Journal of Helminthology

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Genotype characterization of livestock and human cystic echinococcosis in Mazandaran province, Iran

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Short Communication

Cite this article: Gorgani-Firouzjaee T, Kalantrai N, Ghaffari S, Alipour J, Siadati S (2019). Genotype characterization of livestock and human cystic echinococcosis in Mazandaran province, Iran. *Journal of Helminthology* **93**, 255–259. https://doi.org/10.1017/S0022149X1800010X

Received: 16 October 2017
Accepted: 17 January 2018
First published online: 15 February 2018

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Abstract

Echinococcus granulosus is a helminth from the family Taeniidae, which causes cystic echinococcosis (CE) in humans and diverse livestock around the world. The identification of existing genotypes in different regions is a major step towards the prevention and establishment of control programmes for the disease. This study aimed to detect CE genotypes using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of the internal transcribed spacer-1 (ITS1) gene and sequencing of the cytochrome *c* oxidase subunit 1 (Cox1) gene in isolates from the central part of Mazandaran province, northern Iran. Forty isolates were collected from sheep, 17 from cattle and 6 from human formalin-fixed paraffin-embedded tissues (FFPE). The ITS1 and Cox1 genes were successfully amplified by PCR in 41 and 42 samples, respectively. PCR-RFLP and sequencing showed that all isolates had the G1–G3 genotypes in this study. Out of 31 isolates subjected to sequencing for the Cox1 gene, 80.7% had the G1 genotype. G2 (16.1%) and G3 (3.2%) genotypes were observed in five sheep and one cattle samples, respectively. Five human isolates were also sequenced for the ITS1 gene, which showed that all samples belonged to the G1 genotype. Ten haplotypes were determined among the isolates by alignment analysis of the Cox1 gene. In summary, this study demonstrated that G1 was the dominant genotype circulating between humans and livestock in the studied region. Furthermore, high genotypic diversity among the CE isolates was observed.

Introduction

Cystic echinococcosis (CE), an important zoonotic disease which is widespread around the world, is caused by the larval stages of *Echinococcus granulosus*, a helminth from the family Taeniidae. Carnivores, particularly dogs and other canines, serve as definitive hosts. Intermediate hosts, which harbour metacestodes, include a wide range of wild and domestic herbivores. Humans may serve as accidental hosts and the consumption of food contaminated with parasite eggs or the direct transmission of eggs from dogs can lead to infection (Craig & Larrieu, 2006). Past taxonomical and molecular studies on *E. granulosus* demonstrated that this species is composed of several strains that are genetically different from one another (Farhadi *et al.*, 2015). Until now, ten distinct genotypes (G1–G10) have been detected, including the species *E. granulosus* (genotypes G1, G2, G3; sheep and buffalo strains), *E. equinus* (G4; horse strain), *E. ortleppi* (G5; cattle strain) and *E. canadensis* (G6–G10) (Thompson, 2008; Alvarez Rojas *et al.*, 2014). Later investigation by Lymbery *et al.* (2015) demonstrated that the G6 (camel strain) and G7 (pig strain) genotypes are single species which are different from the G8 and G10 genotypes (cervid strains). Hence, the G6/G7 genotypes are named *E. intermedius*, G8 is named *E. borealis* and G10 is named *E. canadensis* (Lymbery *et al.*, 2015).

CE is a cosmopolitan zoonotic disease, with a high prevalence rate in livestock and humans in some parts of America (especially South America), Australia, northern and eastern Africa, southern and central parts of Russia, central Asia, China and parts of the Mediterranean region (Grosso *et al.*, 2012). In Iran, hydatidosis is an important public-health problem and the disease is observed in different parts of the country. In livestock, the total prevalence of CE is estimated to be 5.9% in sheep, 8.8% in cattle, 6.4% in goats, 16.5% in buffalos and 32.7% in camels (Khalkhali *et al.*, 2017). The data on operated cases revealed that, in this country, 1.18–3 cases per 100,000 surgeries in hospitals are related to hydatidosis (Ghaffari, 1999; Fasihi Harandi *et al.*, 2012). The total prevalence of cystic echinococcosis in humans is calculated to be 4.2% in Iran (Khalkhali *et al.*, 2017).

