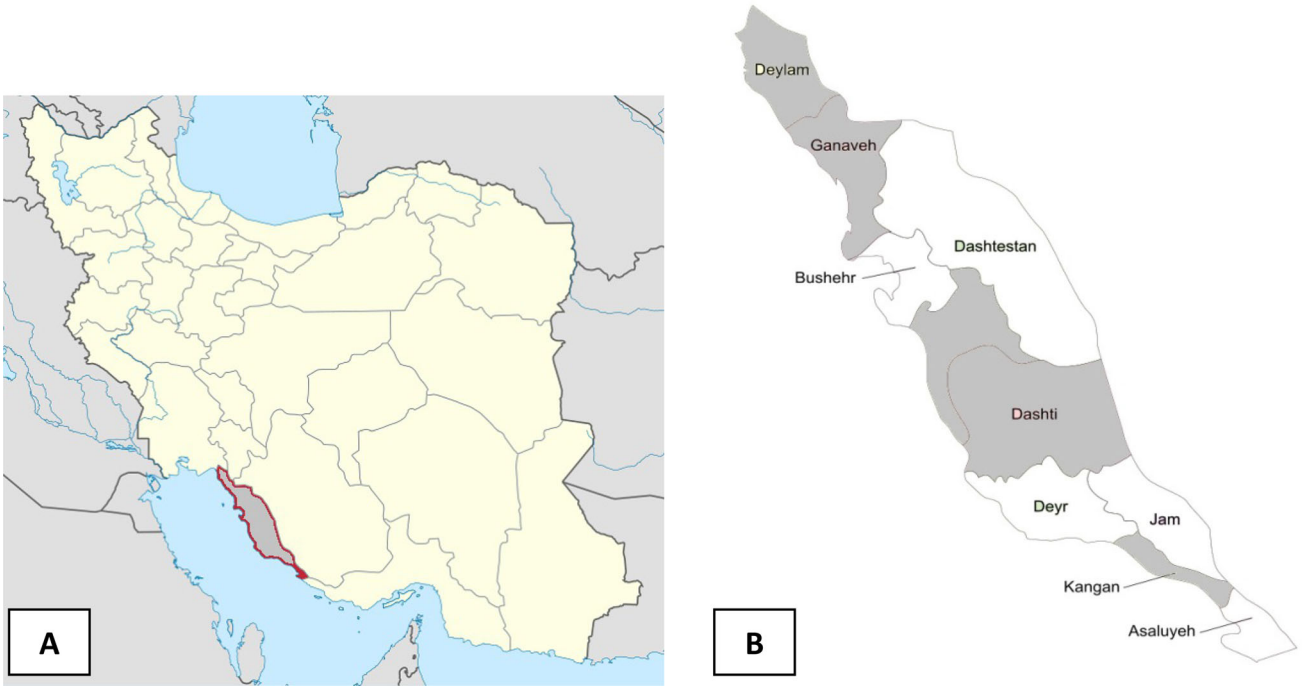

Fig. 1 **a** The location of Bushehr province on the map of Iran; Bushehr province is located in the southwest of Iran and the northwestern margin of the Persian Gulf (red color). **b** Map of Bushehr province by cities. Cities where hydatid cyst samples were collected from slaughterhouses (red color) (colour figure online)

PCR process and gel electrophoresis

The mitochondrial cox1 gene fragment of the parasite was selected as the target whose specific region was amplified by JB3-JB4.5 primer pairs with sequences of 5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′ and 5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′ (Bowles et al. 1992), respectively. Temperature and time programs of 1 × (5′95 °C) + 30 × (30″94 °C +30″56 °C + 30″72 °C) + 1 × (5′72 °C) were used for PCR test. The PCR product was electrophoresed on 2% agarose gel and TAE buffer.

Sequencing strategy and bioinformatics

To prepare the PCR product for sequencing, DNA bands obtained by electrophoresis of PCR products on agarose gel were cut by a Bistouri blade, purified using a Commercial kit (gen all) according to the kit manufacturer's protocol, and sequenced by Bioneer Company (South Korea) using the ABI 3730 automated DNA sequencer (Applied Biosystems). The quality of the electropherograms was checked by MEGA 6.0 software, and the obtained sequences were compared with each other as well as with the sequences on the GenBank database using BioEdit software and BLAST program. The phylogenetic tree was constructed using MEGA 6 software by a Bootstrap Test of phylogeny, and Maximum Composite Likelihood model with 1000 replicates, using ~ 400 bp nucleotide sequence of *cox1* gene".

