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High resolution melting technique for molecular epidemiological studies of cystic echinococcosis: differentiating G1, G3, and G6 genotypes of *Echinococcus granulosus* sensu lato

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Abstract Reliable and rapid genotyping of large number of *Echinococcus granulosus* sensu lato isolates is crucial for understanding the epidemiology and transmission of cystic echinococcosis. We have developed a method for distinguishing and discriminating common genotypes of *E. granulosus* s.l. (G1, G3, and G6) in Iran. This method is based on polymerase chain reaction coupled with high resolution melting curve (HRM), ramping from 70 to 86 °C with fluorescence data acquisition set at 0.1 °C increments and continuous fluorescence monitoring. Consistency of this technique was assessed by inter- and intra-assays. Assessment of intra- and inter-assay variability showed low and acceptable coefficient of variations ranging from 0.09 to 0.17 %. Two hundred and eighty *E. granulosus* s.l. isolates from sheep, cattle, and camel were used to evaluate the applicability and accuracy of the method. The isolates were categorized as G1 (93, 94, and 25 %), G3 (7, 4, and 4 %), and G6 (0, 2, and 71 %) for sheep, cattle, and camel, respectively. HRM results were completely compatible with those obtained from sequencing and rostellar hook measurement. This method proved to be a valuable screening tool for large-scale molecular epidemiological studies.

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Introduction

Cystic echinococcosis (CE) is one of the most important parasitic zoonoses in the world caused by larval stages of *Echinococcus granulosus* s.l., a tapeworm that lives in the small intestine of dogs and other carnivores as definitive host and a wide range of domestic herbivores, e.g., sheep, cattle, buffalo, goat, camel, horse, pig, as well as wild ungulates as intermediate hosts. Human can be infected as an accidental host via ingestion of contaminated vegetables and water and direct contact with dogs. Metacestodes usually infect the liver and lungs of the intermediate host and cause hydatid cysts in those organs (Thompson and McManus 2002). CE imposes great monetary and non-monetary burden on the society in areas where the disease is endemic (Budke et al. 2006; Harandi et al. 2012; Murray et al. 2012).

There are wide molecular genetic, biological, and morphological variations within *E. granulosus* sensu lato. This leads to the erection of several new species and genotypes, designated as G1 to G10 (Nakao et al. 2010). Knowledge about *E. granulosus* species and genotypes has important implications for the epidemiology, control, and prevention of the disease as well as future vaccine and drug designs (Thompson 2008; Maillard et al. 2007). Iran is an endemic area for *E. granulosus*

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s.l., and CE is a well-known disease in this country and it constitutes about 1 % of all surgical admissions (Bagheri et al. 2011). Several molecular and morphological studies in Iran showed the existence of *E. granulosus* sensu stricto (G1–G3) and *Echinococcus canadensis* (G6) (Harandi et al. 2002; Sharbatkhori et al. 2011; Hajialilo et al. 2012; Kia et al. 2010; Cardona and Carmena 2013). Differentiating the most frequent genotypes in a defined endemic region by using a fast, reliable, cost-benefit, and straightforward method is essential.

There are many molecular methods that have been developed to define genotypes of *E. granulosus* such as those based on morphology, developmental biology, and protein and nucleic acid analysis. A wide range of DNA-based methods provides us with in-depth information on *E. granulosus* genomic variation. Random amplified polymorphic DNA, restriction fragment length polymorphism, single strand conformational polymorphism coupled with polymerase chain reaction (PCR) have been the most frequently used tools for characterization of the parasite. However, one or the other of these methods is time consuming, complicated, and not economical or accurate enough. Nucleic acid sequencing is the golden standard for determining the genotypes of *E. granulosus*, but it is not practical to use this tool for large-scale molecular epidemiological studies (Gasser 2006; Lymbery and Thompson 2012).

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, and does not need post-PCR processes so that eliminating hazards of ethidium bromide and gel electrophoresis. This technique has been widely used for diagnosis of single nucleotide mutations in human genetic disorders and for the identification of several infectious agents including protozoan and metazoan parasites (Bastien et al. 2008; Krypuy et al. 2007; Millat et al. 2009).