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Molecular data and the sequencing of different isolates from livestock have proved the presence of various genotypes (G1–G10) of *E. granulosus* around the world (Grosso *et al.*, 2012). G1 is the most commonly identified genotype (72.9%) in humans and animals throughout the world (McManus, 2013). In Iran, restriction fragment length polymorphism (RFLP) and sequence analysis have detected different genotypes, including G1–G3, G6 and G7, in animal and human isolates. To date, no study has been conducted regarding the sequencing of different genotypes of *E. granulosus* and the genetic characterization of CE in the central part of Mazandaran province, northern Iran. Hence, the present study was designed to identify *E. granulosus* genotypes in different host species using polymerase chain reaction (PCR)-RFLP of internal transcribed spacer-1 (ITS1) ribosomal DNA and the sequencing of the cytochrome *c* oxidase subunit 1 (Cox1) mitochondrial gene for the first time in this region.

Materials and methods

Collection and examination of hydatid cysts

In the current study, 63 hydatid cyst samples were collected from December 2016 to March 2017. Fifty-seven livestock samples (40 sheep and 17 cattle) were obtained from a slaughterhouse in Babol, the central part of Mazandaran province. Six archived formalin-fixed paraffin-embedded tissues (FFPE) were obtained from human cases with a history of hydatid cyst surgery from Shahid Beheshti Hospital, Babol, Iran. Regarding the animal samples, the whole content of the cysts from the lung and liver were examined under a light microscope for the presence of protoscoleces. The protoscoleces were washed by double-distilled water and stored at −20°C until used.

DNA extraction and molecular assays

Genomic DNA from protoscoleces was extracted using a Tissue DNA extraction kit (Bio Basic, Markham, Ontario, Canada) according to the manufacturer's instructions. The quality and concentration of extracted DNA was measured with a spectrophotometer (NanoDrop® 2000C, Thermo Scientific, Waltham, Massachusetts, USA). The genomic DNA was stored at −20°C until PCR amplification.

For DNA extraction from FFPE samples, serial sections of 7-mm thickness were obtained from the FFPE blocks. The deparaffinization of sections was performed according to the procedure of Kalantari *et al.* (2016). Deparaffinized tissues were kept at −20°C until used. The DNA extraction of tissue samples was performed using a PCRBio rapid extract kit according to the manufacturer's protocol (PCR BioSystems, London, UK). After evaluating the quality of extracted DNA, the samples were stored at −20°C until used.

PCR-RFLP of the ITS1 gene

The primer pairs used in this study for the amplification of the ITS1 gene were EgF (5′-AGAGCACTTTTGTATGCA-3′) and EgR (5′-ATGGTTGTTATCGCTGCGA-3′), which produce a 460-bp fragment (described by Moghaddas *et al.*, 2015). The DNA amplification was performed in a total volume of 25 µl, under the following temperature conditions: an initial denaturation step at 95°C for 5 min; 35 cycles at 94°C for 45 s (denaturation), 50°C for 45 s (annealing) and 72°C for 45 s (extension); with a final extension step at 72°C for 10 min. After the determination of PCR products in 2% agarose gels, the products were digested with the restriction enzyme *Bsh*1236I according to Moghaddas *et al.* (2015). The products were electrophoresed in 3% agarose gel, inspected under UV light and photographed using a gel documentation system (Vilbert, Lourmat, France).

