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ORIGINAL ARTICLE

Molecular characterization and sequence analysis of *Echinococcus granulosus* from sheep isolates in East Azerbaijan province, northwest of Iran

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Abstract *Echinococcus granulosus* as an etiologic agent of hydatid cyst is one of the most important zoonotic helminthes in the world that causing enormous economic and health losses. The aim of this study was to evaluate genotype of *E. granulosus* isolated from sheep using mitochondrial cytochrome c oxidase subunit 1 (cox1) gene and sequencing method in East Azerbaijan province, northwest of Iran. Nineteen sheep hydatid cyst samples were collected. Genomic DNA was extracted from protoscoleces using commercial DNA extraction kit. Mitochondrial cox1 region was amplified by polymerase chain reaction (PCR) and all isolates were sequenced. Afterward, sequences were analyzed for determination of genotypes by related software. G1 (94.73 %) and G3 (5.27 %) genotypes were identified from the isolates which out of 19 hydatid cysts, 17 samples were G1B, 1 sample G1D and the other one had G3 genotype. Results of this study indicate that common sheep strain (G1); especially G1B is the dominant subtype of *E. granulosus* in East Azerbaijan province.

Keywords *Echinococcus granulosus* · Cox1 · Sheep · Sequencing · Iran

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Introduction

Echinococcus granulosus, as one of the smallest tapeworms of the Taeniidae, infects dogs and wolves, whereas the larval stage (hydatid cyst) expands in several species of wild and domestic mammals and in humans, causing a zoonosis of great veterinary and medical importance, cystic echinococcosis (CE) (Daniel Mwambete et al. 2004). Liver and lung are the main sites to formation hydatid cyst (Ghabouli Mehrabani et al. 2014).

This disease has been reported from the Middle East, Russia, Australia, New Zealand, America and Africa (Schantz 1995). Hydatidosis, as a main public health concern, is endemic in many areas of the Iran. The prevalence of CE in livestock (sheep, cattle, camels and goats) in Iran was reported 6.7 % totally. Also based on serological studies, seroprevalence rate of the infection in human demonstrated from 1.2 to 21.4 % in different areas of Iran (Hajialilo et al. 2012). According to studies, the prevalence of hydatid cyst in sheep and cattle in different regions of the country is 5.1–74.4 and 3.5–38.3 % respectively (Rokni 2009).

This organism shows a vast intraspecific variation associated to host specificity, morphology, epidemiology, biology, physiology and genetic (Daniel Mwambete et al. 2004). Since, ten strains or genotypes (G1–G10) have been described of *E. granulosus* (Piccoli et al. 2013). Some authors have suggested that these genotypes should be clustered into four different species: *E. granulosus* sensu stricto (G1, G2 and G3, or G1–G3 complex), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), and *Echinococcus canadensis* (G6 to G10, or G6–G10 complex) (Knapp et al. 2011). These genotypes are included common strains of sheep (G1); Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel

strain (G6), pig strain (G7 & G9) and deer strain (G8 & G10) (Youssefi et al. 2013).

Different techniques have been applied to the study on genetic variability of *E. granulosus*. Lately, researches have focused their analysis based on the parasite's mitochondrial cytochrome c oxidase subunit 1 (cox1) region as suitable genetic marker (Pour et al. 2011). It was felt necessary to study due to hyperendemicity of disease in Iran and East Azerbaijan region and multiplicity of intermediate hosts which carry the disease. The results about *E. granulosus*' genotyping in different region can be used in studies on the prevention and control, epidemiology, vaccine design, drug sensitivity, life cycle analysis, transmission and disease progression (Ergin et al. 2010).

The aim of this study was to evaluate genotype of *E. granulosus* isolated from sheep using (cox1) gene and sequencing method in East Azerbaijan province, northwest of Iran.

Material and methods

Sample collection

In this study, 19 *E. granulosus* cysts were collected from sheep. The animals originated from various locations within East Azerbaijan province that slaughtered in abattoirs located in the Tabriz. Each animal cyst was processed as an *E. granulosus* isolate. Cyst fluid containing protoscoleces were aspirated from cysts by a separate syringe under sterile conditions and were washed three times with normal saline and stored at −20 °C.

DNA extraction

Genomic DNA was extracted from protoscoleces using commercial DNA extraction kit *(AccuPrep®Genomic DNA Extraction kit Cat.No: K-3032)* according to the manufacturer's instructions and then stored at −20 °C until polymerase chain reaction (PCR) amplification.

