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Genotypic and Phenotypic Characterization of *Echinococcus granulosus* of Iran

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Abstract: Molecular and morphological analyses were used to study phenotypic and genotypic characteristics of two common sheep and camel strains of *Echinococcus granulosus*. Biometric analysis of fifteen characters showed that those 15 morphometric values of camel isolates have significantly higher than sheep isolates. Number of hooks, length and width total of the handle, the blade and the distance between the blade and the guard of large and small hooks were those characters that measured. Molecular analysis of ND1 fragment of mtDNA obtained from G1 and G6 genotypes using PCR-RFLP technique with two commercial restriction endonucleases enzyme AatI and BstHHI were in agreement with the morphological findings. PCR-RFLP band patterns using AatI demonstrated two bands of 170 and 301 bp in sheep strain whereas camel strain remained undigested. Besides PCR-RFLP profiles using BstHHI demonstrated two bands of 137 and 334 bp identical to the G6 genotype and one DNA fragment of 471 bp in G1 genotype. Because of the intraspecific variation of sheep and camel strains using both morphological and molecular tools concomitantly provides more accurate and reliable information about the phenotypic and genotypic characteristics of *E. granulosus* isolates and emphasizes the valuable tools in transmission and epidemiological studies.

Key words: Genotype, *Echinococcus granulosus*, PCR-RFLP, mtDNA, phenotype

INTRODUCTION

Infection with the larval stage of *Echinococcus granulosus*, known as cystic echinococcosis (CE), is a highly endemic zoonosis in the ruminant breeding areas of Iran. The parasite is mainly transmitted in a synanthropic cycle generally involving dogs (large stray populations) and livestock animals (sheep, cattle, goats and camels); (Thompson and McManus, 2001). DNA-based studies have shown that the species *E. granulosus* is composed of heterogeneous groups of genetic variants, defined as strains (McManus, 2002). In various studies, strains were often characterized after Polymerase Chain Reaction (PCR) based methods, has been extensively used to characterize strain grouping within *E. granulosus*. To date, ten distinct genotypes (G1-G10) have been identified for *E. granulosus* (Thompson, 1995; Lavikainen *et al*., 2003; Scott *et al*., 1997). The common sheep G1 strain is widespread and has been mainly identified in Mediterranean and semiarid breeding areas as being carried by sheep and cattle. In contrast, the camel G6 strain is often found in desert areas, carried by camels. Most human infections are due to the common sheep strain, whereas only a few cases have been attributed to the camel strain. For some authors, the definition of all the *E. granulosus* strains should be reassessed. It has not yet been demonstrated that each strain forms a true clade (i.e., a monophyletic taxon) with a real epidemiological relevance (Obwaller *et al*., 2004).

In Iran, two strains, the common sheep and camel, are widespread. The sheep strain, the most infective for humans, has been described in where it has been presumed to play an important role in the transmission of CE to humans (Karimi *et al*., 2007; Dalimi *et al*., 2002). The identification of *E. granulosus* strains or variants has been carried out in different laboratories using different analytical methods (morphology, physiology, biochemistry and molecular genetics), all of which have proved to be useful, particularly when used concomitantly. So, using both morphological and molecular approaches together could provide more accurate and reliable information about the nature and extent of variation within *E. granulosus* (Eckert and Thompson, 1997; McManus and Bryant, 1995; Roratto *et al*., 2006).

In regions of Iran, where two common intermediate host of *E. granulosus* (Sheep and Camel) coexist, differential diagnosis of G1 and G6 strains is important

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due to the different transmission and epidemiological characteristics. Nevertheless, no comparative study has been carried out in Iran, until now with material from regions where both intermediate host of *E. granulosus* (Sheep and Camel) co-exist and material from geographical areas where both species of do not co-exist. Moreover, studies on this question are also needed in human endemic areas. Additionally, research must be carried out to look for phenotypic and genotypic analysis that make the accurate and reliable diagnosis of G1 and G6 strains. The aim of this study was to characterize more thoroughly agents of cystic echinococcosis in the Iran by morphological and genetic approaches. Survey of *E. granulosus* from various localities was undertaken to uncover a strain composition and population structuring in this territory.