PCR amplification of the Cox1 gene

A fragment of the cytochrome *c* oxidase subunit 1 (Cox1) gene was amplified. The forward and reverse primers of the Cox1 gene were JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′), which were selected according to Bowles *et al.* (1992) for the amplification of 450-bp fragments. The PCR amplification was performed in a total volume of 25 µl with the thermal program as follows: an initial denaturation step at 95°C for 5 min; 40 cycles at 94°C for 55 s (denaturation), 54.7°C for 55 s (annealing) and 72°C for 1 min (extension); and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on a 2% agarose gel, and the bands were visualized under UV light and photographed using a gel documentation system (Vilbert).

PCR amplification of the bcl-2 gene

In order to confirm the extracted DNA from the FFPE samples, a fragment of the *bcl-2* gene (cell death regulatory gene) was amplified using primer pairs *bcl-2* forward (5′-TGGCCAGGGTCAGA GTTAAA-3′ and *bcl-2* reverse (5′-TGGCCTCTCTTGCGGAGTA-3′). All stages of PCR amplification were carried out based on the PCR protocols described by Liu *et al.* (2014).

Sequencing

The PCR products of the Cox1 mitochondrial gene were purified and subjected to sequencing with both forward and reverse primers by Bioneer Company (South Korea). The sequencing results were analysed using Chromas (v.2.6.4) software and compared with each other and the reference sequence in the GenBank database by BLAST analysis (www.ncbi.nlm.nih.gov).

The multiple alignments of sequences were performed using the ClustalW method of Mega (v.6) software (Tamura *et al.*, 2011). The phylogenetic analysis of the different haplotypes obtained from this work, together with the reference sequences of *E. granulosus* genotypes (G1–G10) and other *Echinococcus* species, was constructed according to the neighbour-joining (NJ) method using Mega (v.6) software. For the comparison of a relevant outgroup sequence, a sequence of *Taenia saginata* was also selected.

Results and discussion

In total, 63 CE samples, including 40 sheep and 17 cattle along with 6 human isolates, were evaluated. In the animal isolates, 29 samples were obtained from the liver and 28 samples were from the lung. In human samples, 4 samples were obtained from the liver and 1 sample each from the lung and the peritoneal cavity. Amplification of the *bcl-2* gene was used as an internal control for human samples, and a 152-bp fragment was amplified in the all extracted DNA from FFPE tissue samples.

In the present study, PCR-RFLP of the ITS1 gene and digestion with *Bsh*1236I was performed, as previously examined by Moghaddas *et al.* (2015). The application of this method had

https://doi.org/10.1017/S0022149X1800010X Published online by Cambridge University Press

several advantages, including reduction in PCR program time, reduction of digestion time and cost effectiveness. Here, the ITS1 gene was amplified successfully in 5 human and 37 animal samples (9 cattle, 28 sheep). The RFLP with *Bsh*1236I showed that 100% of isolates had G1–G3 genotypes. Our findings are supported by the results of previous studies performed in different parts of Iran (Kia *et al.*, 2010; Hajialilo *et al.*, 2012; Khademvatan *et al.*, 2012; Pezeshki *et al.*, 2012; Dousti *et al.*, 2013; Hanifian *et al.*, 2013).

All human and animal isolates were further evaluated using the Cox1 gene. A 450-bp fragment was amplified in 2 human, 9 cattle and 20 sheep samples. Two-direction sequencing was carried out for these isolates. BLAST analysis showed 100% identity, 100% coverage and 0.0 E-value with reference sequences in GenBank (accession number KX269858.1). All human isolates had G1 genotypes. In cattle, 8 (88.8%) isolates had the G1 genotype and only 1 (11.1%) had the G3 genotype. G1 and G2 genotypes were identified in 15 (75%) and 5 (25%) of the sheep isolates, respectively. The G2 genotype was also observed in one cattle sample and the overall frequency of the G2 genotype was 16.1% (table 1). These outcomes show that G1 is the most common genotype (80.6%). According to several studies, G1 is the most commonly identified genotype in livestock and humans worldwide (Eryıldız & Şakru, 2012; McManus, 2013; Sharma *et al.*, 2013; Alvarez Rojas *et al.*, 2014; Hu *et al.*, 2015; Sharbatkhori *et al.*, 2016). The molecular and sequencing data on CE in Iran shows that the G2 genotype has only been reported in cattle and humans (Rostami *et al.*, 2015; Sharbatkhori *et al.*, 2016). The comparison of our results with those of other studies mentioned above shows that this genotype is infrequent in our country and it is limited to the northern part of Iran. The other genotype detected in the present study was G3, which was observed in one cattle isolate. However, the results of other investigations in Iran revealed that after G1, the G3 genotype is more widespread in our country (Hajialilo *et al.*, 2012; Nikmanesh *et al.*, 2014; Pestechian *et al.*, 2014). Another commonly reported genotype in Iran is G6, which was not observed in our work (Sadjjadi *et al.*, 2013; Moghaddas *et al.*, 2015; Sharbatkhori *et al.*, 2016). The absence of the G6 genotype could be due to the lack of camel breeding and consumption of camel meat in this region. These results indicate that the sheep–dog cycle is the most common transmission route of cystic echinococcosis in the study region.

