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Analysis of the *cox1* gene in *Echinococcus granulosus* from sheep in northeast Iran using PCR high-resolution melting (qPCR-HRM) curve analysis

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Abstract. *Echinococcus granulosus*, the etiologic agent of echinococcosis, is one of the most important zoonotic helminthes worldwide. Knowledge of *E. granulosus* species and genotypes has important implications for epidemiology, control, and prevention of diseases as well as future vaccine and drug designs. There are many molecular methods developed to define genotypes of *E. granulosus*, among them high resolution melting (HRM) analysis, as a new approach, is a single step and closed tube method. It is appropriate for fast screening of large number of isolates. This technique is an accurate, user friendly, cost-effective, fast and simple method, which does not need post-PCR processes. Between March and lst august 2016, of 726 sheep examined in abattoirs in Razavi Khorasan province, Northeast Iran, 109 harboured cystic echincoccosis lesions (liver samples= 65 and lung samples= 44) which were collected for analysis. Total genomic DNA was extracted from each sample and amplified for the presence of polymorphism in the mitochondrial *cox1* gene of *Echinococcus granulosus* using a high resolution melting curve (HRM) method. A total of 109 hydatid cyst samples analyzed by PCR high-resolution melting (qPCR-HRM) curve of the *cox1* gene, all isolates were identified as G1 genotype (sheep strain). G1 is the predominant genotype in sheep in northeast of Iran. The high incidence of the G1 genotype (known to be the predominant *E. granulosus* genotype infecting humans globally) in sheep has considerable implications for hydatid disease control programs in this area.

INTRODUCTION

Hydatid disease or cystic echincoccosis (CE) caused by the larval stage of the zoonotic tapeworm *Echinococcus granulosus*, has a global distribution but is especially prevalent in developing countries (Thompson, 2008). Iran is an important endemic focus of CE where various domestic livestock are commonly infected (Fasihi Harandi *et al.*, 2012). Prevalence of CE have been reported in Iranian ruminants especially in sheep (5.1%–74.4%), goats (2%–20%), camels (25.7%–59.3%), cattle (3.5%–38.3%) and buffalo (11.9%–70%) (Harandi *et al.*, 2002; Dalimi *et al.*, 2002; Rokni, 2009). *E. granulosus* are detected in livestock only at slaughterhouse post-mortem inspection. It can cause considerable economic losses in livestock products (meat, milk, wool and

egg) as well as resulting in high mortality in humans (Azami *et al.*, 2013). The median annual cost associated with CE in Iranian livestock was estimated to be US$132 million with sheep infection responsible for 48% of the total economic losses (Fasihi Harandi *et al.*, 2012).

Currently, ten distinct genotypes (G1-G10) have been characterized within the *E. granulosus* species complex using mitochondrial cytochrome c oxidase subunit 1 (*cox1*) analysis. These different genotypes comprise two sheep strains (G1 and G2), the buffalo strain (G3), the horse strain (G4), the cattle strain (G5), the camel strain (G6), the pig strain (G7), the cervid strain (G8), the human polish strain (G9) and Fennos-canadian cervid strains (G10) (Grosso *et al.*, 2012). These defined genotypes have been included in a taxonomic revision of the *Echinococcus* genus on the basis of mitochondrial sequences resulting in four species: *E. granulosus* sensu stricto (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6, G7, G8 and G10) (Nakao *et al.*, 2013; Hüttner *et al.*, 2008). A more recent study, based on similar criteria, has suggested that the G6 and G7 genotypes represent a single species that is different from both the G8 and G10 genotypes; these authors suggested the names: *E. intermedius* (G6, G7), *E. borealis* (G8) and *E. canadensis* (G10) (Lymbery *et al.*, 2015). *E. granulosus* sensu stricto (G1-G3) have broad geographical distributions and a wide range of host specicity and are responsible (particularly G1) for most livestock and human infections (Nakao *et al.*, 2013). Genetic variation in *E. granulosus* can result in changes in some important phenotypic characteristics such as the life cycle pattern, infectivity and host specicity in intermediate hosts, developmental rate, geographical distribution, antigenicity and transmission dynamics impacting on the control of hydatid disease. As a result, research on genetic variation in the *E. granulosus* population has led to the development of control strategies such as vaccine design, effective treatment and development of diagnostic methods, as well as understanding the pathology and epidemiology of the disease (de la Rue *et al.*, 2006; Bhattacharya *et al.*, 2007; Grosso *et al.*, 2012). QPCR-HRM analysis is used as a reliable and sensitive scanning method for identification and genotyping of parasitic organisms such as *Fascioloides magna*, Hookworm, *Naegleria*, *Cryptosporidium*, *Plasmodium falciparum*, *Leishmania*, *Giardia*, *Dientamoeba fragilis* and *Toxoplasma gondii* (Robinson *et al.*, 2006; Pangasa *et al.*, 2009; Hussein *et al.*, 2009; Cruz *et al.*, 2010; Nasereddin and Jaffe, 2010; Costa *et al.*, 2011; Radvansky *et al.*, 2011; Ngui *et al.*, 2012; Zhang *et al.*, 2012). Our study aimed at determining the genotype(s) of *E. granulosus* hydatid cyst isolates collected from sheep in slaughter houses in Northeast Iran using qPCR-high resolution melting curve (HRM) analysis of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene.