MATERIALS AND METHODS

Parasites: The study was carried out in hydatid cysts isolated from livers and lungs of naturally infected sheep and camel obtained in abattoirs from various regions of Fars provinces, southern regions of Iran (Shiraz, Kazeroun, Marvdasht, Fasa and Lar) between September 2007 and March 2008. The cysts were processed separately and an isolate or sample represented protoscoleces aspirated from an individual hydatid cyst. A small number of the protoscoleces were used for morphometric analysis randomly, while the rest were used for genetic analysis. Cysts were never pooled and processed jointly (Ponce-Gordo and Cuesta-Bandera, 1997). Protoscoleces rostellar hooks were aspirated under sterile conditions together with the hydatid fluid from each cyst and rinsed twice with sterile saline. Each suspension of protoscoleces was sieved to remove the larger debris. Protoscoleces were fixed in 80% (v/v) ethanol or stored at -20°C for further processing.

Morphological analysis: The 32 samples of sheep (16 samples obtained from liver and 16 samples from lung origin) and 26 samples of camel origin (13 samples obtained from liver and 13 samples from lung origin) were analyzed. Biometric characters based on the three large hooks and three small hooks per sample measured. Individual protoscoleces from each suspension was mounted in polyvinyl lactophenol on glass slide with sufficient cover-slip pressure so that the rostellar hooks were spread out but not damaged. Several linear measurements of both large and small hook were analyzed, as indicated in Fig. 1. The measurements were taken by a computer connected to a microscope (Olympus BX50), using image analysis software (Image Pro Plus-Media Cybernetics, US). The characters studied were: total number of hooks (NH), total length (TL), total width (TW), blade length (BL), blade width (BW), handle length (HL), handle width (HW) and distance between blade and guard (BGD). All measurements are in micrometers unless another one has been indicated. Data analysis was carried out with Statistical Package for Social Sciences (SPSS version 9.0). The data were submitted to the Student's t-test. The statistical significance was assessed at p = 0.05.

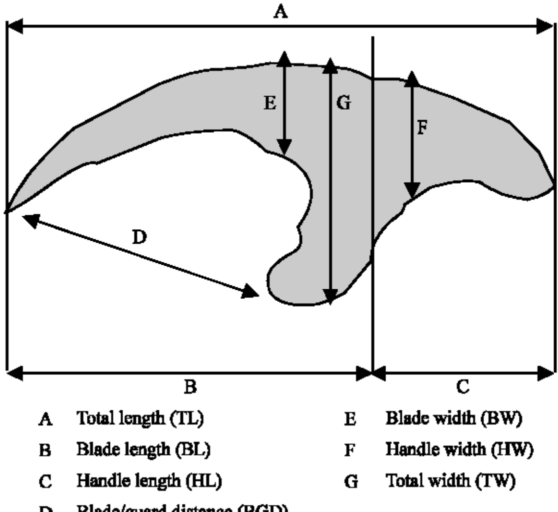

Fig. 1: Diagrammatic representation of a rostellar hook from a protoscolex of *E. granulosus*, showing the measurements taken in this study

Molecular analysis: The protoscoleces were rinsed several times with sterile distilled water to remove the ethanol prior to DNA extraction. Total *E. granulosus* genomic DNA was extracted using extraction procedure (Sambrook and Russell, 2001).

E. granulosus genomic DNA samples were analyzed by PCR-RFLP of the gene coding for NADH dehydrogenase 1 region (ND1) according to Bowles and McManus (1993a) with slight modifications. Primers used were those reported by Bowles and McManus (1993a).

The PCR was performed in a 25 μL volume containing 2 μL genomic DNA, 100 μM each deoxynucleoside triphosphate (dNTP; MBI Fermentas, Vilnius, Lithuania), 20 pmol each of primers (Invitrogen Life Technologies, Paislay, Scotland) and 0.5 U DNA Red Taq Polymerase in 2.5 μL reaction buffer ×10 (Sigma, Saint Louis, MO, USA). The amplification conditions were as follows: an initial step of denaturation (30 sec at 94°C) followed by 40 cycles of successive denaturation (30 sec at 94°C), hybridization (30 sec at 55°C) and elongation (30 sec at 72°C) and then a final elongation of 5 min at 72°C.

