First report of Tasmanian sheep strain (G2) genotype isolated from Iranian goat using the high resolution melting (HRM) analysis

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Abstract

Aim: The present study was aimed to evaluate E. granulosus genotypes isolated from goats using HRM analysis in Isfahan province.

Background: Cystic echinoccosis, so-called hydatidosis, is widespread infection caused by the larval stage of Echinococcus granulosus. This is an important zoonotic disease worldwide, especially in the developing countries such as Iran. To date, molecular studies mainly based on the mitochondrial DNA sequences have identified distinct genotypes termed G1-G10 which can differ in some characteristics such as the growth and infectivity to different intermediate hosts or the survival rate in the definitive hosts that are important for the development of control strategies.

Methods: From August to December 2014, 1341 goats were investigated and hydatid cysts were collected from the liver and lungs of 43 infected goats in Isfahan province abattoirs, Isfahan, Iran. Total genomic DNA was extracted from each sample, amplified for the presence of polymorphism of mitochondrial gene coding for cytochrome c oxidase subunit 1 (CO1), using high resolution melting curve (HRM) method.

Results: The results of HRM analysis using the sequence of CO1 gene for 43 Echinococcus granulosus isolates from goats showed 31, 2 and 10 isolates were identified as G1, G2, and G3 genotypes, respectively.

Conclusion: G1 is the predominant genotype in the isolated goat samples in Isfahan province, and the presence of G2 strain was reported for the first time in goat in Iran.

Keywords: Echinococcus granulosus, G2 genotype, HRM, goat, Iran

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Introduction

Infection of humans and animals with the cestode Echinococcus granulosus (E. granulosus), so-called hydatidosis, is one of the most important and prevalent parasitic diseases in different parts of Iran (1-3). E. granulosus in the domestic animals is detected only at the time of post-mortem inspection at the slaughterhouse. It can cause economic losses in livestock as well as high mortality in humans (4,5). The prevalence of adult worms has been reported from dogs, wolves and jackals in the Middle East and Iran (6). In addition to humans, sheep, goats, buffaloes, camels, horses, cattle and pigs are as intermediate hosts of Echinococcus spp. Metacestode stage of the parasite is routinely found in the viscera of the mentioned animals, especially in their liver and lung (1,7). E. granulosus shows a wide range of intra-specific variation related to host specificity, biology, morphology, epidemiology and genetics (8). To date, molecular studies mainly based on the mitochondrial DNA sequences have identified 10 distinct genotypes termed G1- G10 (9,10). Different genotypes have
been known as sheep strain (G1), Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7), cervid strain (G8), human poli colony strain (G9), and Fennoscandian cervid strains (G10). All defined genotypes were divided into five species: E. granulosus sensu stricto characteristics such as the growth and infectivity to different variations (20,21). The present study was aimed to evaluate that are important for the development of control strategies The population of goats is estimated to be 25,800,000 (15). 43 infected goats in Isfahan province abattoirs, Iran. Out of 43 samples infected with hydatid cyst, 37 were fertile and 7 were infertile. All hydatid cysts were obtained under sterile condition, and then protoscolices and/or the germinal layer were collected from an individual hydatid cyst. In order to perform molecular analysis, the protoscolices and germinal layer were stored in 70% ethanol at -20 °C until DNA extraction.

Materials and Methods

Sample collection
From August to December 2014, 1341 goats were examined and hydatid cysts were collected from the liver and lungs of 43 infected goats in Isfahan province abattoirs, Iran. Out of 43 samples infected with hydatid cyst, 37 were fertile and 7 were infertile. All hydatid cysts were obtained under sterile condition, and then protoscolices and/or the germinal layer were collected from an individual hydatid cyst. In order to perform molecular analysis, the protoscolices and germinal layer were stored in 70% ethanol at -20 °C until DNA extraction.