MATERIALS AND METHODS

Sampling

During March and August 2016, 726 sheep were inspected at slaughterhouse for the presence of hydatid cysts in abattoirs in Razavi Khorasan province, Northeast Iran. One hundred and nine CE cysts (65 in livers and 44 in lungs) were collected among which 101 were fertile. Protoscoleces (from fertile hydatid cysts) and germinal layers (from non-fertile cysts) were rinsed in physiological saline solution. Then, the 109 individual samples were transferred into separate sterile test tubes, covered with 70% (v/v) ethanol and kept at -20°C until DNA extraction.

DNA extraction

Before DNA extraction, the samples were rinsed three times with sterile distilled water to remove ethanol. Genomic DNA was extracted from the protoscoleces and germinal layers using DNA extraction kits (Ge Net Bio, Daejeon, Korea) according to the manufacturer's instructions. The concentration of DNA was determined by Nano Drop and each DNA samples was stored at -20°C until molecular analysis.

QPCR-HRM

For molecular identification, qPCR-HRM of the *cox1* gene was performed on the Rotor-Gene 6000 series software version 1.7 (Corbett, Hilden, Germany) using the following primers: forward JB3 primer (5-TTT TTT GGG CAT CCT GAG GTT TAT-3) and reverse JB4.5 primer (5-TAA AGA AAG AAC ATA ATG AAA ATG-3') as described by Bowles *et al.* (Bowles *et al.*, 1992). PCR amplification was done in a 25 µl final volume containing 12.5 µl master mixes (Type-it HRM PCR Kit, Qiagen, Hilden, Germany), 9.5 µl distilled water, 1µl (10 nmol/µl) each primer, and 2 µl template DNA. Cycling reactions were performed using the following protocol: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 10 sec) and annealing (55°C, 30 sec) and next 72ºC for 27 sec, and a final extension at (72ºC for 5 min). The fluorescence signal was measured at each cycle at the end of each reaction. The melting experiment was performed from 55ºC to 95ºC at intervals (ramps) 0.2ºC/sec with continuous fluorescence monitoring.

All tests were performed in triplicate and T_m analysis was repeated 3 times within run (intra-assay) and 3 times between runs (inter-assay) to confirm the repeatability of the T_m assay by estimating the T_m variation. Three DNA samples which had already been sequenced for *cox1* and identified as G1, G3, and G6 (accession numbers: KC660075, KU697314, KC415063, respectively) were included in each PCR set as positive controls.

DNA sequencing

To confirm the identified genotype and HRM results, 25 PCR products were randomly sequenced. The sequences were compared with available sequences in GenBank using the BLAST algorithms and databases from the National Center for Biotechnology (www.ncbi.nlm.nih.gov). Phylogenetic analysis and phylogeny tree drawing of the sequence data were done by the Maximum Composite Likelihood method using MEGA 5 software.

RESULTS

All 109 CE hydatid cyst samples (isolates) examined using qPCR- HRM analysis of the partial sequences of the *cox1* gene were identified as the G1 genotype (common sheep strain). The melting curve results for controls and samples indicated that the T_m was between 79.7ºC and 80.15ºC (mean T_m=79.92) for G1 and mean T_m=80.42 and 79.33 for the G3 and G6 genotypes, respectively. Melting curve and normalized HRM graph analysis of the *cox1* amplicons for controls and samples are shown in Figure 1.