The specificity of amplications and the size of products were assessed by electrophoresis in 1.5% (w/v) Tris-acetate/EDTA (TAE) agarose gels. Using O'RangeRuler™ 50 bp DNA Ladder (Fermentas). The PCR products were then purified with DNA purification kit (MBST, Iran) as per instruction the manufacturer. The purified PCR products of each sheep and camel ND1 mtDNA PCR products were then digested directly with AatI and BstHHI as per instruction the manufacturer (Fermentas). The restriction fragments were separated by running through 1.5% (w/v) agarose gel stained using ethidium bromide and then photographed upon UV transillumination.

RESULTS

Morphology: The arrangement of hooks with two rows of alternating large and small hooks obtained from sheep and camel specimens were similar. The fifteen morphometric values (NH, LTL, LTW, LBL, LBW, LHL, LHW, LBGD, STL, STW, SBL, SBW, SHL, SHW and SBGD) detected from 174 large hooks and 174 small hooks demonstrated a high degree of phenotypic polymorphism between *E. granulosus* of sheep and camel strains. The total number of protoscoleces of camel isolate was greater than sheep isolate besides the other all morphometric values of camel strain was significantly higher than the other one. For example mean total lengths of large and small hooks of sheep specimens were 23.6 and 19.1 μm whereas camels isolate were 27.5 and 21.3 μm, respectively (Table 1).

Characterization of G1 and G6 genotypes by PCR-RFLP: In the present study we used ND1 mtDNA-PCR linked restriction fragment length polymorphism (PCR-RFLP) to characterize G1 and G6 genotypes of *E. granulosus* DNA isolated from cysts recovered from sheep and camel specifically eleven livers and eleven lungs, in the State of Fars province. The ND1 mtDNA-PCR yielded one amplification product of 471 bp lengths of the G1 and G6 genotypes. The PCR-RFLP patterns produced after digestion of the ND1 fragment with the two restriction endonucleases AatI and BstHHI. Recognition pattern of AatI restriction enzyme (AGGCCT) is at nucleotide interval 168-173 in the sheep NDI nucleotide sequences, but there wasn't recognition site in the G6 sequences. Besides restriction enzyme BstHHI has one restriction site (GCGC) at nucleotide interval 332-335 in the G6 sequence whereas G1 sequences haven't recognition site of BstHHI. These sequences variations, therefore, provide the basis of a PCR-RFLP method to rapidly survey *E. granulosus* isolates and discriminate the two genotypes by PCR-RFLP analysis. In corroboration of the sequence analysis, The RFLP patterns after digestion of the mtDNA-PCR amplicons by AatI demonstrated two bands of 170 and 301 bp in G1 strain whereas G6 strain remained undigested (The 471 bp band, similar to amplicons). Besides PCR-RFLP profiles using BstHHI demonstrated two bands of 137 and 334 bp identical to the G6 genotype and one DNA fragment of 471 bp in G1 genotype (Fig. 2).

Table 1: Morphometric comparison between rostellar hooks (large and small) characteristics of *E. granulosus* metacestode according to common sheep and camel origin

Characters (μm)	Sheep origin (n = 32)	Camel origin (n = 26)
Total number of hooks (NH)	35.1±2.9	36.9±3.9
Large hook: total length (LTL)	23.6±0.8	27.5±2.2
Large hook: total width (LTW)	8.5±0.9	9.6±1.1
Large hook: blade length (LBL)	11.9±0.9	13.9±1.1
Large hook: blade width (LBW)	8.5±1.0	9.6±1.2
Large hook: handle length (LHL)	6.8±1.0	7.8±1.2
Large hook: handle width (LHW)	3.9±0.7	4.6±0.9
Large hook: blade/guard distance (LBGD)	9.7±2.2	10.4±2.2
Small hook: total length (STL)	19.1±1.5	21.3±1.6
Small hook: total width (STW)	7.1±1.0	8.5±1.5
Small hook: blade length (SBL)	7.9±0.9	9.6±0.9
Small hook: blade width (SBW)	7.1±1.2	8.6±1.5
Small hook: handle length (SHL)	6.8±1.3	8.2±1.5
Small hook: handle width (SHW)	3.5±0.6	4.4±1.1
Small hook: blade/guard distance (SBGD)	9.8±2.2	10.3±2.2