Results

A total of 62 samples of hepatic and pulmonary hydatid cysts were collected from animals slaughtered in abattoirs of Bushehr province (34 samples of sheep, 26 samples of goats and 2 samples of cattle) and DNA extracted by phenol–chloroform method was amplified by PCR using primers specific for the cox1 gene. Figure 2 shows the electrophoresis bands of 4 samples of cox1 gene PCR products (Fig. 2).

Forty-nine samples out of a total of 62 samples were analyzed and sequenced by Bioneer Company (South Korea) using the ABI 3730 automated DNA sequencer (Applied Biosystems), of which 42 samples had the genotype of *Echinococcus granulosus* and seven samples had the genotype of *Taenia hydatigena*. The multiple alignments of the nucleotide sequences were also performed with the ClustalW application within BioEdit software version 7.1.". After drawing the phylogenetic tree, it was found that all samples of hydatid cyst belonged to G1 strain (Fig. 3).

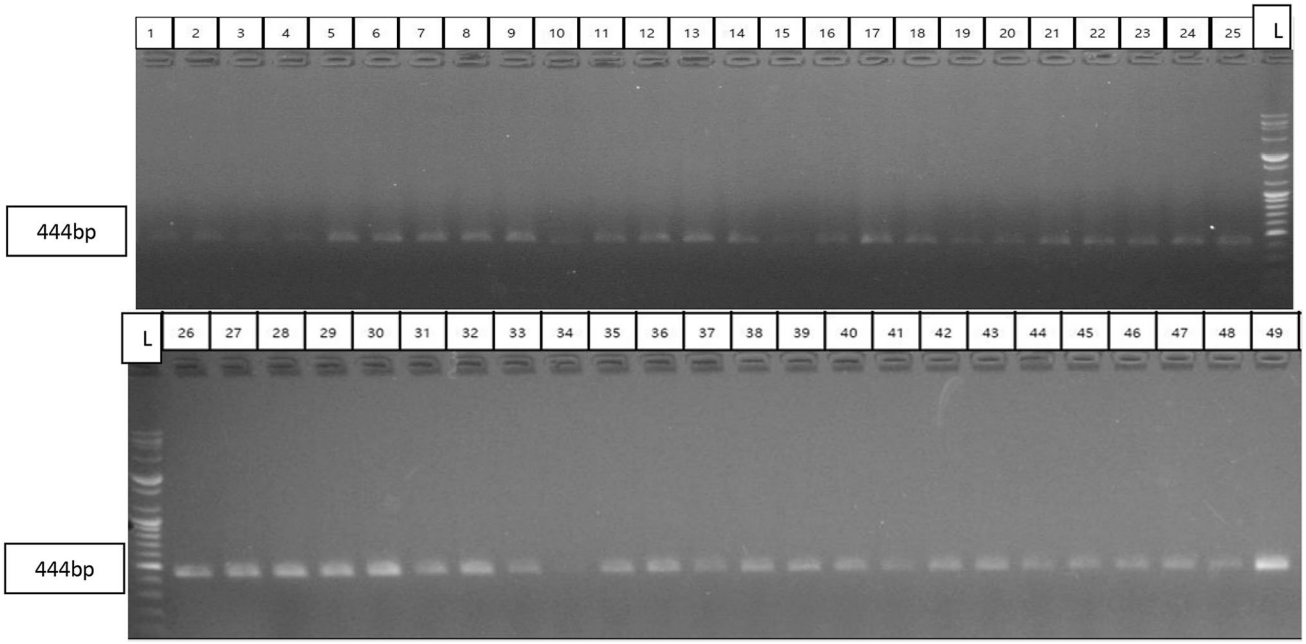

Fig. 2 Electrophoresis of PCR products from *cox1* gene. L: 50 bp molecular marker, 1 to 49: PCR products with a length of 444 bp

