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Original Article

Echinococcus granulosus Sensu Stricto in Dogs and Jackals from Caspian Sea Region, Northern Iran

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Abstract

Background: The aim of the present study was genotyping of *Echinococcus granulosus* isolates from dogs and jackals in Mazandaran Province, northern Iran, and using partial sequence of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*).

Methods: *E. granulosus* isolates (n = 15) were collected from 42 stray dogs and 16 jackals found in south of the Caspian Sea in northern Iran. After morphological study, the isolates were genetically characterized using consensus sequences (366bp) of the *cox1* gene. Phylogenetic analysis of *cox1* nucleotide sequence data was performed using a Bayesian Inference approach.

Results: Four different sequences were observed among the isolates. Two genotypes [G1 (66.7%) and G3 (33.3%)] were identified among the isolates. The G1 sequences indicated three sequence profiles. One profile (Maz1) had 100% homology with reference sequence (AN: KP339045). Two other profiles, designated Maz2 and Maz3, had 99% homology with the G1 genotype (ANs: KP339046 and KP339047). A G3 sequence designated Maz4 showed 100% homology with a G3 reference sequence (AN: KP339048).

Conclusion: The occurrence of the G1 genotype of *E. granulosus sensu stricto* as a frequent genotype in dogs is emphasized. This study established the first molecular characterization of *E. granulosus* in the province.

Introduction

Echinococcus granulosus, the causative agent of a zoonotic disease known as cystic echinococcosis/hydatidosis, is an important cause of morbidity and mortality in humans globally, particularly in regions of extensive livestock husbandry (1). Echinococcosis is endemic in all Middle Eastern countries, including Iran (2). Domestic and wild livestock play the role of intermediate host and can retain the larval stage or hydatid cyst in internal organs, particularly lung and liver. Domestic and wild carnivores, especially dogs, serve as the definitive hosts and harbor adult stage of the parasite in their intestines (3, 4).

A more complete understanding of the transmission cycle of the *E. granulosus* complex in each region will assist the development of preventive and control strategies for echinococcosis. Widespread isolation of adult *E. granulosus* from dogs, jackals and wolves has been reported from different parts of Iran (2). In a comprehensive study conducted in 13 provinces of the country, the prevalence of *E. granulosus* in sheepdogs was 27.2% (5).

To date, investigations employing mitochondrial and nuclear DNA sequences have characterized ten different genotypes (G1–G10) within *E. granulosus* sensu lato (6, 7). These include two ovine strains (G1 and G2), two bovid strains (G3 and G5), an equine strain (G4), a camel strain (G6), two porcine strains (G7 and G9) and two cervid strains (G8 and G10) (8, 9). However, a taxonomic revision made mainly on the basis of mitochondrial data suggests that the *E. granulosus* complex could be split into four distinct species, including *E. granulosus* sensu stricto (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6–G10) (10, 11). *E. felidis* is closely related to *E. granulosus* sensu stricto and is clustered within the *E. granulosus* complex (12). Thompson (9) and Saarma et al. (13) recently recommended that genotypes G6–G10 be divided to two different species based on nuclear sequences and epidemiological criteria. These species include *E. canadensis*, which comprises the cervid genotypes (G8 and G10), and *E. intermedius*, which comprises the camel/porcine genotypes (G6/G7).

Most of the previous genetic characterizations were carried out on larval stages in intermediate hosts because of the high risk of hydatid infection during experimentation with definitive hosts. However, genetic identification of adult worms is necessary to provide more complete knowledge of the genotypes present and the cycles occurring in endemic regions. There are few reports describing the genetic characterization of *E. granulosus* complex in dogs around the world (14-18). In Iran, human hydatidosis is responsible for about 1% of surgical admissions and the range of human infection is 0.6–1.2%. The overall annual cost of cystic echinococcosis in the country has been estimated to be US$232.3 million (19). Most of the molecular characterizations of *E. granulosus* complexes have been performed using the protoscoleces of cystic echinococcoses isolated from humans or different livestock species, including sheep, cattle, goats, camels, and buffalo, and have indicated the presence of different genotypes (G1, G3, and G6) in Iran (20-31). The only previous study on adult *E. granulosus* worms revealed the existence of G1 (75%), G2 (10%), and G3 (15%) genotypes among 20 dog isolates from Lorestan Province, western Iran (18). Mazandaran province in northern Iran could be a hotspot for echinococcosis in the country, as many people live in villages and human and dogs are always in close contact. Very limited information is available concerning *E. granulosus* complexes isolated from definitive hosts in the country, including Mazandaran province, which is located south of the Caspian Sea.