All values are shown as mean and standard deviation. Significant difference ($p<0.05$), Student's t-test

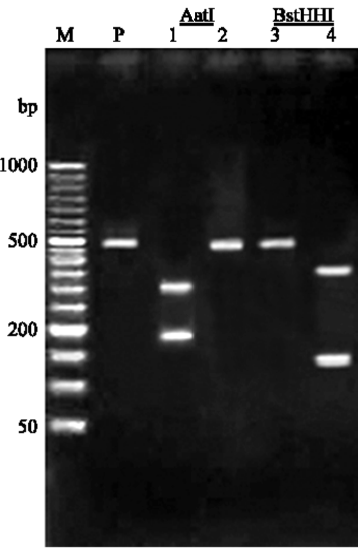

Fig. 2: PCR-RFLP analyses of the mitochondrial NDI gene using the restriction endonuclease AatI and BstHHI. Lane P, undigested NDI PCR product (471 bp); lanes 1-2 and 3-4, representative examples of AatI and BstHHI digested NDI PCR products respectively; lanes 1 and 3, sheep isolates and lane 2 and 4, camel isolates; lane bp (base pairs), molecular weight marker lane (M)

DISCUSSION

It is now generally recognized that the dog tapeworm *E. granulosus*, the cause of cystic hydatid disease, exhibits substantial genotypic and phenotypic diversity. This variability has important implications for the design and development of diagnostic tools of *E. granulosus*. Genotypic variation within *E. granulosus* detected with molecular techniques caused to classify this cestode to ten genotypes as each genotype appears to have specific phenotypic characteristics. This organism shows a great intraspecific variation in relation to host specificity, epidemiology, morphology, developmental biology, biochemistry, physiology, biochemistry and genetics (Thompson and Lymbery, 1988). This variability led to the identification of subspecific variants or strains and some authors have proposed a revision of the taxonomy of the genus *Echinococcus* (Thompson *et al*., 1995, 2002). Based on these variability, several different analytic methods has been carried out for the detection of *E. granulosus* strains and each of them proved to be useful but using both morphological and molecular approaches together, similar to the present study, could provide more accurate and reliable information about the nature and extent of variation within *E. granulosus*.

The main purpose of present study was to detect G1 and G6 strain of *E. granulosus* obtained from Fars, Iran using phenotypic and genotypic analysis. From Iran, only G1 and G6 strains of *E. granulosus* exist, based on abattoir surveys, the mean prevalence of hydatidosis of sheep in different parts of the country has been reported to be 8.1% and corresponding feature for cattle and goats were 12 and 6.5%, respectively (Dalimi *et al*., 2002). Oryan *et al*. (1994) reported much higher numbers for infected sheep (26%) of Fars province in the early 1990s. The other study performed in Shiraz, a city in southwest Iran and the capital of Fars province, during a 5-year period from 20 March 1999 to 19 March 2004 were used to determine the prevalence of hydatidosis in sheep, cattle and goats in the region. A total of 844,039 animals (cattle 131,716; sheep 577,090; goats 135,233) slaughtered in the 5-year period and overall 34,856 (4.1%) livers and 123,402 (14.62%) lungs were condemned. Hydatidosis was responsible for 28.7 and 15.4% of total livers and lungs condemnations, respectively (Ansari-Lari, 2005).

Data obtained from morphological studies, can be unreliable and/or limited if used as the sole distinguishing criterion for sibling species and strains (Thompson and Lymbery, 1988). Thus, using both morphological and molecular genetic approaches concomitantly could provide more accurate and reliable information about the nature and extent of variation within *E. granulosus* isolates (McManus and Bryant, 1995; Gordo and Badera, 1997).