DNA extraction
The samples were rinsed three times with sterile distilled water to remove the ethanol prior to DNA extraction. Total genomic DNA was extracted using the genomic DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer’s instructions with some modifications. Concentration of DNA was determined by Nano Drop and the manufacturer's instructions with some HRM is a reliable, less time-consuming and cost-effective technique for identification of helminthic parasites that can be, so effective and beneficial for genotyping E. granulosus (26,27). In the present study, the CO1 gene was used to identification of E. granulosus genotypes. CO1 gene is one of the best targets to discriminate strains, genotypes and microvariants of Echinococcus spp. (28,29). A few studies have been done on the genotypes of E. granulosus on goats in Iran (15,30). In the present study, molecular results of the samples isolated from goats demonstrated the presence of three E. granulosus strains, including the common sheep strain (G1), Tasmanian sheep strain (G2) and buffalo strain (G3). We isolated G2 strain by using HRM analysis for the first time on livestock in Iran. The G2 genotype occurred DNA sequencing and phylogenetic analysis
To confirm the identified genotypes, 6 samples of different curve were randomly sequenced for cox1. The obtained sequences were compared with previously published sequences of the mitochondrial CO1 gene for E. granulosus genotypes in NCBI using basic local alignment search tool (BLAST) system. Phylogenetic analyses of the sequence data were inferred with maximum likelihood using the Molecular Evolutionary Genetics Analysis (Mega5) software package (version 5.2.1, 2013) (23-25).

Results
Out of 43 hydatid cysts isolated, 28 of them were collected from liver and 15 from lungs; 37 cysts were fertile and 7 infertile. All the isolates identified by HRM were clustered along with the corresponding reference genotypes as shown in Figure 1. HRM analysis using the sequence of CO1 gene for 43 E. granulosus isolates from goats showed G1 genotype was identified in 31 and G3 in 10 isolates. For the first time G2 genotype was detected in the 2 collected isolates (Figure 2). Also, Tm analysis was used for the detection of E. granulosus genotypes (G1, G2 and G3 genotypes) in goats in Isfahan province, Iran (Figure 3).

Discussion
HRM is a reliable, less time-consuming and cost-effective technique for identification of helminthic parasites that can be, so effective and beneficial for genotyping E. granulosus (26,27). In the present study, the CO1 gene was used to identification of E. granulosus genotypes. CO1 gene is one of the best targets to discriminate strains, genotypes and microvariants of Echinococcus spp. (28,29). A few studies have been done on the genotypes of E. granulosus on goats in Iran (15,30). In the present study, molecular results of the samples isolated from goats demonstrated the presence of three E. granulosus strains, including the common sheep strain (G1), Tasmanian sheep strain (G2) and buffalo strain (G3). We isolated G2 strain by using HRM analysis for the first time on livestock in Iran. The G2 genotype occurred
in two liver samples of goats. In two previous studies, G2 strain was isolated from human and dog in Iran (31,32). In the other countries, the G2 strain was isolated from cattle in Italy and sheep in Argentina (33,34). Out of 43 samples from goats, 31 isolates (72.1%) were G1 genotype (sheep strain). The G1 genotype is the dominant strain in both human and animals in Iran and the world (35).

In all studies carried out on goats in Iran, G1 genotype was isolated (36) that is evidence for the goat is a good intermediate host for the sheep strain. In the present study, G3 genotype (buffalo strain) was isolated from 10 (23.2%). G3 genotype has been reported in the intermediate hosts including sheep, goat, cattle, pigs, and human, and definitive hosts in Iran and other countries such as India, Turkey, Pakistan, Italy and Greece (10,37). In a similar study on goats in Isfahan, G3 genotype in 25% of cases was observed using CO1 fragment (38), but in studies on the hydatid cysts isolated from goats in Mazandaran and Lorestan provinces, as well as Varamin city in Iran, G3 genotype was not found (30,36). These findings indicate that the goats can be important intermediate host for

Figure 1. Molecular phylogenetic tree of 10 E. granulosus isolates of goat along with reference isolates based on CO1 gene sequence. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2 parameter model (23). The tree with the highest log likelihood (-814.2241) is shown. Reference accession nos.: G1, M64661; G2, M64662; G3, M64663; G6, M84666.

Figure 2. HRM based on (EVA Green-TM) curve analyses of E. granulosus identified by sequencing (A-C). (A) G1, (B) G3, and (C) G2 genotype.
the buffalo strain in Isfahan province.

In conclusion, HRM analysis is a reliable and rapid technique for screening and discrimination of different species and genotypes within *E. granulosus*. G1 is predominant genotype in goats in Isfahan province, but the presence of G2 strain was detected for the first time in livestock in this area and should be noted in the intermediate hosts.