The aim of the present study was to perform the genetic characterization of *E. granulosus*

isolates from definitive hosts, including dog and jackal, using a partial sequence of the cytochrome *c* oxidase subunit 1 gene (*cox*1). This study will improve our understanding of the identification and evaluation of local strains and of the parasite's life cycle in Mazandaran province, northern Iran.

Materials and Methods

Source of isolates

The small intestines from the carcasses of 42 stray dogs and 16 jackals (*Canisaureus*) killed in car accidents on the roads of Mazandaran Province, northern Iran, were examined for adult worms of the genus *Echinococcus* during the period from September 2013 to March 2014 (6 months).

Adult *E. granulosus* worms were collected from a total of 12 naturally infected dogs and 3 jackals. The worms were removed from each infected animal, transferred to two separate tubes, washed three times with normal saline, and kept in 10% formalin and 70% ethanol at 4 °C until further analysis (32).

DNA extraction and mitochondrial PCR amplification

Before DNA extraction, the worms were thoroughly washed in distilled water to remove ethanol. Genomic DNA was extracted using a DNA mini Kit (Bioneer; Daejeon, Korea) according to the manufacturer's instructions. A partial sequence of the mitochondrial *cox*1 gene was amplified from the genomic DNA isolates using primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAACATAATGAAAA-TG-3') (33). PCR reactions were performed in a final volume of 20 µl containing 50–100 ng (7 µl) of genomic DNA, 25 pmol of each primer, 3.5 mM $MgCl_2$, 250 □M of each deoxynucleoside triphosphate, and 2 units of Taq polymerase.

PCR amplifications were conducted under following conditions: 94 °C for 5 min (initial denaturation) followed by 35 cycles of [94 °C for 60 s (denaturation), 50 °C for 60 s (annealing), and 72 °C for 60 s (extension)] and a final extension at 72 °C for 5 min. Negative (no-DNA) and positive (control DNA) controls were included in each set of PCR reactions.

PCR products (5 ul aliquots) and a 100 bp DNA ladder (Fermentas; Vilnius, Lithuania) were electrophoresed on 1% (w/v) agarose gels and stained with ethidium bromide (0.5 µg/ml). The gels were visualized using a UV transilluminator (UVitec; Cambridge, UK) (Fig. 1).

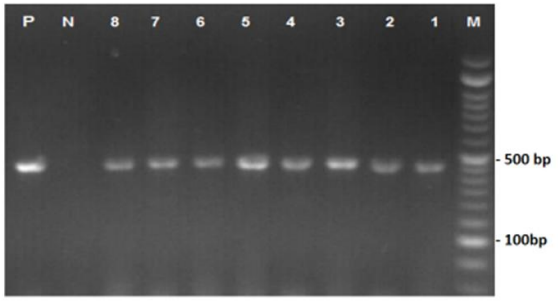

Fig. 1: Agarose gel electrophoresis of cox1–PCR products. Lanes 1 and 2-8 *E. granulosus* isolated from jackal and dogs, respectively. Lane N, negative control; Lane P, positive control, Lane M, 100-bp DNA ladder

Sequencing and phylogenetic analysis

PCR products were subjected to DNA sequencing using the same primers used in the primary PCR. The electropherogram of each sequence was checked manually. The sequences were compared with each other using the program BioEdit and with other sequences in GenBank (34) using the online Blast system (http://blast.ncbi.nlm.nih.gov/). The representative partial *cox*1 gene sequences were submitted to GenBank (accession numbersKP339045 to KP339048).

A phylogenetic analysis was conducted using a dataset containing the *cox*1 sequences representing the isolates detected in this study and key reference sequences from previous

studies (10, 17, 33, 35). This dataset represented all currently recognized *Echinococcus* species and *E. granulosus* genotypes, along with *Taenia saginata* as the outgroup. A phylogenetic tree was constructed with the software package MrBayes v.3.1.2 (http://mrbayes.csit.fsu.edu/index.-php) by employing the Bayesian Inference (BI) method. Posterior probabilities (pp) were inserted for 2,000,000 generations (ngen: 2,000,000; burnin: 20000) employing the Monte Carlo Markov Chain procedure and four simultaneous tree-building chains (nchains: 4), with every 100th tree saved (samplefreq: 100). Treeview X v.0.5.0 software (36) was used to display the trees. All GenBank accession numbers for the sequences inferred from this study and for the reference genotypes/species used in the phylogenetic analysis are shown in Fig. 2.