In the present study, ten different haplotypes were found among the genotypes by alignment analysis. H1–H2 in the G1 group was the most commonly identified haplotype. The nucleotide

Table 1. *Echinococcus granulosus* genotypes and haplotypes obtained from Cox1 gene sequence analysis in different host species, along with reference sequences deposited in GenBank.

Haplotypes	Host (*n*)	Genotype	Profile Cox1 (accession number)	References
H1	Sheep (13) Cattle (5)	G1	EgS2 (MF346705) EgB2 (MF625021)	Present study
H2	Sheep (1)	G1	EgS1 (MF625022)	Present study
H3	Sheep (1)	G1	EgS12 (MF625020)	Present study
H4	Cattle (1)	G1	EgB6 (MF449137)	Present study
H5	Cattle (2)	G1	EgB1 (MF346706)	Present study
H6	Human (1)	G1	EgHum5 (MG099696)	Present study
H7	Human (1)	G1	EgHum4 (MG099695)	Present study
H8	Sheep (4)	G2	EgS11 (MF625018)	Present study
H9	Cattle (1)	G2	EgS6 (MF449131)	Present study
H10	Cattle (1)	G3	EgB3 (MF625019)	Present study
G1	Sheep	–	U50464	Okamoto *et al.* (1995)
G2	Sheep	–	M84662	Bowles *et al.* (1992)
G3	Buffalo	–	M84663	Bowles *et al.* (1992)
G4	Horse	–	M84664	Bowles *et al.* (1992)
G5	Cattle	–	M84665	Bowles *et al.* (1992)
G6	Camel	–	M84666	Bowles *et al.* (1992)
G7	Pig	–	M84667	Bowles *et al.* (1992)
G8	Moose	–	AB235848	Nakao *et al.* (2006)
G10	Reindeer	–	AF525457	Lavikainen *et al.* (2003)
E.multilocularis	Human	–	M84668	Bowles *et al.* (1992)
E.oligarthrus	Rodent	–	M84671	Bowles *et al.* (1992)
E. vogeli	Rodent	–	M84670	Bowles *et al.* (1992)
E. felidis	Lion	–	EF558356	Huttner *et al.* (2008)
Taenia saginata	Cattle	–	GT284836	Okamoto *et al.* (2010)

https://doi.org/10.1017/S0022149X1800010X Published online by Cambridge University Press

