

## Determination of *Echinococcus granulosus* genotypes in livestock slaughtered in Shush County, Southwest Iran using PCR-RFLP

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### Summary

Echinococcosis is a zoonotic disease caused by the larval stages of *Echinococcus* spp. that occurs in most parts of the world. Herein, we aimed to evaluate the genotypes of isolated hydatid cysts from slaughtered animals in Shush county, southwestern Iran. Totally, 96 hydatid cysts were collected, including 11 buffaloes, 13 cattle, 12 goat and 60 sheep. The PCR was done by a primer pair (BDI and 4s) to amplify ITS1 fragment. Four restriction endonucleases including AluI, HpaII, RsaI, and TaqI were used for RFLP products and enzymatic reactions were electrophoresed. Finally, twenty PCR products were sent for sequencing and phylogenetic tree was drawn with MEGA6. Molecular identification of 96 hydatid cysts demonstrated a distinctive 1000 bp fragment in all samples from four animal hosts. RFLP analysis showed similar digestion patterns in all samples. AluI digestion yielded 800 bp and 200 bp fragments, HpaII digestion made 700 bp and 300 bp fragments and RsaI digestion entailed 655 and 345 segments. Moreover, TaqI rendered no digestion pattern on rDNA-ITS1 region. Additionally, *E. granulosus* sensu stricto (G1-3 complex) was the prevailing genotype in all livestock samples, according to PCR-RFLP and sequencing analyses.

**Keywords:** *Echinococcus granulosus*; genotypes; livestock; Shush County; PCR-RFLP

### Introduction

As an ancient zoonotic parasitosis, cystic echinococcosis (CE) is still a landmark neglected tropical disease around the globe, rendered by *Echinococcus granulosus* (*E. granulosus*) larval stage (Cardona & Carmena, 2013; Ito & Budke, 2017; Moro, P. & Schantz, 2009). The endemicity of CE is appointed to various parts of the world, including South America, the Middle East, and Mediterranean zone, northeastern Africa as well as Australia (Ito & Budke, 2017; Rojas *et al.*, 2014). Iran is considered as an endemic area for CE in the Middle East region, specifically owing to traditional animal husbandry and availability of abattoir wastes to dogs (Rokni, 2009). Based on slaughterhouse investigations, the animal prevalence rates in hyperendemic areas ranges from 20 %

to 95 %. Also, it is estimated that US\$ 3 billion is considered for case treatment and livestock losses (Who, 2017). Being a cyclozoonosis, the continuity of the *E. granulosus* life cycle is relied on the ecological interactions between domestic/wild canid populations (definitive hosts) and ungulates (intermediate hosts) in the natural environment. In this regard, there exist two ecological cycles of the hydatidosis: the domestic cycle frequently found in countries with sheep and cattle farming, and sylvatic cycle involving feral carnivores and wild herbivores (Carmena & Cardona, 2014; Otero-Abad & Torgerson, 2013). Although rare, aberrant human infections may occur via accidental ingestion of parasite eggs shed in dog feces in communities with poor sanitation practices (Rokni, 2009; Torgerson *et al.*, 2002).

From phenotypic characters and gene sequences standpoint, *E.*

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Fig. 1. Collected hydatid cyst samples from slaughtered livestock in Shush abattoir.

*granulosus* sensu lato is currently divided into genotypes G1– G10 of which *E. granulosus* sensu stricto (G1-3 complex) are the most frequently implicated genotypes in human infections. *E. felidis* (the former 'lion strain'), *E. equinus* (the 'horse strain', G4), *E. ortleppi* (the 'cattle strain', G5), *E. canadensis* and the latter species, as recognized here, shows the highest diversity and is composed of the 'camel strain', G6, the 'pig strain', G7, and two 'cervid strains', G8 and G10 (Kinkar *et al.*, 2017; Moazeni-Bistgani *et al.*, 2013). G1 is the most eminent and exclusive cause of human and animal infection, cycling between dogs and sheep in vast pastoral lands globally; however, phylogenetic evidence demonstrate that other animals such as goat, cattle, and camel, also, act as minor intermediate hosts for this genotype (LyMBERY, 2017). So far, many molecular tests have been used to discern the genotypic characteristics of *E. granulosus* (Ito & Budke, 2017). Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) is a verified molecular diagnostic for genotype detection, based on sequence-specific endonucleases (Bowles & Mcmanus, 1993;