The coefficient of variation (CV) was calculated by dividing the standard deviation by the arithmetic mean of the measured values of the T_m (CV=standard deviation [SD]/mean value). The intra-assay CVs represent the mean CVs for the results obtained from the replicates of all the *E. granulosus* genotypes (controls and samples) in the same run. The inter-assay CVs represent the mean CVs for the results obtained from 3 separate runs (Table 1).

The sequencing results of 25 PCR products of individual samples confirmed that all were correctly differentiated by HRM analysis (Figure 2).

DISCUSSION

Iran is the third most populous and second largest country in the Middle East and has the fourth largest sheep population in the world (Fasihi Harandi *et al.*, 2012). Sheep are the most important intermediate hosts for *E. granulosus* in Iran (Ahmadi and Dalimi, 2006). In this study, in sheep, a CE prevalence of 15.0% (109/726 animals) was evident from surveillance of a number of slaughterhouses in Razavi Khorasan province. Due to the high CE rates, particularly in this province, and the high numbers of sheep in Iran, genotyping of sheep cysts is essential. Accordingly, genotyping of isolates of *E. granulosus* hydatid cysts from the 109 infected sheep was

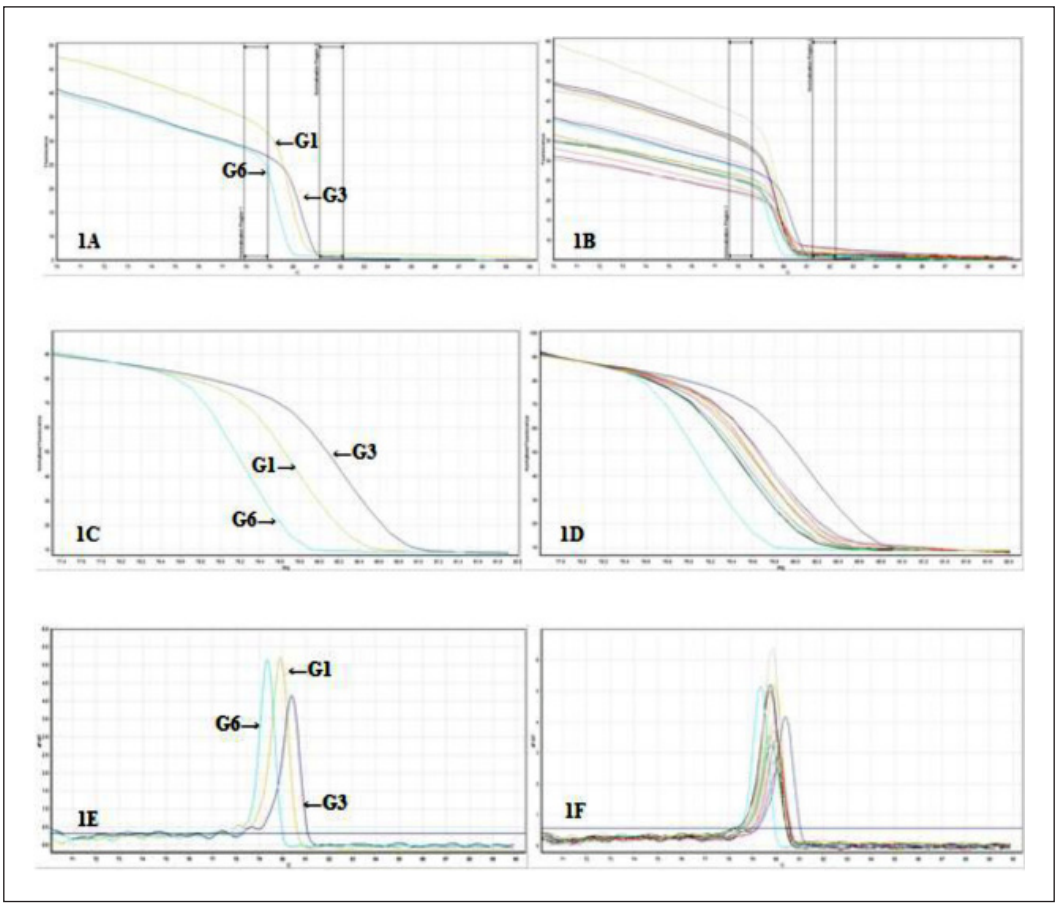

Figure 1. Genotyping of *E. granulosus* G1, G3, and G6 genotypes using HRM curve analysis. (A, B) representative profiles in the melting curve analysis of *cox1* amplicons for controls and samples. (C, D) Normalized HRM graph analysis for the controls and samples. (E, F) Melt curve analysis for genotype controls and samples.