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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.

**References**

1. Rostami Nejad M, Nazemalhosseini Mojarad E, Nochi Z, Fasih Harandi M, Cherahipour K, Mowlavi GR, et al. *Echinococcus granulosus* strain differentiation in Iran based on sequence heterogeneity in the mitochondrial 12SrRNA gene. J Helminthol 2008; 82: 343-7.
2. Rostami Nejad M, Taghipour N, Nochi Z, Nazemalhosseini Mojarad E, Mohebhi SR, Fasih Harandi M, et al. Molecular identification of animal isolates of *Echinococcus granulosus* from Iran using four mitochondrial genes. J Helminthol 2012; 86: 485-92.
3. Dalimi A, Motamedi G, Hosseini M, Mohammadian B, Malaki H, Ghamari Z, et al. Echinococcosis/ hydatidosis in western Iran. Vet Parasitol 2002; 105: 161-71.
4. Singh BB, Sharma JK, Tuli A, Sharma R, Bal MS, Aulakh RS, et al. Prevalence and morphological characterisation of *Echinococcus granulosus* from north India. J Parasit Dis 2014; 38: 36-40.
5. Ahmadi N, Meshkehkar M. An abattoir-based study on the prevalence and economic losses due to cystic Echinococcosis in slaughtered herbivores in Ahwaz, south-western Iran. J Helminthol 2011; 85: 33-9.
6. Sharbatkhori M, Mirhendi H, Harandi MF, Rezaeian M, Mohebali M, Eshraghian M, et al. *Echinococcus granulosus* genotypes in livestock of Iran indicating high frequency of G1 genotype in camels. Exp Parasitol 2010; 124: 373-9.
7. Thompson R, McManus DP. Towards a taxonomic revision of the genus *Echinococcus*. Trends Parasitol 2002; 18: 452-7.
8. Thompson R. The taxonomy, phylogeny and transmission of *Echinococcus*. Exp Parasitol 2008; 119: 439-46.
9. Lavikainen A, Lehtinen M, Meri T, Hirvelä-Koski V, Meri S. Molecular genetic characterization of the *Fennoscandian cervid* strain, a new genotypic group (G10) of *Echinococcus granulosus*. Parasitology 2003; 127: 207-15.
10. Sharbatkhori M, Harandi MF, Mirhendi H, Hajialilo E, Kia EB. Sequence analysis of cox1 and nad1 genes in *Echinococcus* granulosus G3 genotype in camels (*Camelus dromedarius*) from central Iran. Parasitol Res 2011; 108: 521-7.
11. Grosso G, Gruttaduria S, Biondi A, Marventano S, Mistratta A. Worldwide epidemiology of liver hydatidosis including the Mediterranean area. World J Gastroenterol 2012; 18: 1425.
12. Nakao M, Lavikainen A, Yanagida T, Ito A. Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae). Int J Parasitol 2013; 43: 1017-29.
13. Hüttner M, Nakao M, Wassermann T, Stiefert L, Boomker JD, Dinkel A, et al. Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. Int J Parasitol 2008; 38: 861-8.
14. Soriano S, Pianciani N, Pianciola L, Mazzeo M, Lazzarini L, Saiz M, et al. Molecular characterization of *Echinococcus* isolates indicates goats as reservoir for *Echinococcus canadensis* G6 genotype in Neuquén, Patagonia Argentina. Parasitol Int 2010; 59: 626-8.
15. Rajabloo M, Hosseini SH, Jaloussian F. Morphological and
molecular characterisation of *Echinococcus granulosus* from goat isolates in Iran. Acta Trop 2012; 123: 67-71.

16. Azami M, Anvarinejad M, Ezatpour B, Alirezaei M. Prevalence of hydatidosis in slaughtered animals in Iran. Turkiye Parazitol Derg 2013; 37: 102-6.

17. Ahmadi N. Hydatidosis in camels (*Camelus dromedarius*) and their potential role in the epidemiology of *Echinococcus granulosus* in Iran. J helmintol 2005; 79: 119-25.