A T_m-based real-time PCR method has been developed by Maurelli et al. (2009) to discriminate between genotypes within *E. granulosus* sensu stricto, i.e., G1, G2, and G3. However, this method is not as accurate as HRM. Therefore, in the present study, we tried to assess HRM analysis as an alternative to DNA sequencing for detecting nucleotide variations and genotyping large number of *E. granulosus* s.l. isolates from various animal intermediate hosts in Iran.

Materials and methods

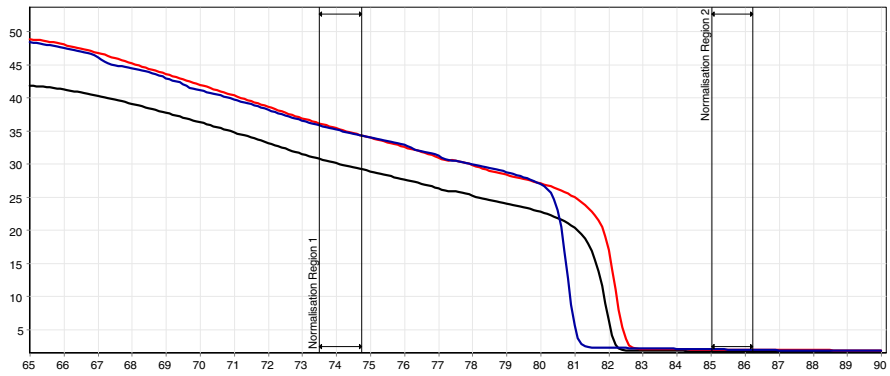
Collection of samples Specimens of larval stages of *E. granulosus* were collected from liver and lungs of 300 sheep, cattle, and camel hosts during routine veterinary inspection of animal carcasses in abattoirs from different parts of Iran. All the specimens were transferred to the Parasitology Lab of the School of Medicine, Kerman University of Medical Sciences. The protoscoleces were aspirated and washed three times with normal saline and stored at −20 °C until use.

DNA extraction A pack of protoscoleces was homogenized with an equal volume of distilled water. DNA extraction was carried out using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, samples were incubated overnight in 200 μL tissue lysis buffer and 20 μg/mL proteinase K at 56 °C. DNA extracted from protoscoleces was stored at −20 °C until used. The same buffer solution was used for all DNA samples because different buffers can affect melting temperature.

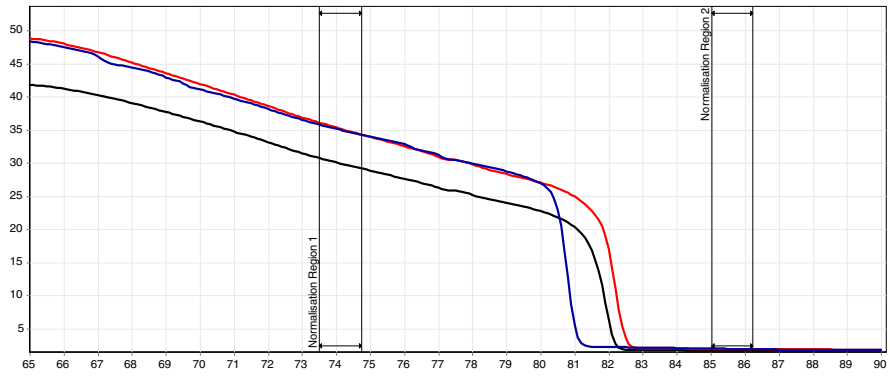
DNA amplification, melting curve analysis, and high resolution melting curve analysis A target sequence of mitochondrial DNA coding for cytochrome *c* oxidase subunit 1 (*CO1*) gene has been amplified by real-time PCR. The PCR reaction was performed in a volume of 25 μL using 1× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 10 pmol of each primer with final concentration of 400nM, and 25 ng DNA.

Two primers, JB3 (forward), 5′-TTTTTTGGGCATCCT GAGGTTTAT-3′, and JB4.5 (reverse), 5′-TAAAGAAAG AACATAATGAAAATG-3′, were used to amplify a fragment of 390 bp of *CO1* gene (Bowles et al. 1992) under the following conditions: 15 min at 95 °C (initial denaturation), followed by 35 cycles of 95 °C for 10 s (denaturation), and 54 °C for 15 s (annealing) and 72 °C for 10 s using 36-well Rotor-Gene Q Real-Time PCR System (Qiagen Inc.). After an initial step of 95 °C for 1 min and 40 °C for 1 min, melting curve was obtained by increasing the temperature from 70 to 86 °C at the default of Rotor Gene melting rate (0.1 °C each step) with continuous fluorescence monitoring. This enables the detection of down to single base pair variation among amplicons. Optical measurements (fluorescence signals) in the green channel (using a digital filter, with excitation at 479 nm and detection at 510 nm) were measured. In each PCR run, samples with no DNA template were used as negative controls. Three DNA samples that have already sequenced for CO1 and identified as G1, G3, and G6 (accession numbers: HM563013, HM563017, HM563018, respectively) were included in each PCR set as positive controls.