Dousti *et al.*, 2013; Mcmanus, 2002). Alterations in the genetic variants of *E. granulosus* populations, their host diversity, and public health importance would emphasize the molecular discernment of *E. granulosus* genotypes (Craig *et al.*, 2007; Moazeni-Bistgani, Taghipoor *et al.*, 2013; Siles-Lucas & Gottstein, 2001). Herein, we investigated the prevalence of *E. granulosus* genotypes in slaughtered livestock of Shush city, Khuzestan province, Southwest of Iran.

## Materials and Methods

### Study area and sample collection

Shush city is located in Khuzestan province, Southwest of Iran, have 3,577 km<sup>2</sup> area and dry and hot weather, with air temperatures ranging +1 and +53°C. During March - November 2017, a total number of 96 hydatid cysts were collected from slaughtered livestock at Shush abattoir, including 11 buffaloes, 13 cattle, 12 goats and 60 sheep (Fig. 1). Protoscolices of *E. granulosus* were removed from hydatid cyst contents and prepared as previously described by Smyth *et al.* and Balbinotti *et al.* (Balbinotti *et al.*, 2012; Smyth & Davies, 1974) Briefly, the hydatid cysts were aspirated and examined microscopically for cyst fertility. Protoscolices that aspirated from fertile cyst were rinsed multiple times with sterile 0.9 % sodium chloride solution and maintained in 70 % ethanol for molecular purposes.

### DNA extraction and PCR amplification

At first, all stored samples were washed twice with PBS to remove ethanol. The DNA extraction procedure was accomplished using PrimePrep genomic DNA isolation kit from tissue (GeNet Bio, South Korea) based on the manufacturer's protocol and the genomic DNA was kept at -20°C for PCR reaction. The concentration of each DNA sample was measured by NanoDrop (Thermo, USA) evaluation at A260. In each sample, an Internal Transcribed

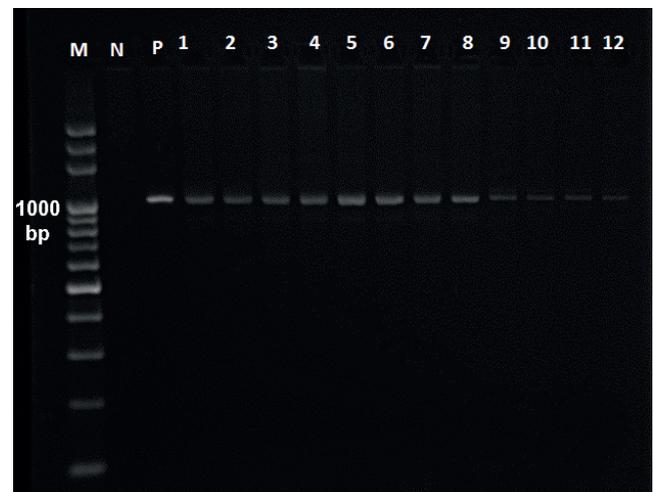


Fig. 2. Distinctive 1000 bp amplified fragment for *E. granulosus* ITS1 in conventional PCR.