18. Rokni M. Echinococcosis/hydatidosis in Iran. Iran J Parasitol 2009; 4: 1-16.

19. Hoghoughi N. A study of the prevalence of *Echinococcus granulosus* in dogs and hydatid cyst in sheep, goats, cattle and Man in Isfahan. Pahlal Med J 1971; 2: 670-6.

20. Maurelli MP, Rinaldi L, Capuano F, Perugini AG, Cringoli G. Development of a real-time PCR for the differentiation of the G1 and G2/G3 genotypes of *Echinococcus granulosus*. Parasitol Res 2009; 105: 255-9.

21. Dinkel A, Njoroge EM, Zimmermann A, Wälz M, Zeyhle E, Elmahdi IE, et al. A PCR system for detection of species and genotypes of the *Echinococcus granulosus* complex, with reference to the epidemiological situation in eastern Africa. Int J Parasitol 2004; 34: 645-53.

22. Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol Biochem Parasitol 1992; 54: 165-73.

23. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16: 111-20.

24. Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008; 9: 299-306.

25. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28: 2731-9.

26. Ngui R, Lim YA, Chua KH. Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. PloS one 2012; 7: e41996.

27. Rostami S, Talebi S, Babaei Z, Sharbatkhori M, Ziaali N, Rostami H, et al. High resolution melting technique for molecular epidemiological studies of cystic echinococcosis: differentiating G1, G3, and G6 genotypes of *Echinococcus granulosus* sensu lato. Parasitol Res 2013; 112: 3441-7.

28. McManus D. The molecular epidemiology of *Echinococcus granulosus* and cystic hydatid disease. Trans R Soc Trop Med Hyg 2002; 96: S151-7.

29. Pour AA, Hosseini SH, Shayan P. Comparative genotyping of *Echinococcus granulosus* infecting buffalo in Iran using cox1 gene. Parasitol Res 2011; 108: 1229-34.

30. Yousefi M, Tabaripour R, Fallah Omrani V, Spotin A, Esfandiari B. Genotypic characterization of *Echinococcus granulosus* in Iranian goats. Asian Pac J Trop Dis 2013; 3: 362-6.

31. Rostami S, Torbaghan SS, Dabiri S, Babayi Z, Mohammadi MA, Sharbatkhori M, et al. Genetic characterization of *Echinococcus granulosus* from a large number of formalin-fixed, paraffin-embedded tissue samples of human isolates in Iran. Am J Trop Med Hyg 2014; 92: 588-94.

32. Parsa F, Fashi Harandi M, Rostami S, Sharbatkhori M. Genotyping *Echinococcus granulosus* from dogs from Western Iran. Exp Parasitol 2012; 132: 308-12.

33. Rosenzvit M, Zhang LH, Kamnetzky L, Canova S, Guarnera E, McManus D. Genetic variation and epidemiology of *Echinococcus granulosus* in Argentina. Parasitology 1999; 118: 523-30.

34. Casulli A, Manfredi MT, La Rosa G, Cerbo ARD, Genchi C, Pozio E. *Echinococcus ortleppi* and *E. granulosus* G1, G2 and G3 genotypes in Italian bovines. Vet Parasitol 2008; 155: 168-72.

35. Sánchez E, Cáceres O, Náquira C, García D, Patiño G, Silvia H, et al. Molecular characterization of *Echinococcus granulosus* from Peru by sequencing of the mitochondrial cytochrome C oxidase subunit I gene. Mem Inst Oswaldo Cruz 2010; 105: 806-10.

36. Sharafi SM, Rostami-Nejad M, Moazzeni M, Yousefi M, Saneie B, Hosseini-Safa A, et al. *Echinococcus granulosus* genotypes in Iran. Gastroenterol Hepatol Bed Bench 2014; 7: 82.

37. Varcasia A, Canu S, Kogkos A, Pipia AP, Scala A, Garippa G, et al. Molecular characterization of *Echinococcus granulosus* in sheep and goats of Peloponnesus, Greece. Parasitol Res 2007; 101: 1135-9.

38. Pestechian N, Hosseini Safa A, Tajedini M, Rostami-Nejad M, Mousavi M, Yousofi H, et al. Genetic diversity of *Echinococcus granulosus* in center of Iran. Korean J Parasitol 2014; 52: 413-8.