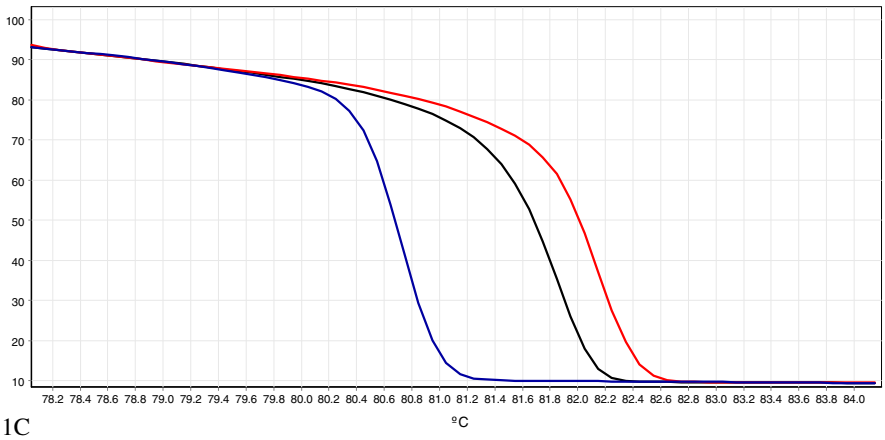
For genotyping, melting profiles were analyzed with the software Rotor Gene. This software analyzed the HRM curve data to recognize changes in the shape of the curve. One sample for each of *E. granulosus* G1, G3 and, G6 genotypes was characterized by DNA sequencing. This was identified to the software as a standard sample, i.e., three standard samples representing G1, G3, and G6 genotypes. Analysis program was done as follows: In the first step, derivative of the



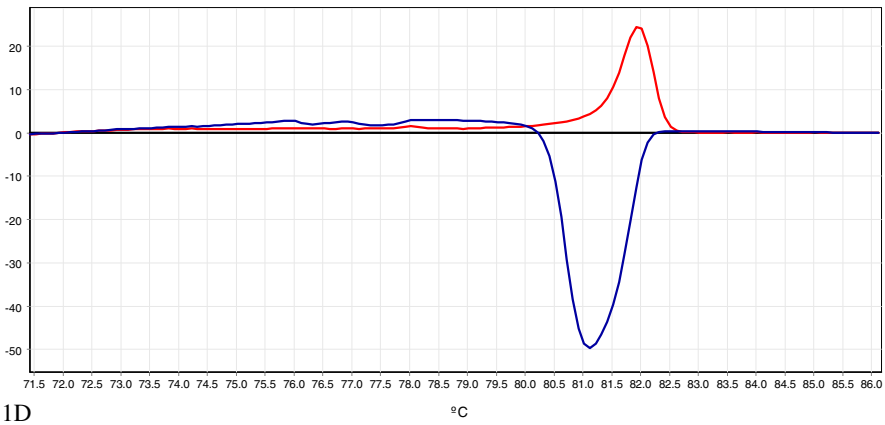
1A



1B



1C



1D

Fig. 1 Genotyping of *E. granulosus* G1, G3, and G6 genotypes using HRM curve analysis. **a** derivative melting curve (−dF/dT) for *E. granulosus* genotypes, G1 (*black*), G3 (*red*), or G6 (*blue*). **b** representative profiles in the melting curve analysis of CO1 amplicons for the same data. **c** normalized HRM curve analysis for the same data. **d** HRM differential plot for the same data (G1 genotype as a baseline)