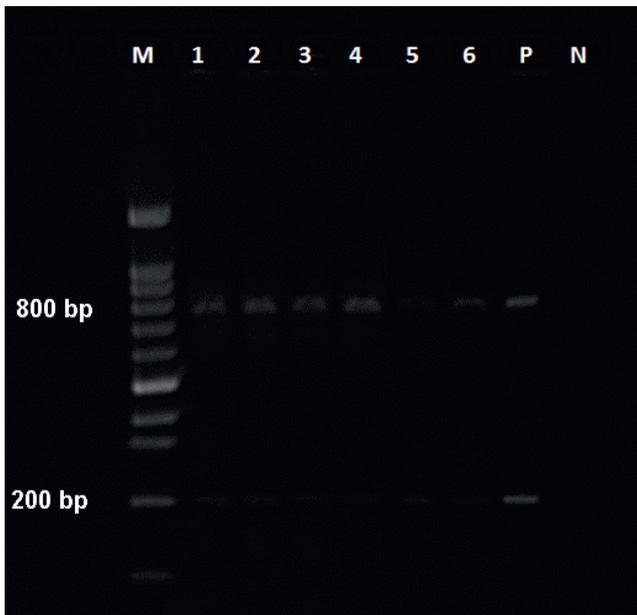


Fig. 3. Enzymatic digestion with Alul. Lanes 1,2: sheep; lanes 3,4: cattle; lane 5: buffalo; lane 6: goat; lanes 7 and 8: positive and negative controls, respectively.

Spacer 1 (ITS1) fragment was amplified by conventional PCR, using a specific primer pair:

BDI (5'-GTCCTAACAAAGGTTTCCGTA-3') for the 18S region.

4S (5'-TCTAGCGTTCGAA(G/A)TGTCGATG-3') for the 5.8S region.

A 25 µl PCR mixture was prepared for each sample, containing 12.5 µl of Master Mix (Ampliqon, Denmark), 3 µl of extracted DNA, ten pmol of each primer and 6.5 µl of sterilized water. The following PCR program was carried out in an automated thermo cycler (FlexCycler, Analytik Jena, Germany): an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec, followed by a final extension step at 72°C for 5 min. Finally, the PCR products were separated by 1.5 % agarose gel electrophoresis and displayed under ultraviolet (UV) illumination.

#### Enzymatic digestion

To perform PCR-RFLP, four specific restriction endonucleases, enclosing Alul (AG/CT), HpaII (C/CGG) (Jena Bioscience, Jena, Germany), RsaI (GT/AC) and TaqI (T/CGA) (Thermo Scientific, Massachusetts, United States) were used. In case of digestion with RsaI and TaqI, the procedure was done in 50 µl reaction mixture, including 5 µl of the universal buffer, one µg of pure DNA or PCR product, 10 U of enzyme and PCR-grade water for the rest. The prepared mixtures were incubated in a thermocycler, being set to 37°C for RsaI and 65°C for TaqI, both for 2 hours. Also the digestion mixture for Alul and HpaII included Tango buffer (2 µl), PCR product (10 µl), enzyme (1-2 µl) and nuclease-free water (18 µl), which incubated at 37°C for an hour. All digested products were visualized using 2 % agarose gel electrophoresis and under UV condition.

#### Sequencing and phylogenetic analysis

Twenty PCR products were submitted to be sequenced by an ABI-3730XL capillary machine (Macrogen Inc., South Korea) in two directions using forward and reverse primers. The sequencing results were interpreted and compared to other GenBank registered sequences using the BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple alignments was exerted by ClustalX and Bioedit software to align and compare obtained nucleotide sequences. Neighbor-joining approach using MEGA 6 bioinformatics software was used to create the phylogenetic tree.