Polymerase chain reaction (PCR)

To amplification of the cox1 gene fragment we used primers (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and (5′TAAAGAAAGAACATAATGAAAATG-3′) as forward and reverse respectively (Ergin et al. 2010). Twenty microliter reaction volumes containing Taq DNA polymerase (1 U), from each dNTP (dATP, dCTP, dGTP, dTTP) 250 µM, Tris–HCL (pH 9.0) 10 mM, KCL (30 mM), Mgcl2 (1.5 mM), Template DNA (50 ng) 7 µl and (10 Pmol) 1 µl of each primer were used and amplified by PCR under the following temperature conditions: initial denaturation 94 °C (5 min) and then denaturation 94 °C (30 s), annealing 56 °C (45 s), extension 72° (35 s), in 35 cycles and final extension 72° (10 min). PCR products were electrophoresed on 1.5 % agarose gel after staining with safe stain and then 440 bp band of cox1 gene was seen under UV light using transilluminator.

Sequencing and phylogenetic analysis

Nineteen PCR products were purified by gel extraction kit (*AccuPrep®Gel Purification Kit Cat No: K-3035*) according to the manufacturer's instructions and sequenced by Genetic Analyzer 3130 ABI. Sequences were compared with each other and available reference sequences in GenBank using Chromas and Sequencher softwares and BLAST program. Reference sequences of *E. granulosus* genotypes (G1–G10) and *Echinococcus vogeli* (as outgroup) were inferred from previous publications (Schneider et al. 2010; Bowles and McManus 1993) and the National Center for Biology Information (http://www.ncbi.nlm.nih.gov/). After multiple alignments by ClustalW, phylogenetic analyses of the sequences data were performed using cox1 sequences and phylogeny tree was drawn using sequences obtained in this study as well as reference sequences of all described *E. granulosus* genotypes by MEGA4 software. GenBank accession numbers for the sequences obtained from this study and the reference genotypes are shown in Table 1.

Results

PCR amplification and sequencing was successfully performed on hydatid cyst isolates. After electrophoresis on PCR product, 440 bp bands were seen in all samples under UV light clearly. G1 (94.73 %) and G3 (5.27 %) genotypes were identified from the isolates which out of 19 sheep hydatid cysts, 17 samples were G1B, 1 sample G1D and the other one had G3 genotype.

Three sequences (one of each subtype) were submitted to the GenBank and registered under following accession numbers KJ540227, KJ540229 and KJ540231. Phylogenetic tree based on analysis of sequences is shown in Fig. 1. Isolates were grouped into two distinct clusters related to the G1–G3 complex and the G6–G10 complex. In this study, most of the isolates (94.73 %) were identified as G1 (sheep strain) and were clustered with the G1 reference genotype. Also isolate identified as G3 (buffalo strain) (5.27 %) clustered with the G3 reference sequence.

Discussion

The results of this study indicated that the G1 genotype of *E. granulosus* (sheep strain) was the most commonly

Table 1 Haplotypes of *Echinococcus granulosus* obtained from East Azerbaijan province and other investigations based on cox1 gene

E. granulosus Haplotype	Host	Accession number	Reference
S1	Sheep	–	This study
S2	Sheep	–	This study
S5	Sheep	–	This study
S7	Sheep	–	This study
S8	Sheep	–	This study
S9	Sheep	KJ540227	This study
S10	Sheep	–	This study
S11	Sheep	–	This study
S13–S18	Sheep	–	This study
S19	Sheep	KJ540229	This study
S20	Sheep	KJ540231	This study
S21–S23	Sheep	–	This study
G1A	Livestock-human	AF458871	(Kamenetzky et al. 2002)
G1B	Sheep-cattle	FN646370	(Beato et al. 2010)
G1B	Pig	KC660075	(Monteiro et al. 2014)
G1B	Sheep	DQ062857	(Varcasia et al. 2007)
G1B	Human	JX854029	(Sharma et al. 2013)
G1C	Livestock-human	AF458873	(Kamenetzky et al. 2002)
G1D	Homo sapiens	DQ356880	(Bart et al. 2006)
G1D	Livestock-human	AF458874	(Kamenetzky et al. 2002)
G1E	Livestock-human	AF458875	(Kamenetzky et al. 2002)
G2	Sheep	KC109652	(Adwan et al. 2013)
G3	Sheep	EF545563	(Vural et al. 2008)
G3	Sheep	DQ269943	(Bhattacharya et al. 2007)
G4	Horse	M84664	(Bowles et al. 1992)
G5	Spotted deer	JX068638	(Boufana et al. 2012)
G6	Camel	HM626406	(Sharifiyazdi et al. 2011)
G7	Pig	JQ356718	(Umhang et al. 2013)
G8	Alces alces	AB777910	(Konyaev et al. 2013)
G9	Homo sapiens	KC415063	(Sharma et al. 2013)
G10	Reindeer	AF525457	(Lavikainen et al. 2003)
Out group	Rodent	M84670	(Bowles et al. 1992)
E. vogeli			

