Molecular Characterization of *Echinococcus granulosus* Isolated from Sheep and Camel in Iran

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Summary

Forty-eight isolates of hydatid cyst collected from sheep and camels slaughtered in different areas of Iran were analyzed by polymerase chain reaction and restriction fragment length polymorphism techniques. The results indicated that the isolates could be categorized into two distinct and uniform genotype grouping, the camel/dog strain group (Genotype 6) and cosmopolitan common sheep strain group (Genotype 1) of *E.granulosus.* These isolates can potentially act as a risk factor for human health in the country.

***Key words*:** *Echinococcus granulosus*, hydatid cyst, strain, rDNA-ITS1, PCR-RFLP

Introduction

Cystic hydatid disease, caused by *Echinococcus granulosus* is prevalent in most parts of Iran, especially in rural areas where offal from slaughterhouses is incorrectly disposed of /or where slaughtering is practiced on farms. Various surveys throughout the country have indicated that hydatid cyst is commonly found in sheep, camels, cattle and goats (Mobedi *et al* 1970, Eslami 1990, Moghaddar *et al* 1992, Oryan *et al* 1994, Mobedi & Dalimi 1994, Dalimi *et al* 2002). Furthermore, human cases are regularly observed in the country and widespread recovery of adult worms has been

reported from dogs, jackals and wolves (Mobedi *et al* 1973, Eslami 1990, Mobedi & Dalimi, 1994, Mehrabani *et al* 1999, Dalimi *et al* 2002).

In Iran, sheep are the most common and important intermediate host of *E.granulosus*. The prevalence of infection and cyst fertility rates in sheep are high (Eslami 1990, Oryan *et al* 1994, Hosseini & Eslami 1998, Mehrabani *et al* 1999, Dalimi *et al* 2002). Camels are found in the majority arid regions and commonly infected with *E.granulosus*, possessing a high cyst fertility rate (Mobedi *et al* 1970, Moghaddar *et al* 1992, Hosseini & Eslami 1998, Dalimi *et al* 2002). Numerous studies have provided evidence that *E.granulosus* harboured complex of different strains throughout the world (McManus & Smyth 1986, Bowles & McManus 1993a). These can effect on a wide variety of criteria that impact on the epidemiology, pathology, control and prevention of cystic hydatid disease (Thompson & Lymbery 1988). Furthermore, there is evidence to suggest that some strains are more infective for human than others (Eckert &Thompson 1997). Molecular approaches especially polymerase chain reaction (PCR)-based technique, have been used extensively to characterize strain grouping within *E.granulosus*. To date, in this regard nine distinct genotypes (G1-G9) have been identified (Bowles *et al* 1992, Bowles & McManus 1993b, Bowles & McManus 1993c, Bowles *et al* 1994, Scott *et al* 1997). Strain characterization of the *E.granulosus* in Iran was previously done using mitochonderial DNA markers and rDNA-ITS1 region as a genetic marker (Zhang *et al* 1998a, Fasihi 2000).

The objective of the present work was to determine the molecular characterization of *E.granulosus* isolated from sheep and camels of different regions of Iran.

Materials and Methods

Parasite. Forty-eight isolates of hydatid cysts of *E.granulosus*, including 25 isolates from sheep slaughtered at Tehran, Zanjan, Esfahan, Sari and Ahwaz and 23 isolates from camels slaughtered at Tehran, Esfahan and Yazd were collected. Protoscolices were aspirated from cysts and fixed in 75%(v/v) ethanol. The protoscolices were

rinsed three times with sterile distilled water to remove the ethanol prior to DNA extraction.

PCR. Total genomic DNA was prepared from individual isolates using standard extraction procedure (Sambrook *et al* 1989). The PCR amplification was performed using primer pair BD1 and 4S described by Bowles and McManus (1993c) in the rDNA-ITS1 region of the parasite. The Genius (Techne®, UK) thermal cycler was applied for PCR amplification. PCR amplification was performed in 30µl volumes containing sample DNA (20-100ng), 20mM Tris-HCl (pH8.4), 50mM KCl, 3mM $MgCl_2$, 140µM of each dNTP (Gibco BRL), 20pmol of each primers BD1 and 4S (Primm®, Italy) and 1 unit of Taq DNA polymerase (Gibco BRL). PCR conditions were as follows: 33 cycles, 95ºC for 30s (denaturation), 55ºC for 30s (annealing), and 72ºC for 30s (extension). PCR products were electrophoresed at 90V for 30min through 1.5%(w/v) Tris-borate/EDTA (TBE) agarose gels and stained with ethidium bromide.