Results

Twelve (28.6%) of the 42 stray dogs and 3 (18.7%) of the 16 jackals were infected with *Echinococcus granolosus*. A fragment of about 450 bp within mitochondrial cox1 region was successfully amplified from 8 and 1 *Echinococcus* isolates obtained from dogs and a jackal, respectively (Fig. 1). All 9 isolates were subjected to sequencing and a consensus sequence fragment of 366 nocleotides was used for the sequence analysis. Alignments of the sequences with those of known *E. granulosus* genotypes revealed the presence of the G1 and G3 genotypes. G1 genotype was found in 5 dog and 1 jackal isolates. G3 genotype was observed in 3 dog isolates. Four representative sequences profiles from this study, designated Maz1 to Maz4, were submitted to GenBank under accession numbers KP339045 to KP339048. A consensus phylogenetic tree indicating the relationships among the isolates from the present study and the reference sequences from *E. granulosus* genotypes is shown in Fig. 2. All isolates grouped with the reference sequences of the G1-G3 complex, with maximal statistical support (PP = 1).

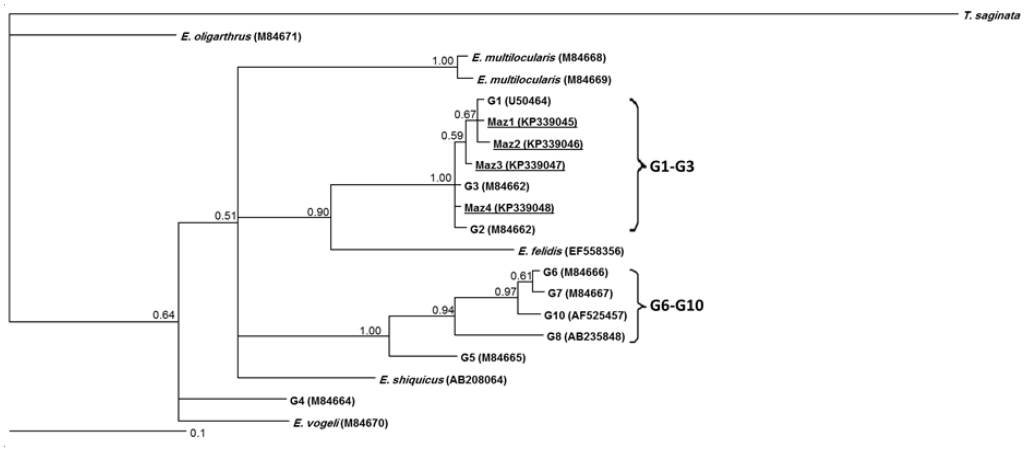

Fig. 2: Phylogenetic tree of *E. granulosus* isolates from Caspian Sea area, Mazandaran Province, northern Iran (shown as underlined) and reference sequences for *E. granulosus sensu lato* and other species of *Echinococcus* using the (BI) method. The relationships were obtained according to phylogenetic analysisof cox1 sequence data using Bayesian Inference method. All sequence profiles defined as Maz 1–4 represent genotypes G1–G3 (G1–G3 complex, *E. granulosus* sensu stricto).Theaccession numbers of sequences are indicated in parenthesis. The scale bar indicates distance. *Taenia saginata* sequence was taken from reference Bowles and McManus 1994. Nodal support is given as a pp value

Discussion

Mazandaran Province has a wet and humid climate and contains pastures suitable for the traditional rearing of domestic livestock. The presence of stray dogs infected with *E. granulosus* creates the potential for transferring the parasite. The prevalence of cystic echinococcosis has been reported to range from 3.3% to 63.3% in farm dogs in Iran, with an infection rate of 43.3% in Mazandaran Province (5). Another study from western Iran reported frequency of 19.1% and 2.3% for echinococcosis in dogs and golden jackals, respectively (37). A recent study in East Azerbaijan Province in northwestern Iran reported a frequency of 20% for *E. granulosus* among 80 stray dogs (38). In the present study, the infection rate was 28.6% among dogs, which is somewhat higher than previous reports from this province (5). In addition, an infection rate of 18.7% was obtained for jackals in the present study, which is much higher than the previously reported rate in western Iran (2.3%) (37). Despite the high prevalence of the disease, there have been no studies characterizing the *E. granulosus* strains in definitive hosts within the study area. Thus, the current study is the first specific identification, using morphological and molecular methods, of *E. granulosus* in isolates from definitive hosts (dog and jackal) from the north of Iran.