identified genotype from sheep in East Azerbaijan province, Iran. These finding suggest that sheep–dog cycle occur in this region. G1 is the dominant genotype found in world livestock (Moro and Schantz 2008). However in some North African countries, such as Sudan, G6 is the dominant genotype which found in sheep, goats and camels (Hajialilo et al. 2012).

In order to genotyping of sheep hydatid cysts using sequencing with cox1 gene, G1 is reported as predominant genotype in different countries such as Peru, China, Turkey, Italy and Tunisia that are consistent with this study (Sánchez et al. 2010; Ma et al. 2008; Utuk et al. 2008; Busi et al. 2007; Varcasia et al. 2006; M'rad et al. 2005).

In a study that was done by (Pezeshki et al. 2013) in the Ardabil province, out of 19 sheep hydatid cysts, 18 samples G1 and 1 sample G3 genotype were reported. The results of our study are quite similar with this study that could be due to the proximity of Ardabil with East Azerbaijan province in terms of parasite life cycle. This indicates that the G3 genotype could be as one of the etiologic factors in this area. Therefore, to determine transmission cycle, the study of G3 genotype reservoirs as intermediate hosts in this region is essential. In a study in Egypt (Abdel Aaty et al. 2012), 42 sheep hydatid cyst samples were genotyped that all samples were reported as G6 genotype. Also in Pakistan (Latif et al. 2010), one-