RFLP. PCR products were digested for 6-8h with the 4-base cutting restriction endonucleases *Alu1, Hpa11, Rsa1* and *Taq1* using buffers recommended by the manufacturer (Boehriger Mannheim). Generally, 3-5µl of PCR product was used, the total volume was increased to 20µl for digestion with especial buffer and this reaction volume was digested with 5 units of the mentioned restriction enzymes. PCR-RFLP products (restriction fragments) were separated by vertical electrophoresis at 100V for 180min through 5% TBE polyacrylamid gel, stained with nitrate silver and photographed.

Results

Two ITS1 fragments are amplified from DNA of the *E.granulosus* of sheep (1Kb and 0.9Kb) and camel (1.1Kb and 1Kb) strains are shown in figure 1. PCR products of all isolates showed similar patterns.

A comparison of PCR-RFLP patterns of sheep and camel isolates with the *Alu1, Hpa11, Rsa1* and *Taq1* is shown in figure 2. Although all isolates of sheep or camel produced identical patterns with the individual enzymes but the sheep isolates were

shown clearly different from the camel isolates. PCR products of the sheep isolates digested by *Alu1, HpaII, Taq1* and *Rsa1* produced 4 (aprox. 700, 250, 150, 85 bp), 5 (aprox. 880, 850, 325, 225, 85 bp), 3 (aprox. 825, 235, 192 bp) and 5 (aprox. 615, 300, 290, 155, 118 bp) bands respectively. Meanwhile for the camel isolates *Alu1, HpaII, Taq1* and *Rsa1* produced 5 (aprox. 700,400, 230, 150,72 bp), 5 (aprox. 885, 590, 300, 200, 85 bp), 4 (aprox. 825, 235, 192, 62 bp) and 8 (aprox. 615, 322, 295, 234, 175, 118, 80, 62 bp) bands respectively.

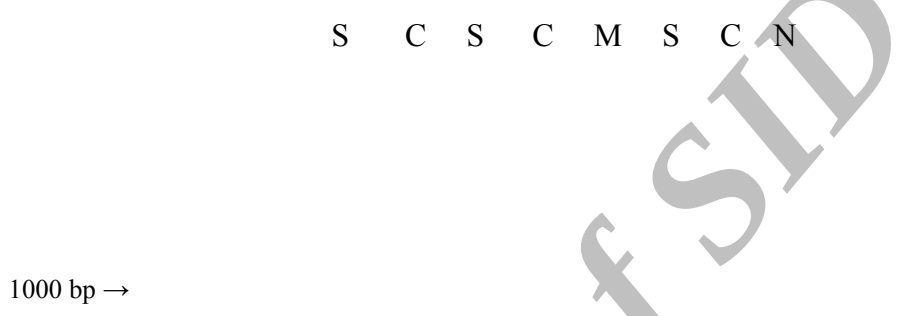

Figure 1. *PCR-amplified ITS1 fragments from sheep (G1 genotype) and camel (G6 genotype) DNA isolates of Echinococcus granulosus. Lane S, sheep origin; lane C, camel origin; lane N, negative control; lane M, DNA molecular weight marker (X-174/Hae III:1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp, CinnaGen Inc.)*

Discussion

Cystic hydatid disease is one of the most important zoonoses in Iran. The extent of the disease prevalence has been highlighted by retrospective surveys of some workers in Iran (Mobedi *et al* 1970, Mobedi *et al* 1973, Eslami 1990, Moghaddar *et al* 1992, Oryan *et al* 1994, Mobedi & Dalimi 1994, Mehrabani *et al* 1999, Hosseini & Eslami 1998, Dalimi *et al* 2002). Some investigators in some parts of the world have carried out molecular studies of the parasite. The mitochondrial and nuclear DNA analysis have previously been used successfully in molecular surveys on *E.granulosus* (Hope *et al* 1992, Bowles *et al* 1992, Bowles & McManus 1993b, Bowles & McManus 1993c, Washira *et al* 1993, Bowles *et al* 1994, Scott *et al* 1997, Zhang *et al* 1998b). Bowles *et al* (1992) sequenced the CO1 gene of 56 isolates and seven variant

sequences (G1-G7) were found for *E.granulosus*. Bowles and McManus (1993b) have used partial sequences of mitochondrial NADH dehydrogenase 1 (ND1) gene for 59 isolates and found the same groupings of variant for the CO1 gene with slightly less resolution.