In the present study, the genetic characterization of eight dog and a jackal isolates of *E. granulosus* employing partial *cox*1 sequences revealed that the G1–G3 complex (*E. granulosus* sensu stricto) is present in Mazandaran Province, Northern Iran. The data indicate that 66.7% and 33.3% of the isolates belong to the G1 and G3 genotypes, respectively. In a previous study, all of the 6 sheep and 8 goat isolates of *E. granulosus* from Noor in Mazandaran Province were of the G1 genotype (39). A pervious study using partial sequences of the *cox*1 and *nad*1 genes indicated that all 5 cattle and 10 sheep isolates from Golestan Province, the eastern neighbor of Mazandaran Province, belonged to the G1 genotype (22). Another study conducted in Golestan Province using an ITS1-RFLP analysis showed that all of the isolates from human, sheep, and cattle belonged to the G1 genotype and all of the camel isolates belonged to the G6 genotype (40). However, ITS1-RFLP lacks the ability to distinguish the G1- G3 genotypes (25). This issue can explain results of another study in Ahvaz province, southwestern Iran that reported G1 genotype in all 141 sheep, 104 cattle, 84 goats and 5 human isolates of *E. granulosus* using ITS1-RFLP (41). In a recent study of *E. granulosus* isolates from human, sheep and cattle in Zanjan Province located in northwestern Iran, using partial cox1 sequence, 93.35 and 4.65% of isolates belonged to G1 and G3 genotypes, respectively (42). Another study of *E. granulosus* isolates in East Azerbaijan province, northwestern Iran, indicated G1, G3 and G6 genotypes among 16 infected dogs (38).

In this study, G1 was the most prevalent genotype among the isolates, indicating that the sheep–dog cycle is the dominant cystic echinococcosis cycle in the area. This is in concordance with a study in Isfahan province, central Iran indicating G1 as the most prevalent genotype of *E. granulosus* affecting human, sheep, cattle, goats and occasionally camels (43). In addition, other studies have indicated the predominance of G1 genotype in different parts of Iran (18, 44). G1 is the most common genotype reported in animals and human throughout the world (45-47), although, the G6 genotype is the most prevalent in sheep, cattle, camels and humans in some countries of North Africa, such as Mauritania and Sudan (48-51).

All of the isolates in the present study, designated Maz1 to Maz4, formed a strongly supported clade (pp = 1.00) with the 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 the

G1–G3 "complex" as a separate species and do not confirm the hypothesis that G2 or G3 are separate species (13, 17, 22, 52).

This study records the occurrence of G1 and G3 genotypes in dogs as definitive host by sequencing a portion (366 bp) of the *cox*1 gene. Bowles & McManus (53) first reported the G3 genotype as an Indian buffalo strain of *E. granulosus*. However, there have been many reports of the presence of the *E. granulosus* G3 genotype in intermediate hosts, such as humans, sheep, goat, cattle, buffalo, the Nile lechwe (*Kobus megaceros*), and pigs (54-58). Moreover, some authors have demonstrated the presence of the G3 genotype in intermediate hosts such as sheep, cattle, camel, buffalo and humans in some parts of Iran (25, 26, 28, 31).

Our results are in agreement with previous studies in Iran indicating that G1 is the predominant *E. granulosus* genotype in intermediate and definitive hosts (18, 23, 27, 39, 43, 44). The occurrence of *E. granulosus* complex has been described in fecal samples of wild canids in northeast Iran, but no genotypic characterization were performed on the isolates (59).

Conclusion

The existence of two genotypes of *E. granulosus* sensu stricto in dogs and the absence of the G6 genotype in this study warrant more research on the nature of the interactions between different genotypes in dogs and other carnivores as the definitive hosts. More research is also needed to clarify the transmission dynamics of G3 genotypes in the area and to develop appropriate strategies for the prevention and control of the disease.

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