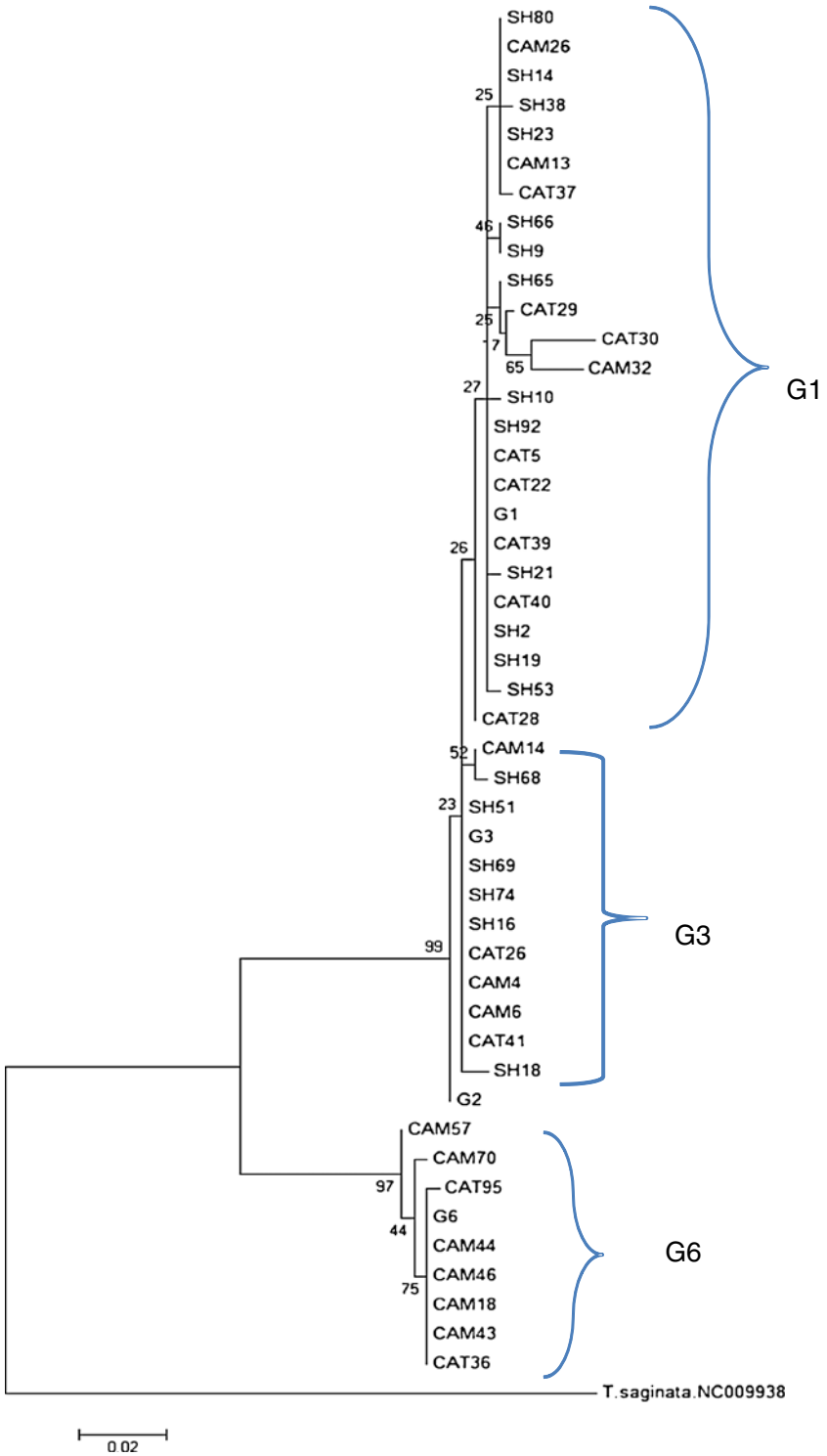


Fig. 2 Molecular phylogenetic tree of 43 *E. granulosus* isolates of sheep, cattle, and camel along with reference isolates based on CO1 gene sequence. The evolutionary history was inferred using the maximum likelihood method based on the Kimura 2-parameter model (Kimura 1980). Reference accession numbers: *G1*, M64661; *G3*, M64663; *G6*, M84666

fluorescence signal was plotted against the temperature to show the melting peak (Fig. 1a). Then, the original melting curve data were normalized in order to uniform values by setting two linear regions (pre- and post-melting transition). These regions were defined as two lines of an upper (100 %) fluorescence line and a lower (0 %) baseline (Fig. 1b, c). The final step is the analysis of difference graph by setting the curves from a base curve and generating a difference plot curve (Fig. 1d).