Table 1. Mean T_m (°C) and SD calculated and intra- and inter-assay coefficient of variations of the G1, G3, and G6 genotypes of *E. granulosus*

E. granulosus genotypes	Mean T_m (°C)	SD	Intra-assay CV (%)	Inter-assay CV (%)
G1	79.95	0.13	0.07	0.16
G3 †	80.45	0.03	0.03	0.04
G6 †	79.35	0.04	0.03	0.05

†Only control.

undertaken using HRM analysis of the mitochondrial *cox1* gene. Research on genotypic variation in *E. granulosus* is important, particularly in endemic regions such as Iran where several different species of intermediate host occur and there is the possibility of interaction between different cycles of transmission (Thompson, 2008; Fasihi Harandi *et al.*, 2012). Regarding the presence of various parasitic phenotypes in the region, the study of parasitic infection characteristics in animal models should be

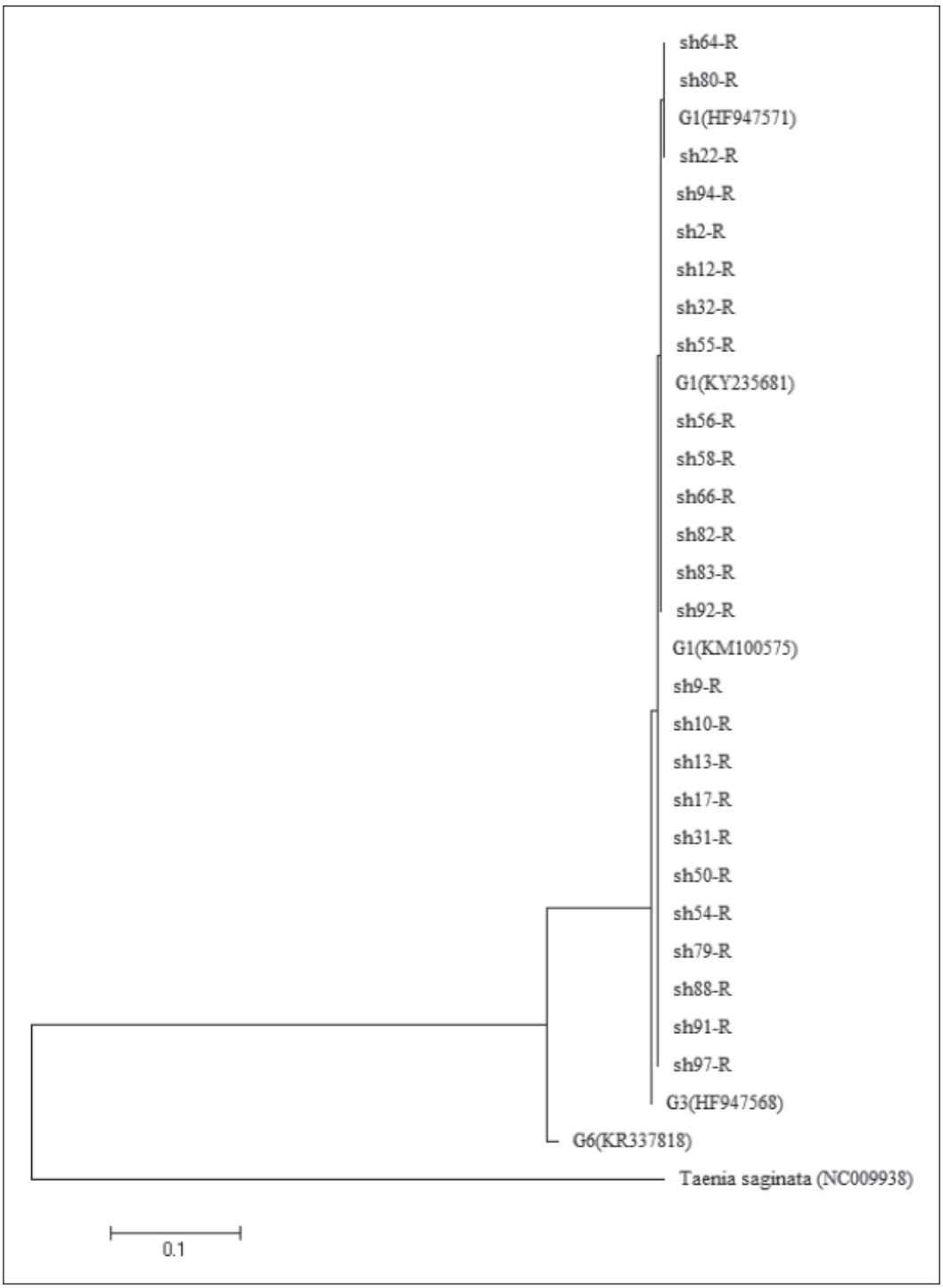

Figure 2. Phylogenetic analysis of 25 *E. granulosus* isolates from sheep in northeast of Iran based on the *cox1*sequence. Reference sequences under accession numbers, G1 (HF947571, KY235681 and KM100575), G3 (HF947568) and G6 (KR337818) were used for phylogenetic analysis. *Taenia saginata* (NC009938) was used as the outgroup.

considered in order to study the disease control program.

HRM technique is more cost-effective and less time-consuming method than conventional PCR and can be used effectively and beneficially for genotyping *E. granulosus* (Ngui *et al.*, 2012; Rostami *et al.*, 2013; Hosseini-Safa *et al.*, 2016).

In Italy, three genotypes (G1 and G2/G3) of *E. granulosus* (Maurelli *et al.*, 2009) were differentiated by qPCR analysis of the mitochondrial gene. Also, seven species of *Echinococcus* (*granulosus*, *ortleppi*, *vogeli*, *oligarthra*, *multilocularis*, *canadensis* and *Taenia hydatigena*) were identified by HRM of the *cox1* gene in Brazil (Santos *et al.*, 2013).

In Iran *cox1* HRM analysis on sheep, goats, cattle and camels hydatid isolates indicated the presence of four genotypes G1, G2, G3 and G6 (Rostami *et al.*, 2013; Pestechian *et al.*, 2014; Hosseini-Safa *et al.*, 2016).