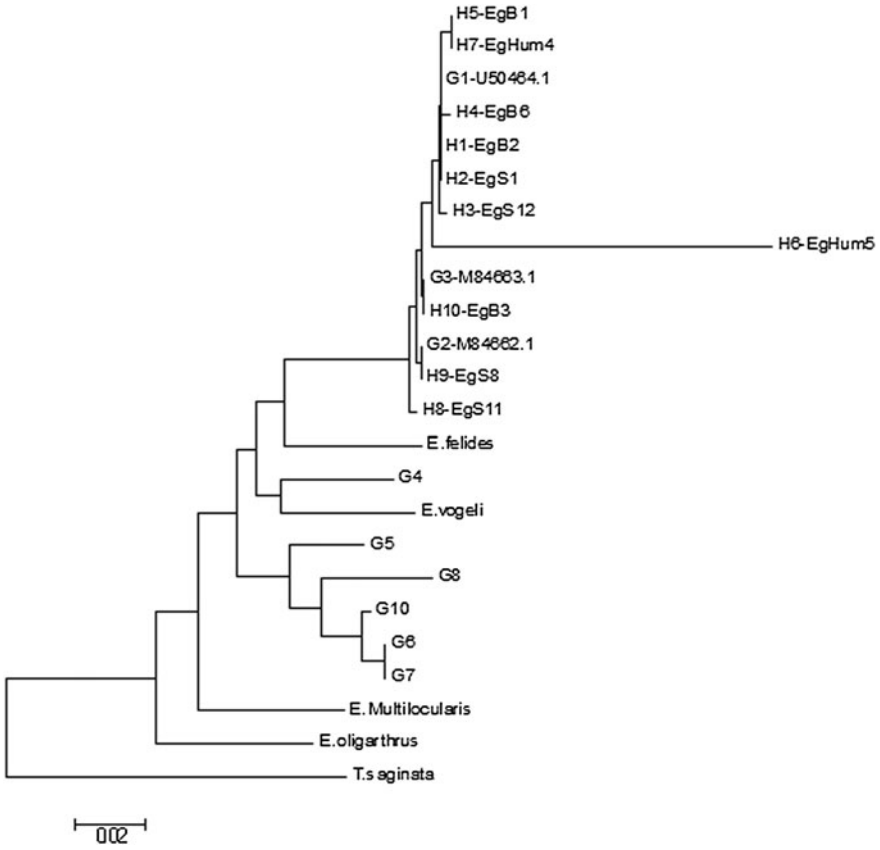

Fig. 1. Genetic relationships between *E. granulosus* isolates from CE in human and animal samples in the present study and reference sequences of G1–G10 genotypes. The phylogenetic tree was performed by neighbour-joining algorithm, with *Taenia saginata* as the outgroup. Haplotypes 1–10 were clustered in the G1–G3 genotype complex of *E. granulosus*.

sequence of H1–H2 had 100% similarity with haplotypes reported previously from Jordan (G01; AB491414), Europe (EG1; JF513058) and Iran (Golc1; KT074941) (Yanagida *et al.*, 2012; Sharbatkhori *et al.*, 2016). The haplotype H3 in our study was similar to the EG4 haplotype detected by Casulli *et al.* (2012), which is the common haplotype in Eastern European populations. The H4 haplotype (KM513627, KT074945) is similar to the haplotypes reported in Golestan province, Iran (Casulli *et al.*, 2012; Sharbatkhori *et al.*, 2016). Other haplotypes found in the current study were not similar to haplotypes detected in Iran and other parts of the world, which could indicate that these are new haplotypes.

The genetic relationship of all ten haplotypes with reference sequences obtained from GenBank, assessed by the NJ algorithm using Mega (v.6) software, is illustrated in fig. 1.

In conclusion, the molecular analysis of *E. granulosus* showed that the common sheep strain G1 is predominant in the northern part of Iran, affecting human, sheep and cattle populations. In addition, infected livestock can act as potential reservoirs for human infection. Also, the results of our study showed high genotypic diversity among these isolates. Finally, the results presented here can help improve the implementation of control programmes against cystic echinococcosis and the promotion of public health.

Acknowledgements. The authors would like to thank all the people who helped with this study. We would also like to thank Ms Taraneh Ghaffari for language editing.

Financial support. This study was supported by the Babol University of Medical Science (grant number 9604415).

Conflict of interest. None.

Ethical standards. This study was approved by the Babol University of Medical Science, ethical code number MUBABOL.HRI.REC.1396.154 3943.

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