Assessment of intra- and inter-assay variability Firstly, T_m variation within (intra-assay) and between (inter-assay) PCR

amplifications was estimated to confirm the repeatability (consistency) of the melting temperature. For this aim, we analyzed five replicates for each selected genotype in a same run (intra-assay) then it was done in five different days (inter-assay). Coefficient of variation (CV) for every T_m profile was calculated by dividing the standard deviation (SD) by the means of the measured values of T_m (CV=SD/mean value). Statistical tests were performed using the software package SPSS v.18.0 (SPSS Inc., Chicago, IL, USA).

DNA sequencing and phylogenetic reconstruction To confirm and validate HRM results, 43 PCR products from randomly selected isolates of each genotype category were sequenced in an ABI-3730XL capillary machine by Macrogen Inc. (South Korea). Sequence data were adjusted manually, and complete alignment was carried out using the softwares BioEdit and Clustal W (Hall 1999; Thompson et al. 1997). The results were compared with published reference sequences and HRM analysis results.

Phylogenetic analyses of the sequence data were inferred with maximum likelihood using the software Mega5 (Kimura 1980; Kumar et al. 2008; Tamura et al. 2011). The phylogenetic tree was run using sequences obtained in this study as well as reference sequences available for *E. granulosus* G1 (accession number, M84661), G2 (accession number, M84662), G3 (accession number, M84663), and G6 (accession number, M84666) genotypes as shown in Fig. 2. *Taenia saginata* CO1 (NC009938) was applied in the model as an out-group. Confidence values for every branch of the trees were determined by 1,000 bootstrap replication.

Morphometric study Biometric characters based on larval rostellar hook size were carried out on all the 43 sequenced isolates. Each individual protoscolex was crushed on a slide under a stereomicroscope. For each isolate, total lengths of two large and two small hooks per rostellum for each of ten protoscoleces were measured using a calibrated eyepiece micrometer under high power magnification. All the measurements were compared to the corresponding sequencing and melting curve results.

Table 1 Mean T_m (°C) and SD calculated and intra- and inter-assay coefficient of variations of 280 G1, G3, and, G6 genotype isolates of *E. granulosus*

E. granulosus genotypes	Mean T_m	SD	Intra-assay CV (%)	Inter-assay CV (%)
G1	82.26	0.13	0.121058	0.1572
G3	82.46	0.12	0.092601	0.143692
G6	81.30	0.14	0.125605	0.168707

Results

As shown in Table 1, HRM and melting curve results indicated that the mean T_m of G1, G3, and G6 isolates were 82.26, 82.46, and 81.30, respectively (Fig. 1a). Assessment of intra- and inter-assay variability showed low and acceptable CVs (Table 1). For HRM analysis, firstly, all graphs were manually normalized and then converted to a difference plot format with G1 standard as a baseline. Original, normalized, and differential HRM graphs were shown in Fig. 1b–d, respectively.

Sequencing results confirmed that all the isolates were correctly differentiated by HRM. All the isolates identified by HRM were clustered along with the corresponding reference genotypes as shown in Fig. 2. Phylogenetic tree was divided into two main clades. The first clade contained two subclades corresponding to the G1 and G3 genotypes (*E. granulosus* sensu stricto), and the second one corresponding to the isolates identified as G6 (*E. canadensis*) along with the individual reference genotypes.

PCR amplifications were successfully performed on all 300 isolates, but 20 cattle isolates did not yield suitable PCR products mainly due to the cyst sterility and/or calcification. According to HRM analysis performed on 280 *E. granulosus* isolates, 192, 15, and 73 isolates were identified as G1, G3, and G6 genotypes. Sheep, cattle, and camel isolates were genotypically categorized as G1 (93, 94, and 25 %), G3 (7, 4, and 4 %), and G6 (0, 2, and 71 %), respectively. Linear measurements of larval hooks were done on 43 samples from livestock. The mean total lengths of large and small hooks were 22.0 and 18.5 μM for *E. granulosus* sensu stricto and 25.5 and 22.0 μM in *E. canadensis* (G6) isolates, respectively. Range of hook size measurements provided further confirmation that all results obtained by HRMA method and those of sequencing are in agreement with each other.

