Research Brief

Genotyping *Echinococcus granulosus* from dogs from Western Iran

Farzad Parsa, Majid Fasihi Harandi, Sima Rostami, Mitra Sharbatkhori

Research highlights:
- This study established the first record of *E. granulosus* G2 genotype in Iran.
- This study presents the first global report of this genotype in dogs as definitive host.
- The presence of G1 genotype of *E. granulosus* as dominant genotype in dogs is emphasized.

Genetic relationships of *Echinococcus granulosus* dog isolates from western Iran and reference sequences for *E. granulosus* sensu lato and other species of *Echinococcus* from previous studies as well as *Taenia saginata* as the outgroup.

ARTICLE INFO

Article history:
Received 23 March 2012
Received in revised form 23 June 2012
Accepted 25 July 2012
Available online 3 August 2012

Keywords:
- Echinococcus granulosus
- G2 genotype
- Dog
- Iran
- Mitochondrial genome

ABSTRACT

Cystic echinococcosis is a zoonotic infection caused by the dog tapeworm, *Echinococcus granulosus*. In the present study, adults of *E. granulosus* (n = 20) were collected from 71 dogs from Western Iran and were genetically characterized using DNA sequencing of the partial mitochondrial cytochrome c oxidase subunit 1 (cox1) and NADH dehydrogenase 1 (*nad1*). Consensus sequences were obtained for cox1 (366) and *nad1* (471) genes. Phylogenetic analysis of concatenated *nad1* and cox1 nucleotide sequence data was performed using Bayesian Inference approach. Overall, the dog isolates indicated nine different sequences in cox1 and seven in *nad1* genes. Three genotypes (G1 [75%], G2 [10%] and G3 [15%]) were identified from the isolates. The G2 sequences indicated 100% homology with reference G2 sequence in both cox1 (Genbank accession number M84662) and *nad1* (AJ237633) genes. G3 sequences showed 100% homology with G3 reference sequence in *nad1* (AJ237633), but displayed two different cox1 profiles, each having 99% homology with reference G3 sequence (M84663). In the phylogenetic tree all of the isolates were grouped into a distinct cluster corresponding to the G1–G3 complex with relevant reference sequences. The presence of G1 genotype (sheep strain) of *E. granulosus* sensu stricto as dominant genotype in dogs is emphasized. To the best of our knowledge, this study established the first record of *E. granulosus* sensu stricto, G2 genotype in Iran.

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1. Introduction

_Echinococcus granulosus_, the causative agent of cystic echinococcosis, is an important cause of morbidity and mortality in humans worldwide, particularly in sheep-raising countries (Dakkak, 2010). Carnivores, especially dogs, play a role for definitive hosts and harbor adult parasites in their intestine, while herbivores serve as intermediate host and can harbor the larval stage or hydatid cyst in any internal organ, particularly lung and liver (WHO/OIE Manual, 2002; Eckert and Deplazes, 2004).

In order to develop preventive and control strategies for echinococcosis, a better knowledge of transmission cycle of _E. granulosus_ complex is necessary. A significant genetic variation has been detected within _E. granulosus_ complex from different species of intermediate hosts in different geographical areas and several strains have been characterized (Thompson, 2008). To date, ten different genotypes (G1–G10) have been described for _E. granulosus_ complex, based on the analyses of mitochondrial and nuclear genetic data (Bowles et al., 1992, 1994; Scott et al., 1997; Lavikainen et al., 2003; Thompson, 2008; Saarima et al., 2009). Recently, a taxonomic revision of the genus has been made mainly on the basis of mitochondrial data, in which _E. granulosus_ complex splitted into four distinct species as follows: _E. granulosus_ sensu stricto (G1–G3), _E. equinus_ (G4), _E. ortleppi_ (G5) and _E. canadensis_ (G6–G10) (Nakao et al., 2007; Moks et al., 2008; Knapp et al., 2011). Also _E. felidis_ is closely related to _E. granulosus_ sensu stricto, and is grouped within _E. granulosus_ complex (Huttner and Romig, 2009). However, based on more complex data, including nuclear sequences and the epidemiological criteria, it was recommended by Thompson (2008) and Saarima et al. (2009) that genotypes G6–G10 could be broken into two different species namely _E. canadensis_, the cervid genotypes (G8 and G10), and _E. intermedius_, the camel/pig genotypes (G6/G7).