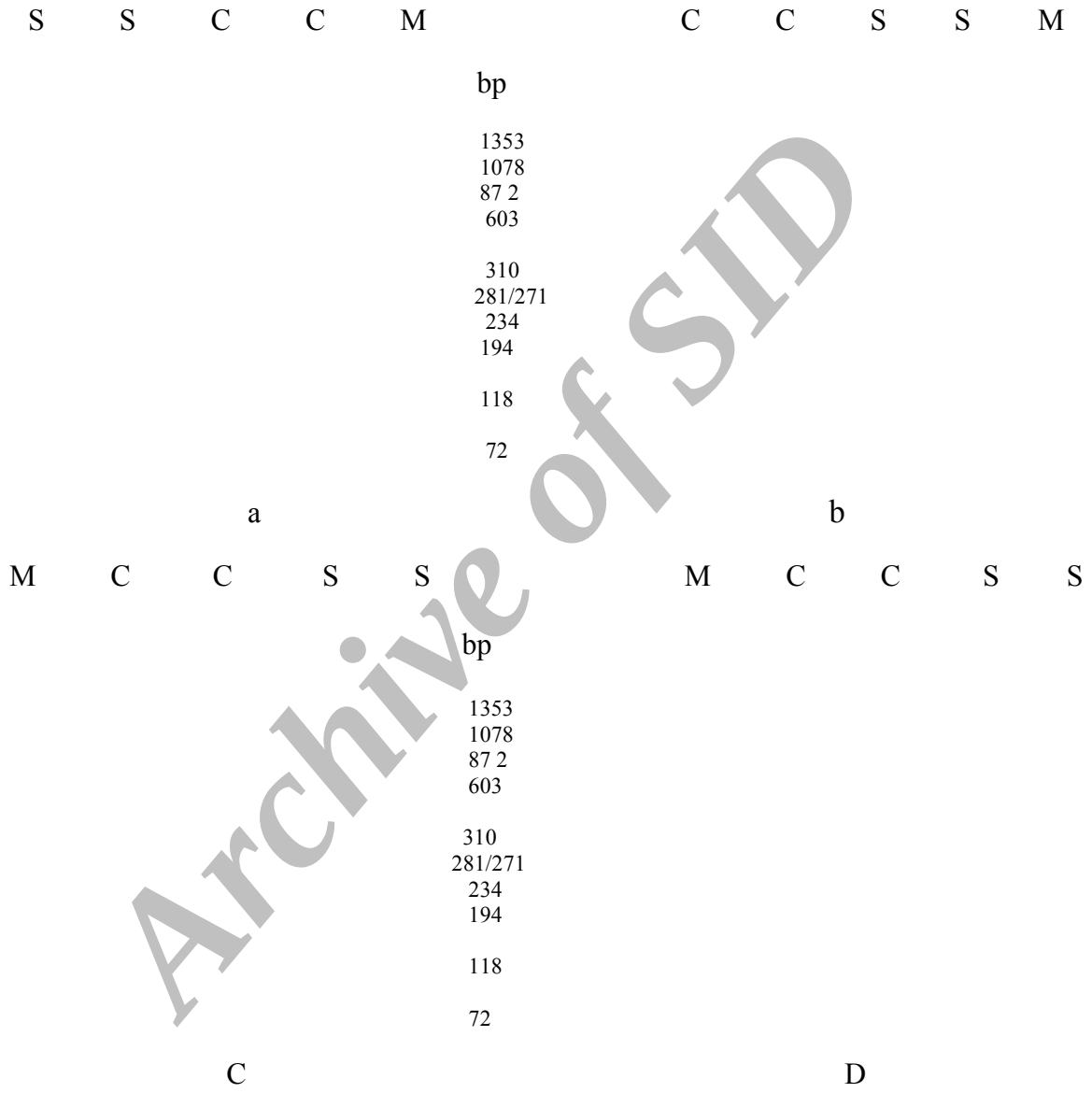

Figure 2. *Comparison of ITS1-PCR-RFLP patterns for Echinococcus granulosus, sheep (G1 genotype) and camel (G6 genotype) isolates using restriction endonucleases Alu1 (panel A), HpaII (panel B), Rsa1 (panel C) and Taq1 (panel D). Lane S, sheep strain; lane C, camel strain; lane M, DNA molecular weight marker (X-174/Hae III: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp, CinnaGen Inc)*