Discussion

HRM analysis is increasingly used as a reliable method for identification and diagnosis of parasitic infections. Recently, the method has been adopted for characterization and genotyping of parasitic organisms, e.g., *Leishmania*, *Cryptosporidium*, *Giardia*, *Plasmodium*, and *Toxoplasma* (Andriantsoanirina et al. 2009; Costa et al. 2011; Nasereddin and Jaffe 2010; Pangasa et al. 2009; Zhang et al. 2011). HRM is being applied for quick and reliable identification of helminth parasites (Ngui et al. 2012). Here, we report the first application of HRM analysis for genotyping *E. granulosus* sensu lato in an endemic region of cystic echinococcosis.

CE is an important parasitic zoonosis with a global distribution. The disease has remarkable economical and medical impacts on the communities in endemic countries. Genotype identification of the causative agent is essential for planning

successful prevention and control campaigns. Different PCR-based methods have been widely employed for characterization of *E. granulosus* isolates of human and animals (Bowles et al. 1992; Bowles and McManus 1993; Breyer et al. 2004; Ma et al. 2012; Addy et al. 2012). Due to the drawbacks of all these methods, in the present study, we successfully used HRM analysis as a molecular epidemiological tool to discriminate existing genotypes of *E. granulosus* sensu lato in Iran.

Conventional melting curve analysis has been used by Maurelli et al. (2009) to distinguish between G1 and G2/G3 genotypes using 12S rRNA gene amplification. However, T_m analysis was not reliable and informative enough to distinguish genotypes with very close T_ms. In the present study, the mean differences of T_m as detected by conventional melting curve were 0.96 (G6 and G1), 1.16 (G6 and G3), and 0.2 (G1 and G3). Therefore, due to T_m overlaps, the method was only capable of distinguishing G6 from either of G1 or G3 genotypes. Precise melting curves obtained from small temperature increments of HRM enabled us to successfully discriminate all three genotypes with good reproducibility and sensitivity.

In this study, HRM analysis is applied to a large number of *E. granulosus* s.l. isolates. Comparing HRM results with those of sequencing showed the accuracy and validity of HRM. In Iran, many molecular studies have been done during the past decade using PCR sequencing (Hajialilo et al. 2012; Sharbatkhori et al. 2009; Sharbatkhori et al. 2011; reviewed in Cardona and Carmena 2013). The sample size in most of the studies was relatively small primarily due to high cost of sequencing. In CE endemic areas, large-scale molecular epidemiological studies need a fast, sensitive, and inexpensive technique as an alternative to sequencing. However, we do not believe HRM analysis as being as competent as DNA sequencing, but as a screening tool in molecular epidemiological studies, it is much cheaper and quicker especially for analyzing large number of *E. granulosus* isolates and in developing counties where research funds are limited. We believe that HRM analysis is a reliable and rapid method for screening large number of specimens and for discrimination of different species and genotypes within *E. granulosus* sensu lato.

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References

Addy F, Alakonya A, Wamae N et al (2012) Prevalence and diversity of cystic echinococcosis in livestock in Maasailand, Kenya. Parasitol Res 111(6):2289–2294

Andriantsoanirina V, Lascombes V, Ratsimbasoa A et al (2009) Rapid detection of point mutations in *Plasmodium falciparum* genes associated with antimalarial drugs resistance by using high-resolution melting analysis. J Microbiol Methods 78(2):165–170

Bagheri R, Haghi SZ, Amini M et al (2011) Pulmonary hydatid cyst: analysis of 1,024 cases. Gen Thorac Cardiovasc Surg 59(2):105–109

Bastien R, Lewis TB, Hawkes JE et al (2008) High throughput amplicon scanning of the TP53 gene in breast cancer using high resolution fluorescent melting curve analyses and automatic mutation calling. Hum Mutat 29(5):757–764

Bowles J, Blair D, McManus DP (1992) Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol Biochem Parasitol 54(2):165–173

Bowles J, McManus DP (1993) NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. Int J Parasitol 23(7):969–972

Breyer I, Georgieva D, Kurdova R et al (2004) *Echinococcus granulosus* strain typing in Bulgaria: the G1 genotype is predominant in intermediate and definitive wild hosts. Parasitol Res 93(2):127–130

Budke CM, Deplazes P, Torgerson PR (2006) Global socioeconomic impact of cystic echinococcosis. Emerg Infect Diseases 12(2):296

Cardona GA, Carmena DA (2013) Review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. Vet Parasitol 192(1–3):10–32