In the present study we found that all 15 morphometric characters significantly different between sheep and camels specimens obtained from different regions of Fars province. On the other hand morphometric analysis found to be useful tool for differential diagnosis of two common G1 and G6 strains of *E. granulosus* from Iran. Data obtained from biometric analysis in our study are similar to those reported from other investigators of Iran (Ahmadi, 2004; Hosseini and Eslami, 1998) but differed to those reported from some other country. For example the number of hooks, blade length of large and small hooks of protoscoleces from sheep of our study are similar to the those of the common sheep strain of Australia, Great Britain and Spain, whereas the total length of large and small hooks of Iranian sheep isolates were significantly shorter than those of the common sheep strain from Australia, Great Britain and Spain (Kumaratilake and Thompson, 1984; Gordo and Badera, 1997). LTL and LBL characters of Iranian camels were significantly different from the Egyptian ones. According to Eckert *et al*. (1989) these differences suggest that probably there is an inter-group heterogeneity between Middle East (Iranian) and African (Egyptian) camel isolates, this matter supported from our findings because of two morphometric value, LTL and LBL significantly differed between Iranian and Egyptian samples.

Recently, an increasing number of investigators have focused their analysis on the parasite's mitochondrial NADH dehydrogenase 1 (ND1) and cytochrome oxidase 1 (CO1) regions as genetic markers (Bowles *et al*., 1994; Zhang *et al*., 1998). The use of mitochondrial DNA sequencing is mainly based on its fast evolution highlighting its importance in differentiation and discrimination of closely related organisms, as in the case of *Echinococcus* strains, which seem to be highly homogenous evolutionary units (Bowles and McManus, 1993a, b). Bowles and McManus (1993b) have used partial sequences of the mitochondrial NADH dehydrogenase 1 (ND1) gene for 59 isolates and found the same groupings of variant for the CO1 gene with a slightly lower resolution. Several approaches using molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA-polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP-PCR) have been used in order to show the high degree of differentiation in the genus *Echinococcus* (Karimi *et al*., 2007; Thompson and McManus, 2001; Siles-Lucas *et al*., 1994). However, it has been shown that genetic variability within strains is present mainly in the *E. granulosus* G1 genotype or sheep strain (Haag *et al*., 1999; Kamenetzky *et al*., 2002; Jenkins *et al*., 2005).

The existence of two sheep and camel strains of *E. granulosus* from Iran using molecular techniques based on DNA nucleotide and predicted amino acid sequence of CO1 and ND1 genes was reported (Zhang *et al*., 1998). In the study of Fasihi Harandi *et al*. (2002), by using both molecular (PCR-RFLP of ITS1 region) and morphological analysis, the same result was obtained.

We found that ND1 mtDNA analyzed using PCR-RFLP technique with commercial AatI and BstHHI restriction endonucleases enzyme could be a useful tool for accurate differentiation between G1 and G6 genotypes of *E. granulosus*. Each of the two restriction enzymes AatI and BstHHI could digest ND1-PCR products of one strain and no affected on the other one. On the other hand ND1-PCR products of sheep and camel strains were digested with AatI and BstHHI respectively. These matters demonstrated that two common sheep and camel isolates had two PCR-RFLP patterns which were in agreement with the results presented by Bowles *et al*. (1995), Bowles and McManus (1993a, b), Zhang *et al*. (1998) and Fasihi Harandi *et al*. (2002).

It was concluded that although morphometric analysis as described in this study could differentiate G1 and G6 strains of *E. granulosus* obtained from Fars province, because of existence morphometric variation between Iranian samples and the other country can be unreliable and/or limited if used as the sole distinguishing criterion for sibling species and strains. Thus, using both morphological and molecular genetic approaches concomitantly could provide more accurate and reliable information about the nature and extent of variation within *E. granulosus* isolates. This result is agrees from some investigators such as: McManus and Bryant (1995), Gordo and Badera (1997) and Fasihi Harandi *et al*. (2002). Whereas some investigators have used molecular and morphological studies separately for characterizing the *E. granulosus* strains (Kumaratilake *et al*., 1986; Eckert *et al*., 1989; Hosseini and Eslami, 1998; Kumaratilake and Thompson, 1984; Bowles *et al*., 1995; Zahang *et al*., 1998; Washira *et al*., 1993).

However, present results reinforce previous study of the phenotypic and genotypic characteristics and would be firm bases to develop genetic analyses and to diagnose causal agents of human hydatidosis From Iran.

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