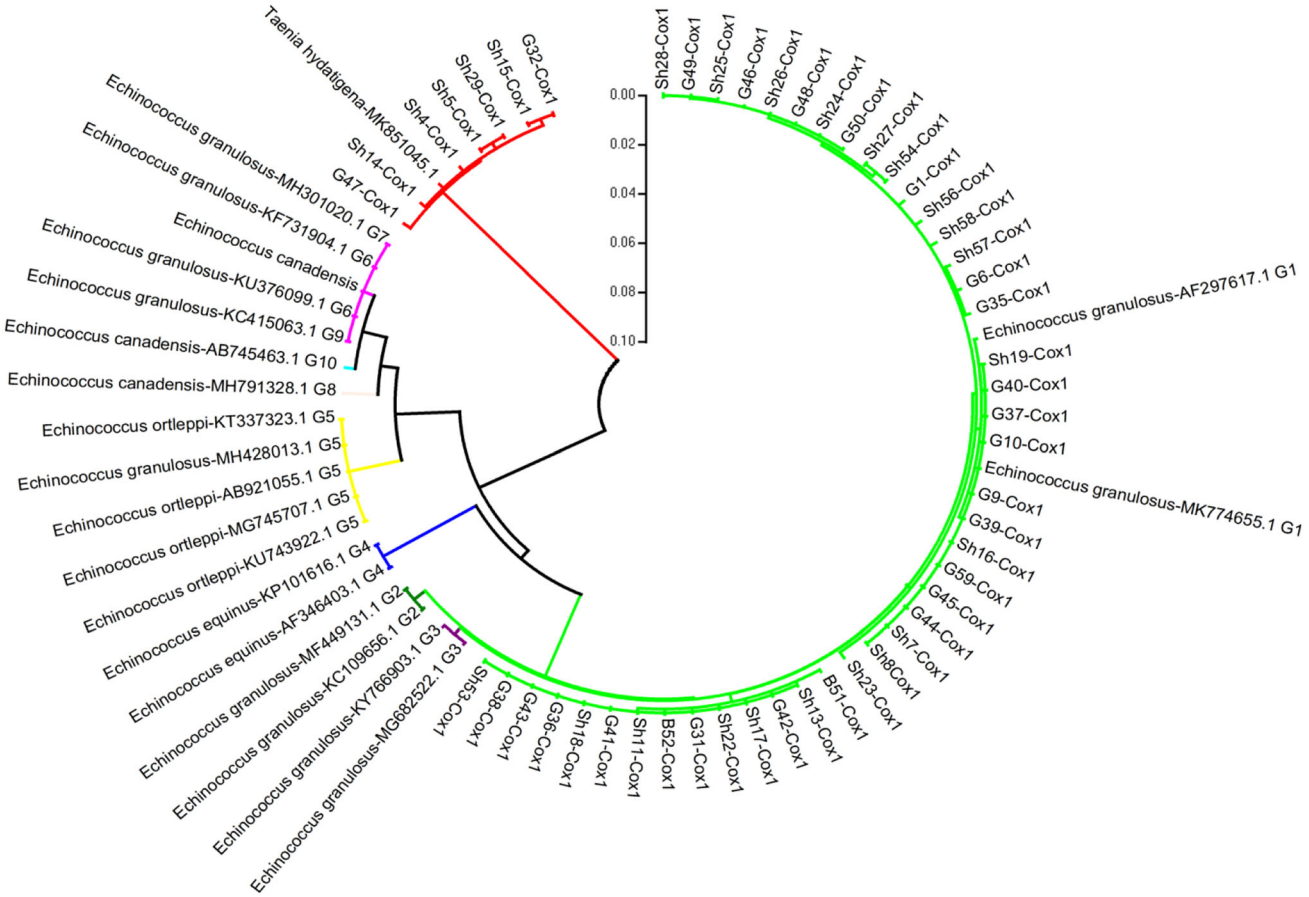

Fig. 3 The phylogenetic tree was constructed using MEGA 6 software by a Bootstrap Test of phylogeny, and Maximum Composite Likelihood model with 1000 replicates, using ~ 400 bp nucleotide sequence of *cox1* gene"

Discussion

Hydatidosis is one of the health problems of many developing countries and various studies indicate a high prevalence of this disease in the Middle East, including Iran (Ahmadi 2005; Sadjjadi 2006; Rokni 2009). The health and economic losses caused by hydatidosis are significant globally and include the treatment cost and resulting disability, as well as the costs imposed on the livestock industry. A study in Iran has shown that the total annual cost of care and treatment of this disease is about 232.3 million dollars (Harandi et al. 2012). Recent molecular studies have shown great genetic diversity in the strains of *Echinococcus* genus. Such a genotypic diversity affects various characteristics of the parasite including life cycle and transmission, biochemical characteristics, pathogenicity and sensitivity to relevant drugs (Yanagida et al. 2012; (Bowles et al. 1992; Thompson and McManus 2001; Thompson 2017). Therefore, it is important to identify the different genotypes of this parasite in each geographical area in order to develop health and prevention programs, as well as design and manufacture of protective vaccines, diagnostic tests with high sensitivity and specificity, and provide effective treatment protocols (McManus 2002; McManus et al. 2003; Busi et al. 2007; Nakao et al. 2013).