Since, there is a high risk of hydatid disease during experiments on dogs, usually genetic characterizations are performed on larval stages in the intermediate hosts. However, genetic identification of adult worms is required as well to provide a better understanding of existing cycles and genotypes in endemic areas. There are only few studies on genetic characterization of _E. granulosus_ complex in dogs around the world (Abbasi et al., 2003; Stefanic et al., 2004; Mathis and Deplazes, 2006; Hüttnner et al., 2008). Widespread recovery of adult _E. granulosus_ has been reported from dogs, jackals and wolves throughout Iran (Sadjadi, 2006). In a comprehensive study conducted in 13 provinces of Iran, the prevalence of _E. granulosus_ in sheepdogs was 27.2% (Eslami and Hosseini, 1998). In Iran, previous molecular studies on _E. granulosus_ complex have been performed on larval stages of _E. granulosus_ isolated from human or different livestock species including sheep, goat, cattle, buffalo and camels, revealing the existence of various genotypes (G1, G3 and G6) in the country (Zhang et al., 1998; Harandi et al., 2002; Ahmadi and Dalimi, 2006; Rostami Nejad et al., 2008; Kia et al., 2010; Sharbatkhori et al., 2010; Parsa et al., 2011; Sharifyazdi et al., 2011).

Lorestan province in Western Iran could be one of hotspots for echinococcosis in the country as many people live as nomads and human and dogs are always in close contact. Very limited information is available for _E. granulosus_ complex in Lorestan province (Rostami Nejad et al., 2008; Parsa et al., 2011), mainly from intermediate hosts of the parasite. The aim of this study was to genetically characterize _E. granulosus_ isolates from dogs from Lorestan province using the partial sequence data of mitochondrial cytochrome c oxidase 1 (cox1) and NADH dehydrogenase 1 (nad1) genes to gain a better understanding of the parasite’s life cycle in the studied area.

2. Materials and methods

2.1. Source of isolates and DNA extraction

Based on a ethical approval from the Municipality committee, from April to November 2011, 71 stray dogs, from western province of Lorestan, were humanely euthanized. Following necropsy the intestines of dogs were examined for adult worms of _E. granulosus_. The worms removed from each infected dog transferred into a separate tube and washed three times with normal saline and stored in 70% ethanol until further examination. Before extracting genomic DNA, the worms were thoroughly washed in distilled water to remove ethanol. Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

2.2. Mitochondrial PCR amplification

A partial sequence for each of cox1 and nad1 mitochondrial genes was amplified separately from individual genomic DNA isolates using the primer sets JB3/JB4.5 (Bowles et al., 1992) and JB11/JB12 (Bowles and McManus, 1993) for cox1 and nad1 genes, respectively. PCRs were performed in a final volume of 50 µl containing 4 µl (50–100 ng) genomic DNA, 25 pmol of each primer, 3.5 mM MgCl2, 250 µM of each dNTP's and 2 units Taq polymerase. Amplifications were conducted under following PCR conditions: 94 °C for 5 min as an initial denaturation, 94 °C (30s), 50 °C (45s), 72 °C(35s) for 35 cycles and a final extension at 72 °C for 10 min. For each set of PCRs negative (no-NA) controls were included. Six microliter aliquots of PCR products were electrophoresed on agarose gel (1.5%, W/V) and stained with ethidium bromide (0.5 µg/ml). A 100 bp ladder (Fermentas, Vilnius, Lithuania) was used as a DNA size marker. The gels were visualized by UV trans-illuminator (UVitec, Cambridge, UK).