Furthermore, Bowles and McManus (1993c) examined 149 isolates from different intermediate hosts, including sheep and camels using PCR-RFLP methods on rDNA-ITS1. They reported different strains of the parasite isolated from sheep, camel,

horse, cattle and pig. Although of diverse host and geographical origin, all "sheep strain" isolates examined gave very similar patterns after restriction enzyme digestion. In another study conducted by Bowles *et al* (1995), three nucleotide data sets (CO1, ND1 and ITS1) have been investigated in order to resolve relationships among species and strains of the genus *Echinococcus*. They provided evidence that *E.granulosus* is not a monophyletic taxon and strains within this species fall into groups which might merit recognition as separate species. Bowles *et al* 1994, examined four isolates (designated G8) of the cervid strain or "northern form" of *E.granulosus* using PCR-RFLP analysis of the nuclear ITS1 region. Scott *et al* (1997) have used mitochondrial (ND1) sequences and ITS1-PCR-RFLP patterns to characterize human and pig isolates of *E.granulosus* in Poland. *E.granulosus* DNA from the Polish patients amplified a single ITS1 fragment in PCR and distinct ITS1-RFLP patterns were obtained after restriction digestion. They concluded that, the form of hydatid isolated from the polish patients represented a distinct or new genotype group (designated G9) of *E.granulosus*. According to the survey by Zahang *et al* (1998), DNA variation within regions of the mitochondrial CO1 and ND1 genes of 28 isolates of *E.granulosus* had confirmed the transmission of 2 strains in Xijiang, namely the common sheep/dog (G1 genotype) and the camel/dog (G6 genotype) forms. Molecular methods also used to confirm the presence and reveal the host preferences of sheep (G1genotype) and camel (G6 genotype) strains in Kenya (Washira *et al* 1993) and to show a single strain (G1) of *E.granulosus* cycles in domestic and sylvatic hosts on mainland Australia (Hope *et al* 1992).

In the preliminary study of Zahang *et al* (1998), DNA nucleotide and predicted amino acid sequence, obtained for CO1 and ND1 genes of 16 isolates of *E.granulosus* from Iran, were categorized into two distinct and uniform genotype groupings, namely sheep and camel strains. In study of Fasihi (2000) molecular characterization of 151 isolates (38 human and 113 animal isolates) was carried out using PCR-RFLP technique. In the study most of the sheep, cattle, goat and human isolates showed sheep strain identity and most of the camel isolates characterized as camel strain. In our study by using PCR-based RFLP approach targeting the internal

transcribed spacer 1 (ITS1) of the rDNA, the similar results were obtained. 25 isolates from sheep and 23 isolates from camels produce 2 different (large and small) ITS1 PCR products. The result is identical with Bowles & McManus (1993c) as well as Bowles *et al* (1995) surveys. By using restriction endonucleases enzymes, two different PCR-RFLP profiles of *E.granulosus* DNA of the common sheep and camel isolates were produced. The complexity of the RFLP patterns obtained with the sheep and camel isolates suggests that a number of distinct ITS1 types are present. Consequently, the forms of *E.granulosus* that occur in sheep and camels in Iran are different. It is, therefore important that the epidemiological significance of these strains, especially the one derived from camels should be considered. In areas where camels occur together with other livestock animals, humans are likely to be exposed to the camel strain as well as the sheep strain through contact with carnivorous definitive hosts, especially dogs. Both the sheep and camel strains were present in Turkana and their life cycle patterns overlap in intermediate as well as definitive hosts (Washira *et al*1993). It is puzzling that there is longstanding epidemiological evidence from several areas in the Middle East, which has suggested that camels are an important reservoir for human infection (Eckert & Thompson 1997, Eckert *et al* 1989).

In *E.granulosus*-endemic areas of Iran it is evident that, the majority of *E.granulosus*-infected livestock animals can potentially act as reservoirs of human infection. This has important implication for hydatid control and public health.

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