The results of our study showed that 43 samples of hydatid cysts related to sheep, goats and cattle studied in Bushehr province belonged to G1 genotype of *E. granulosus*. This result is consistent with the findings of many studies in other parts of Iran as well as global studies, and G1 sheep strain is the only genotype present or the dominant genotype among all intermediate hosts of this parasite. Rostami Nejad et al. (2012) in their study of different regions of Iran have emphasized the presence of two strains of G1 and G6 (with a predominance of G1) in different intermediate hosts including cattle, sheep, goats, camels and buffalo. In a study conducted by Yakhchali and Mardani (2013) to determine the dominant strain of *Echinococcus granulosus* in West Azerbaijan Province (Iran), the sequencing results showed that all samples of cattle, sheep and goats had G1 genotype and the G3 strain was the only genotype found in the studied buffaloes. In a study by Haniloo et al. (2013) on 49 sheep, 28 cattle and 9 human samples from Zanjan province (Iran) using PCR–RFLP method to amplify the ITS1 fragment, the dominant genotype found in all samples was introduced to be G1. Similar studies performed in Lorestan, Isfahan, and Chaharmahal and Bakhtiari provinces (Iran) as well as Northern provinces of Iran on livestock samples by PCR–RFLP method and amplification of ITS1 fragment introduced G1 genotype as the only genotype found in the studied samples (Yousefi 2008; Gholami et al. 2009; Kia et al. 2010; Parsa et al. 2011).

Furthermore, seven of the samples studied in the present study had the genotype of *Taenia hydatigena*. *T. hydatigena* is non-zoonotic and the lifecycle of this *Taenia spp.* is between canids as definitive hosts and sheep or goats as intermediate hosts containing the metacestode larval stage called Cysticercosis (Ohiolei et al. 2019). In a molecular evaluation carried out by Alvi et al. (2020) based on mitochondrial *cox1* gene in eastern Punjab province of Pakistan, the prevalence of *T. hydatigena* metacestodes obtained 4.40%.

In a recent comparative study by Barazesh et al. (2019) on livestock in the Azerbaijan region of Iran and the Van province in Turkey, which has always been considered as another endemic region in the Middle East alongside Iran and Iraq, the results showed that the G1 strain was predominant, and the G3 strain was obtained in only one case and the G1/G3 strain in three samples. Numerous studies in other countries have reported similar results. In two separate studies from different parts of China by Xue-Yong et al. (2018) and Guo et al. (2019), and Odongo et al. (2018) in Kenya, the G1 genotype was identified as the dominant genotype. Hammad et al. (2018) in Iraq, where is located in the neighborhood of Iran and is considered as another endemic region of the disease in the Middle East, have mentioned the G1 genotype in sheep and G3 in buffaloes with the highest prevalence. This was the first report of the presence of genotypes other than G1 in Iraq.

In some previous studies, genotypes other than G1 have been reported as the dominant genotype; Ahmed et al. (2018) in Sudan examined 50 cattle samples and reported the prevalence rates of G6, G5 and G1 genotypes as 88%, 8% and 4%, respectively. Moudgil et al. (2019) in India obtained G7 genotype by examining hydatid cysts in sheep and goats. Abbas et al. (2016) in Egypt examined 500 samples of cattle hydatid cysts, and were able to identify two genotypes of *Echinococcus*, which included G5 (*E. ortleppi*) and G1. This study is the first report of *E. ortleppi* genotype 5 (G5) isolates from cattle in Egypt.

Due to the fact that a significant number of animals slaughtered in slaughterhouses in Bushehr province, mainly from the western provinces of the country (Kermanshah, Lorestan and Khuzestan) enter the province and the predominant genotype in those areas is G1 strain, the identification of G1 genotype in the samples analyzed in this study is not far-fetched.

The findings of this study show that G1 is the only genotype or dominant genotype of *Echinococcus granulosus* in this province. However, the genotype of hydatid cysts isolated from the body of patients as well as *Echinococcus* tapeworms in the intestine of canidae need to be examined to obtain additional information and identify

the complete transmission cycle of this parasite. All of this information can be used to develop programs to prevent disease and arrest the parasite transmission cycle, thereby reducing the incidence of human infection.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This research was financially supported and approved by the Ethical Committee of Bushehr University of Medical Sciences with Ethics No. IR.BPUMS.REC.1397.108.

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