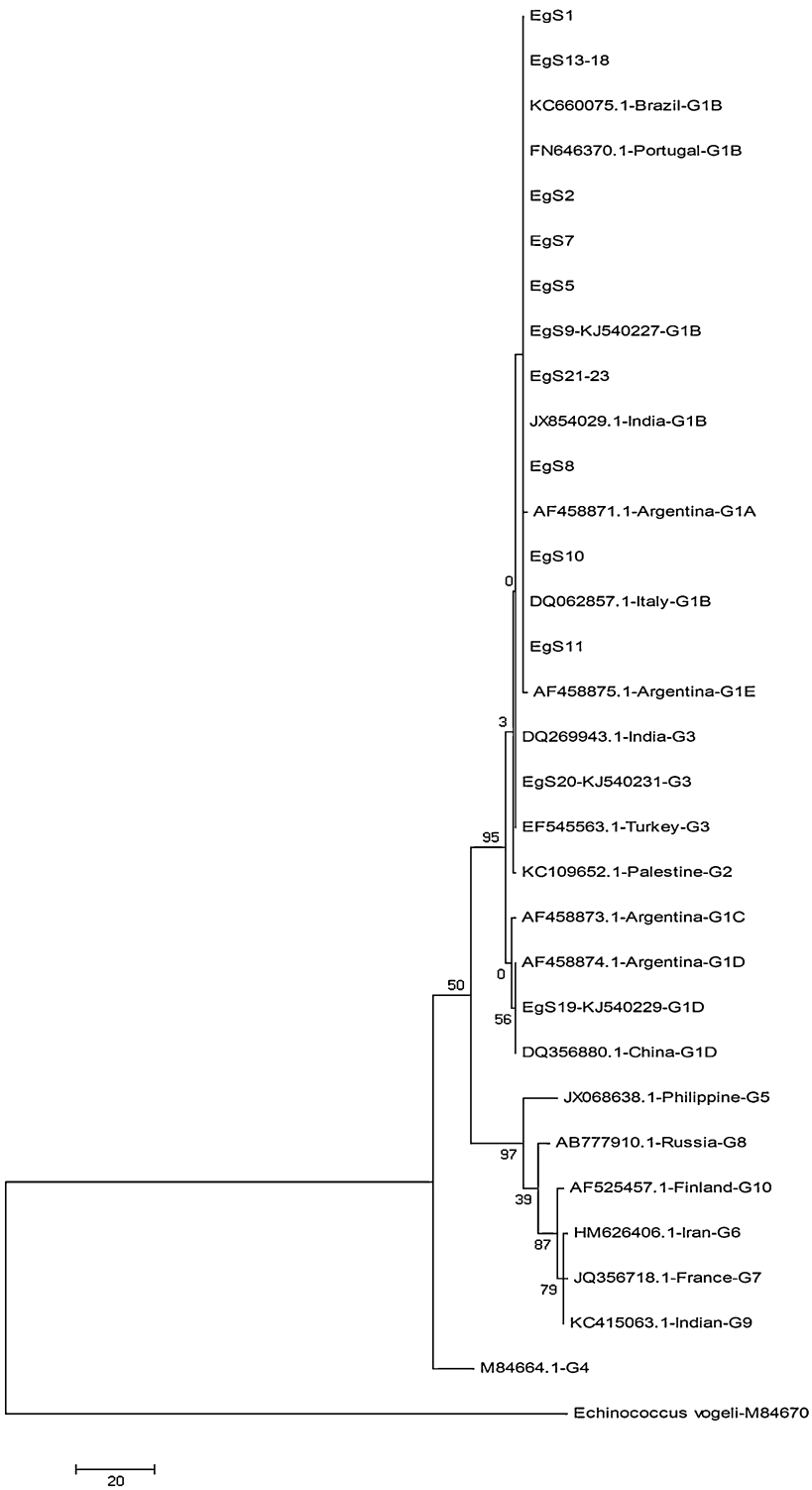

Fig. 1 Phylogeny tree of *Echinococcus granulosus* sheep isolates from Tabriz, Iran and reference sequences for G1–G10 genotypes of *E. granulosus* as well as *Echinococcus vogeli* as the outgroup

third of samples were G1 genotype and in Indian study (Pednekar et al. 2009), out of 8 cysts, 6 samples were reported G3 genotype and 2 samples had G1 genotype. So far, 10 genotypes (G1–G10) have been reported from different intermediate hosts of *E. granulosus* species complex in the world (Thompsom 2008). Due to distribution of intermediate hosts in different parts of the world, there is probability to the difference of genotypes in different regions and their prevalence rate. In other words, the predominant or high prevalence of one genotype in an area indicates the significance and key role of its intermediate hosts in parasite life cycle.

Consequently, all prevention and control proceedings of disease are based on the dominant genotype in a region.

PCR–RFLP, SSCP and Semi nested-PCR as other molecular methods for genotyping of *E. granulosus* were not able to separate correctly genotypes of G1–G3 (*E. granulosus* sensu stricto) and G6–G10 (*E. Canadensis*) (Simsek et al. 2011; Jabbar et al. 2011). In a Greek study in 2007 out of 20 sheep and goat cysts all sheep samples were G1 and goat samples have G6/G7 genotypes by Semi nested-PCR method, while in sequencing, 18 sheep samples, G1 and two samples were G3 genotype. The entire goat samples were reported as G7 genotype (Varcasia et al. 2007). In a study in Turkey, all bovine and sheep samples were reported G1–G3 genotype with SSCP method (Simsek et al. 2011). Also in study conducted in Iran on bovine, sheep and human samples, all samples were reported G1–G3 genotype using PCR–RFLP method (Dousti et al. 2013). As a result, because of exact separation between genotypes of sequencing method, this method is preferable than another methods for genotype determination.

In order to genotype of *E. granulosus*, there are used various molecular methods based on mitochondrial and nuclear DNA genes but according to results of conducted studies, mitochondrial genes are more preferable than other methods for *E. granulosus* genotyping. Information obtained from mitochondrial DNA can help researchers to solve parasite taxonomy problems (Gillespie and Pearson 2001; Schantz 2003).

In the present study, isolates were clustered in major group related to the G1–G3 genotype complex. One identified isolate as G3 genotype was grouped together with the G3 reference genotype (Fig. 1) and being the G1 isolates, providing further evidence that the G1 and G3 genotypes should be considered as *E. granulosus* sensu stricto (Hajialilo et al. 2012).

This study demonstrated that, G1 genotype is transmitted from sheep in Iran. Additionally, this study showed that G1 especially G1B subtype is the predominant among sheep in Northwest of Iran and sheep–dog cycle have interactions in this region.

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Conflict of interest The authors declare that there is no conflict of interest.

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