Costa JM, Cabaret O, Moukoury S et al (2011) Genotyping of the protozoan pathogen *Toxoplasma gondii* using high-resolution melting analysis of the repeated B1 gene. J Microbiol Meth 86(3):357–363

Gasser RB (2006) Molecular tool advances, opportunities and prospects. Vet Parasitol 136(2):69–89

Hajialilo E, Harandi MF, Sharbatkhori M et al (2012) Genetic characterization of *Echinococcus granulosus* in camels, cattle and sheep from the south-east of Iran indicates the presence of the G3 genotype. J Helminthol 86(3):263

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Harandi MF, Budke CM, Rostami S (2012) The monetary burden of cystic echinococcosis in Iran. PLoS Negl Trop Dis 6(11):e1915

Harandi MF, Hobbs RP, Adams PJ et al (2002) Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. Parasitology 125(Pt 4):367–373

Kia EB, Rahimi H, Sharbatkhori M et al (2010) Genotype identification of human cystic echinococcosis in Isfahan, central Iran. Parasitol Res 107(3):757–760

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16(2):111–120

Krypuy M, Ahmed A, Etemadmoghadam D et al (2007) High resolution melting for mutation scanning of TP53 exons 5–8. BMC Cancer 7(1):168

Kumar S, Nei M, Dudley J et al (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9(4):299–306

Lymbery AJ, Thompson RCA (2012) The molecular epidemiology of parasite infections: tools and applications. Mol Biochem Parasitol 181(2):102–116

Ma J, Wang H, Lin G et al (2012) Molecular identification of *Echinococcus* species from eastern and southern Qinghai, China, based on the mitochondrial cox1 gene. Parasitol Res 111(1):179–184

Maurelli MP, Rinaldi L, Capuano F et al (2009) Development of a real-time PCR for the differentiation of the G1 and G2/G3 genotypes of *Echinococcus granulosus*. Parasitol Res 105(1):255–259

Millat G, Chanavat V, Rodriguez-Lafrasse C et al (2009) Rapid, sensitive and inexpensive detection of SCN5A genetic variations by high resolution melting analysis. Clin Biochem 42(6):491–499

Maillard S, Benchikh-Elfegoun MC, Knapp J et al (2007) Taxonomic position and geographical distribution of the common sheep G1

and camel G6 strains of *Echinococcus granulosus* in three African countries. Parasitol Res 100(3):495–503

Murray CJL, Vos T, Lozano R et al (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global burden of disease study 2010. Lancet 380(9859):2197–2223

Nakao M, Yanagida T, Okamoto M et al (2010) State-of-the-art *Echinococcus* and *Taenia* phylogenetic taxonomy of human-pathogenic tapeworms and its application to molecular diagnosis. Infect Genet Evol 10(4):444–452

Nasereddin A, Jaffe CL (2010) Rapid diagnosis of Old World *Leishmaniasis* by high-resolution melting analysis of the 7SL RNA gene. J Clin Microbiol 48(6):2240–2242

Ngui R, Lim YAL, Chua KH (2012) Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. PLoS One 7(7):e41996

Pangasa A, Jex AR, Campbell BE et al (2009) High resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. Mol Cell Probes 23(1):10–15

Sharbatkhori M, Fasihi Harandi M, Mirhendi H et al (2011) Sequence analysis of cox1 and nad1 genes in *Echinococcus granulosus* G3 genotype in camels (*Camelus dromedarius*) from central Iran. Parasitol Res 1–7

Sharbatkhori M, Mirhendi H, Jex AR et al (2009) Genetic categorization of *Echinococcus granulosus* from humans and herbivorous hosts in Iran using an integrated mutation scanningphylogenetic approach. Electrophoresis 30(15):2648–2655

Tamura K, Peterson D, Peterson N et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Thompson JD, Gibson TJ, Plewniak F et al (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25(24):4876–4882

Thompson RCA (2008) The taxonomy, phylogeny and transmission of *Echinococcus*. Exp Parasitol 119(4):439–446

Thompson RCA, McManus DP (2002) Towards a taxonomic revision of the genus *Echinococcus*. Trends Parasitol 18(10):452–457

Zhang P, Liu Y, Alsarakibi M et al (2011) Application of HRM assays with EvaGreen dye for genotyping *Giardia duodenalis* zoonotic assemblages. Parasitol Res 1–7