#### Ethical Approval and/or Informed Consent

The study protocol No: IR.AJUMS.REC.1396.410 was approved by the Ethics Committee on Research in School of Medicine, Ahvaz Jundishapur University of Medical Sciences

#### Results

In total, 96 hydatid cyst isolates were collected from slaughtered domestic animals of Shush abattoir. Molecular identification of hydatid cysts by conventional PCR showed a distinctive 1000 base pair (bp) fragment in all samples from four animal hosts (Fig. 2). The PCR-RFLP analysis of ITS1 segment of *E. granulosus* cysts demonstrated similar digestion patterns in all samples. Alul digestion yielded 800 bp and 200 bp fragments (Fig. 3), HpaII digestion made 700 bp and 300 bp fragments (Fig. 4), and RsaI digestion entailed 655 bp and 345 bp segments (Fig. 5). Moreover, TaqI rendered no digestion pattern on rDNA-ITS1 region (Fig. 6). Also, molecular sequencing disclosed that all 20 hydatid cysts were *E. granulosus* sensu stricto genotype and no other strains were discovered.

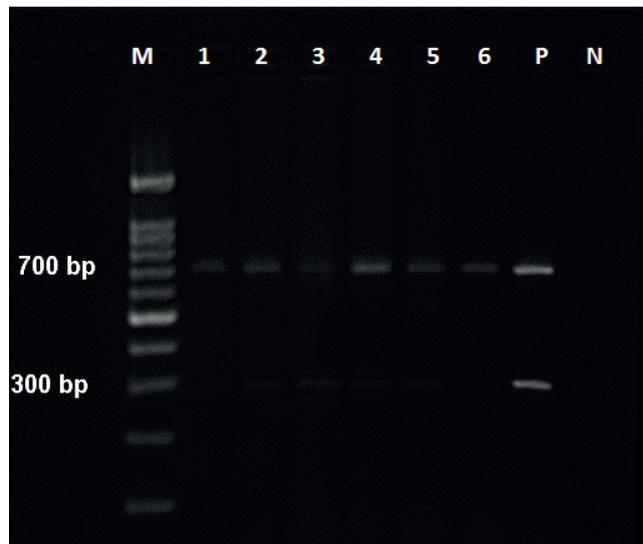


Fig. 4. Enzymatic digestion with HpaII. Lanes 1,2: sheep; lanes 3,4: cattle; lane 5: buffalo; lane 6: goat; lanes 7 and 8: positive and negative controls, respectively.



Fig. 5. Enzymatic digestion with RsaI. Lanes 1-4: sheep; lanes 5-7: cattle; lanes 8, 9: buffalo; lanes 10, 11: goat; lanes 12 and 13: positive and negative controls, respectively.

## Discussion

Hydatidosis is a major public health issue in endemic countries such as Iran (Rokni, 2009). Determination of variations in *E. granulosus* genotypes is an interesting standpoint of hydatidosis research, to better understand ecological processes and life cycle patterns. The accurate taxonomy of *E. granulosus* isolates can be revealed by a wide array of molecular approaches (Carmena & Cardona, 2014; Cucher *et al.*, 2015; Mcmanus & Bowles, 1996). The ITS segment of rDNA, particularly ITS1, has been proved to be an impressive genetic marker for *Echinococcus* genotyping studies (Fadakar *et al.*, 2015; Siles-Lucas *et al.*, 2017). Concerning Bowles *et al.*, a 1000 bp band was observed in all 96 specimens by amplification of rDNA-ITS1 fragment, highlighting the *E. granulosus* identity (Bowles *et al.*, 1995; Bowles & Mcmanus, 1993). Additionally, *E. granulosus sensu stricto* was the prevailing

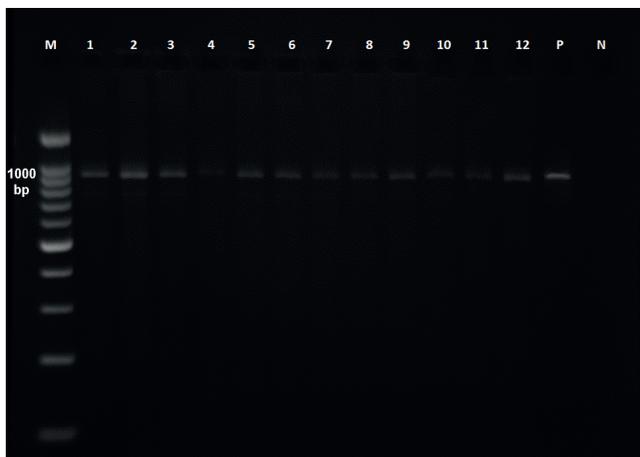


Fig. 6. Enzymatic digestion with TaqI. Lanes 1-4: sheep; lanes 5-7: cattle; lanes 8, 9: buffalo; lanes 10, 11: goat; lanes 12 and 13: positive and negative controls, respectively.