The genotyping survey presented here of *E. granulosus* isolates targeting the *cox1* gene revealed that the predominant genotype in the sheep samples was the G1 genotype (common sheep strain). This outcome is in line with several previous studies conducted in different regions of the world including Ethiopia, Tunisia, Peru, Palestine, China, Turkey and Spain (Daniel Mwambete *et al.*, 2004; M'Rad *et al.*, 2005; Ma *et al.*, 2008; Utuk *et al.*, 2008; Moro *et al.*, 2009; Adwan *et al.*, 2013; Tigre *et al.*, 2016). Also, in concordance with earlier studies in Iran, G1 was the most prevalent genotype (Sharbatkhori *et al.*, 2010). In Tabriz (northwest Iran), Noor and Golestan (northern Iran), Isfahan (central Iran) and Aleshtar (western Iran), all the examined sheep samples were from the G1genotype, consistent with the results of the current study (Jamali *et al.*, 2004; Rostami Nejad *et al.*, 2008; Sharbatkhori *et al.*, 2010). In other studies of *E. granulosus* isolates from sheep in Isfahan and Kerman (Southeastern Iran), in addition to the G1 genotype, the G3 and G6 genotypes have been reported (Pestechian *et al.*, 2014; Spotin *et al.*, 2016).

In conclusion, qPCR-HRM analysis is a reliable and rapid technique for undertaking molecular and epidemiological studies on *E. granulosus* due to its efficiency to analyze large samples of both human and animal isolates. As this study and others have shown, G1 is the predominant genotype infecting sheep in northeast Iran. The high prevalence of the G1 genotype in sheep in northeast Iran has considerable implications for the application of hydatid control programs in this region.

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Conflict of interest

The authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias this paper.

Ethical approval

The presented study was conducted in accordance with Helsinki declaration (1974) and recommendation of the college of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran, an entity as associated with the International Council for Animal Laboratory Sciences.

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