2.3. Sequencing and phylogenetic analysis

All cox1 and nad1 PCR products were subjected to automated sequencing by the Illumina Genome Analysis System, employing the same primers used in the primary PCR. The electropherogram of each sequence was checked by eye, and the sequences were submitted to GenBank (accession numbers JN604097 to JN604112). In order to compare the cox1 and nad1 sequences determined herein with those of reference sequences representing 10 currently known genotypes (G1–G10) of _E. granulosus_ (sensu lato), sequences for the cox1 and nad1 genes were obtained from the public database i.e., GenBank (http://www.ncbi.nlm.nih.gov/). Upon pairwise comparison, the amounts of sequence difference (D), were obtained using the method as previously described by Chilton et al. (1995).

For phylogenetic analysis, the dataset of the concatenated cox + nad1 sequences representing all haplotypes detected was compiled, together with key reference sequences (comprising concatenated cox1 + nad1 sequences from previous studies (Bowles et al., 1992; Bowles and McManus, 1993, 1994; Gasser et al., 1999; Hüttnner et al., 2008; Lavikainen et al., 2008; Nakao et al., 2007) and representing all currently recognized _Echinococcus_ species and _E. granulosus_ ‘genotypes’, and employing Taenia saginata as the outgroup; see Table 1). In every case, each pair of concatenated sequences represented the same isolate (i.e., both the cox1 and nad1 sequences were derived from the same isolate). A phylogenetic tree was constructed by employing Bayesian Inference (BI) method using the program MrBayes v.3.1.2 (http://}
Among 71 stray dogs examined, twenty (28.2%) were found infected with *E. granulosus* (9 males and 11 females). For all of *E. granulosus* isolates, fragments of about 450 and 500 bp were successfully PCR-amplified within *cox1* and *nad1* genes, respectively. For all amplicons, consensus sequences of 366 and 471 nucleotides were obtained for each strain, 15%) in the studied area. Nine sequences were found in *E. granulosus* (9 males and 11 females). For all of *E. granulosus* isolates, fragments of about 450 and 500 bp were successfully PCR-amplified within *cox1* and *nad1* genes, respectively. For all amplicons, consensus sequences of 366 and 471 nucleotides were obtained for each strain, 15%) in the studied area. Nine sequences were found in *E. granulosus* (G1–G3 complex) from Iranian camels using sequence analysis of mitochondrial *cox1* and *nad1* genes (Sharbatkhori et al., 2009, 2011). Later this genotype was also reported from buffalos (Amin Pour et al., 2011), sheep, cattle and again in camels (Hajialillo et al., 2012; Sharifyazdi et al., 2011) in Iran.

In Lorestan province, hydatid surgeries constitute about 0.02–0.15% of all surgical operations (Rostami Nejad et al., 2007) and the prevalence of cystic echinococcosis has been reported ranging from 20% to 30.9% in dogs (Eslami and Hosseini, 1998; Dalimi et al., 2002). In the present study, the infection rate among dogs was 28.2% which is in concordance with previous studies (Dalimi et al., 2002; Eslami and Hosseini, 1998). In spite of high prevalence of the disease, only a few studies have employed molecular tools for the characterisation of *E. granulosus* in the study area (Parsa et al., 2011; Rostami Nejad et al., 2008). The present study presents the first report on the molecular characterisation of *E. granulosus* isolates from its definitive hosts (dog) using mitochondrial loci in Lorestan province, Iran.

In the present study genetic characterization of twenty dog isolates of *E. granulosus* employing mitochondrial *cox1* and *nad1* sequences revealed that the G1–G3 complex (*E. granulosus sensu* L.)