genotype in all livestock samples, according to PCR-RFLP and sequencing analyses. Reportedly, previous investigations across the country have demonstrated the sheep strain as the major detected genotype of CE in production animals (Ahmadi & Dalimi, 2006; Harandi *et al.*, 2002; Moghaddas *et al.*, 2015; Pezeshki *et al.*, 2013; Pour *et al.*, 2011; Sharbatkhori *et al.*, 2010; Sharbatkhori *et al.*, 2016; Zhang *et al.*, 1998). Khademvatan *et al.* reported G1 as the only cause of hydatid cyst in 329 examined livestock (sheep, cattle, and goat) of southwestern Iran, using PCR-RFLP with AluI, MspI and RsaI restriction endonucleases (Khademvatan *et al.*, 2013). Based on a genotyping study in Isfahan province, animal hydatid cysts from sheep, camel, cattle, and goat were characterized using PCR-RFLP with the same primers and enzymes as our study; it was demonstrated that the sheep strain is the most prevalent isolate among these animals (Shahnazi *et al.*, 2011). In a neighbor province, hydatid isolates were gathered from sheep, goat, and cattle; likewise ours, the authors used TaqI, AluI, RsaI and HpaII and showed G1 as the frequent strain (Parsa *et al.*, 2011). In contrast with our study that used ITS1, Sharbatkhori *et al.* used cytochrome C oxidase subunit 1 (*cox1*) and NADH dehydrogenase 1 (*nad1*) mitochondrial genes for sequencing and detected G1 (78.3 %) in all animal hosts such as sheep, goat, cattle, camel and buffalo in Golestan province (Sharbatkhori, Tanzifi *et al.*, 2016). Our results are compatible with these findings. Also, there exist convincing molecular proofs all over the globe suggesting G1 infection of sheep, cattle, goat, and buffalo, enclosing investigations in Iraq (Hammad *et al.*, 2018), Pakistan (Latif *et al.*, 2010), India (Singh *et al.*, 2012), Argentina (Andresiuk *et al.*, 2013), Ethiopia (Tigre *et al.*, 2016) and Greece (Chaligiannis *et al.*, 2015). The current investigation likewise several studies (Khademvatan, Yousefi *et al.*, 2013; Moro, P. L. *et al.*, 2009; Parsa, Haghpanah *et al.*, 2011; Pour, Hosseini *et al.*, 2011; Sharbatkhori, Tanzifi *et al.*, 2016; Varcasia *et al.*, 2006) have also isolated G1 from other animals rather than sheep, and goat, such as cattle/buffalo, suggesting the dominance of this genotype in these animals. Although, it is noticeable that G1 frequently renders infertile hydatid cysts in cattle (Hüttner & Romig, 2009). This finding also emphasizes the interaction of different ecological cycles of CE and warrants further researches to disclose the epidemiology and transmission dynamics of hydatidosis in Iran.

Regarding our results, *E. granulosus sensu stricto* was the predominant genotype in slaughtered sheep, goat, cattle and buffalo of this area, southwestern Iran, similar to other parts of the country. This strain is supposed as the most known genotype of *E. granulosus* with potential serious sequela in humans. Obtained results from this study would shed light for local and nation-wide CE preventive measures, consisting of improved diagnostics and better therapeutics.

## Conflict of Interest Statement

We declare that we have no conflict of interest.

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