### Table 1

<table>
<thead>
<tr>
<th><em>E. granulosus</em> haplotypes isolated from dogs in Iran</th>
<th><em>Echinococcus</em> genotypes/ species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1: Lorc1 (JN604097)</td>
<td>E1 (G1)</td>
<td>Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H2: Lorc1 (JN604097)</td>
<td>E2 (G2)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H3: Lorc1 (JN604097)</td>
<td>E3 (G3)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H4: Lorc1 (JN604097)</td>
<td>E4 (G4)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H5: Lorc1 (JN604097)</td>
<td>E5 (G5)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H6: Lorc1 (JN604097)</td>
<td>E6 (G6)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H7: Lorc1 (JN604097)</td>
<td>E7 (G7)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H8: Lorc1 (JN604097)</td>
<td>E8 (G8)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H9: Lorc1 (JN604097)</td>
<td>E9 (G9)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
</tr>
<tr>
<td>H10: Lorc1 (JN604097)</td>
<td>E10 (G10)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
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<tr>
<td>H11: Lorc1 (JN604097)</td>
<td>E11 (G11)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
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<tr>
<td>H12: Lorc1 (JN604097)</td>
<td>E12 (G12)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
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<tr>
<td>H13: Lorc1 (JN604097)</td>
<td>E13 (G13)</td>
<td>Bowles et al. (1992) and Bowles and McManus (1993)</td>
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strictrum) were present in Lorestan Province, Western Iran. A pervious study conducted in this region using a specific 125rRNA PCR showed that all sheep and goat isolates belonged to G1 genotype (Rostami Nejad et al., 2008). Another study, using ITS1-RFLP, indicated the presence of G1 in isolates originated from sheep, goats and cattle (Parsa et al., 2011). However G1–G3 genotypes are indistinguishable by ITS1 PCR-RFLP. In this study G1 was the most prevalent genotype of the isolates. These findings suggest that sheep–dog cycle is the dominant cycle of CE in the area. G1 is the most frequently genotype identified in livestock and human throughout the world (Breyer et al., 2004; Capuano et al., 2007; Casulli et al., 2008; Moro and Schantz, 2009; Sánchez et al., 2010) although in some countries of north Africa such as Sudan and Mauritania, G6 is the most common genotype in sheep, cattle, camels and human (Bardonnet et al., 2002; Omer et al., 2010).

All isolates in the present study, designated as haplotypes 1 to 13 (H1–H13) formed a strongly supported clade (pp = 1.00), together with reference sequences representing E. granulosus genotypes G1–G3 (E. granulosus sensu stricto) to the exclusion of E. felidis (pp = 1.00). These findings provide further support for considering G1–G3 “complex” as a separate species and do not confirm the hypothesis that G2 is a separate species (Abushhewa et al., 2010; Hüttnier et al., 2008; Lavikainen et al., 2003; Saarma et al., 2009; Vural et al., 2008).

The present study records the occurrence of G2 and G3 genotypes in dogs as definitive host. By sequencing partial cox1 (366 bp) and nad1 (471 bp) genes, Bowles et al. (1992) first reported G2 genotype as Tasmanian sheep strain of E. granulosus complex. The percentage of nucleotide differences in pairwise comparison of G1 and G2 is 0.8% for both partial cox1 and nad1 genes (Bowles et al., 1992; Bowles and Mcmanus, 1993). To date, G2 genotype has been identified in human and various animals such as sheep, cattle, buffalo and camel from South America, Europe, Africa and Asia. It seems that this genotype has a wider spectrum of intermediate hosts and dispersed to a wide range of geographical areas from its original location in Tasmania. It is not clear if G2 genotype is distributed around the world by global livestock trade or via dogs as companion animals.

The presence of E. granulosus complex has been reported in faecal samples of wild canids in Northeast of Iran but no genotype data have been provided for the isolates (Beiromvand et al., 2011). The existence of all three genotypes of E. granulosus sensu stricto in dogs and the absence of G6 genotype in this study justify more research on the nature of interactions of different genotypes in dogs and other definitive hosts. More investigations are also needed to elucidate transmission dynamics of G2 and G3 genotypes in the region.

Acknowledgments

This research project (code 1403) was funded by Islamic Azad University, Borujerd branch. We would like to thank president of the university, Dr. Ahmad Seif, and also vice president for research, Dr. Mohammad Jafar Mahdian.

References


Fig. 1. Genetic relationships of Echinococcus granulosus dog isolates from western Iran and reference sequences for E. granulosus sensu lato and other species of Echinococcus from previous studies as well as Taenia saginata as the outgroup. The relationships were inferred based on phylogenetic analysis of concatenated cox1 + nad1 sequence data (H1–H13 in Bold type) using Bayesian inference. All haplotypes represent genotypes G1–G3 (G1–G3 complex, E. granulosus sensu stricto). The accession numbers and source of sequences are shown in Table 1. Nodal support is given as a pp value.
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