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Genetic Variability of Antigen B8/1 among *Echinococcus granulosus* Isolates from Human, Cattle, and Sheep in Fars Province, Southern Iran

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Abstract

Background: Cystic echinococcosis (CE), known as hydatid cyst, is a zoonotic parasitic infection caused by the larval stage of *Echinococcus granulosus* (*E. granulosus).* Antigen B, the major component of hydatid cyst fluid, is encoded by members of a multigene family. The present study aimed to evaluate the genetic diversity of the gene encoding antigen B8/1 (*EgAgB8/1*) among the main intermediate hosts of *E. granulosus.*

Methods: Twenty-eight hydatid cyst isolates (10 sheep, 9 human, and 9 cattle) were collected in Fars province, Iran. DNA was extracted from each cyst and PCR, followed by DNA sequencing was used to identify potential *EgAgB8/1* sequence variation and polymorphism. A phylogenetic tree was constructed using MEGA 7.0 software and the maximum likelihood method.

Results: Using *EgAgB8/1* primers, an approximately 315 bp band was amplified from all the isolates. The PCR products were sequenced, and the sequences were deposited in GenBank (accession numbers, KY709266-KY709293). The polymorphism variation among the isolates was 0.0, while intra-species variation within the isolates and related sequences in GenBank was 0.5-1%. Analysis of the phylogenetic tree revealed that the isolates from humans, sheep, and cattle all cluster in one group and are homologous to the *EgAgB8/1* M1 allele.

Conclusions: Findings of this study revealed close similarity between the *EgAgB8/1* of human, sheep, and cattle *E. granulosus* isolates. However, differences were found between the *EgAgB8/1* sequences in our study and those reported from other CE endemic areas. Whether such similarities and differences exist in other subunits AgB subunits require further study.

Keywords: Antigen B1, *Echinococcus granulosus*, Fars province, Genetic Variation, Iran.

Introduction

Cystic echinococcosis (CE), or hydatid cyst, an important zoonotic parasitic disease in humans and livestock, is caused by the metacestode stage of *Echinococcus granulosus* (*E. granulosus*) (1, 2). Dogs and carnivores are the main definitive hosts while sheep, cattle, and humans are intermediate hosts. Humans become infected by ingesting the tapeworm eggs in definitive host feces (3-6). In the intermediate host the larva forms CE, a unilocular cyst filled with hydatid fluid (7).

Hydatid cyst fluid (HCF) is a mixture of host and parasite antigens. One of the most abundant antigens of HCF, with a diagnostic value, is antigen B (AgB). Antigen B is the most important antigenic source for the immunodiagnosis of human CE (8-10). The role of AgB in hydatid disease is not completely known, but it is involved in an evasion mechanism that allows parasite establishment in the intermediate host. It also is an immune modulator able to skew Th1/Th2 cytokine ratios toward a non-protective Th2 immune response (11, 12).

Antigen B is a 230 kDa lipoprotein that contains both neutral and polar lipids, including

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fatty acids and sterols (13). In electrophoresis gels it dissociates into subunits of 8, 16, and 24 kDa (14).

Because AgB is highly immunogenic, it is a suitable candidate for serological diagnosis of CE. The 8 kDa subunit is considered as the most appropriate antigen for the diagnosis of CE and has been extensively used in native, recombinant, and synthetic forms for that purpose (15, 16).

Antigen B is encoded by a variably-expressed multigene family, with at least five major gene clusters named *EgAgB1-5*. These genes express mature 8 kDa proteins named AgB8/1-AgB8/5 (17). Given that the immunogenicity of the AgB subunits is due to their protein moieties, variation in AgB expression resulting from sequence differences between the subfamilies, might have diagnostic importance. It has been found that the putative protein isoforms encoded by *AgB8/1-AgB8/5* vary by 44–81% in their amino acid sequences (17).

A recent study showed that AgB8/1 is the major protein component of AgB in the *E. granulosus* G7 genotype (*E. canadensis*), followed by AgB8/4 and AgB8/3. AgB8/5 was the least abundant (0.3%) subunit in this genotype (18). It was also found that AgB2 is not present in HCF of *Echinococcus* G7 isolates, while all five subunits were detectable in bovine fertile HCF (18).

Considering the fact that polymorphisms in AgB subunits may affect their diagnostic performance, the current study aimed to determine the extent of *EgAgB8/1* genetic variability in *E. granulosus* isolates collected from three different intermediate hosts by PCR and DNA sequence analysis.

Materials and Methods

Sample collecting

Twenty-eight hydatid cyst isolates (10 sheep, 9 human, and 9 cattle) were collected. Animal cysts were collected from a slaughterhouse in Fars province in southern Iran, while the human hydatid cysts were collected from patients in university-affiliated hospitals in Shiraz, the capital of Fars province. Germinal layers of the human or cattle hydatid cysts and protoscolices of sheep cysts were isolated and stored in 70% ethanol at -20 ˚C until use.

DNA extraction and PCR amplification

Total genomic DNA from either protoscolices or germinal layers of sheep and human samples were extracted using a manual procedure. The protoscolices (50 µl) were rinsed several times with distilled water to remove the ethanol. After five freeze-thaw cycles with 300 µl of lysis buffer (50 ml of Tris-HCl (100 Mm), pH = 8; 1 mM of EDTA, pH= 8; 1% Tween 20), 15 µl of proteinase K was added to each sample and incubated overnight at 37 ºC, followed by phenol/chloroform/ethanol/acetate extraction. Absolute ethanol was used to precipitate the DNA. Precipitated DNA was re-suspended in 100 µl of nuclease-free double-distilled water and stored at -20 ºC until use. For DNA extraction from germinal layers of human hydatid cysts, the tissues were homogenized before extracting the DNA as explained for the protoscolices.

For the cattle hydatid cysts, total genomic DNA was extracted using a commercial tissue DNA extraction kit (Genomic DNA Extraction, Yekta Tajhiz, Iran) according to the manufacturer's instructions.

Two PCR primer pairs were used to amplify a 280 bp fragment of *E. granulosus* mitochondrial 12S rRNA using G1 Forward: 5'– GCTTTTGTGTGGATTATGCG-3' and G1 Reverse: 5'– TCAAACCAGACATACACCAA-3' primers (19).

The thermocycler was programmed for one cycle of initial denaturation at 94 ºC for 4 min, followed by 35 cycles of denaturation at 94 ºC for 45 sec, annealing at 57 ºC for 30 sec, extension at 72 ºC for 35 sec, and a final extension at 72 ºC for 4 min.

Amplification of *EgAgB8/1* was performed as originally described by Zhang et al. (17), using each of two primers, *EgAgB8/1* F 5′ TCTCGCTCTGGCTCTCGTC 3′ and *EgAgB8/1* R5′ CTTCAGCAATCAACCCTCTGA 3′.

The PCR was performed in a final volume of 25 µl, containing 1 µl of DNA template, 0.5 µl of each primer (20 pmol), 12.5 µl of master mix (Ampliqon, Denmark), and 10.5 µl of double-distilled water. The Mastercycle Gradient thermocycler (Eppendorf, Germany) was programmed by an initial denaturing step (at 94 ºC for 4 min), followed by 35 cycles of denaturation at 94 ºC for 45 sec, annealing at 62 ºC for 30 sec,

extension at 72 ºC for 40 sec, and a final extension at 72 ºC for 4 min. The PCR product was electrophoresed in a 1.5% agarose gel and stained with Gel-red (*Biotium*, United States, California, Fremont).

The amplicons were excised from the agarose gel, purified with a DNA Gel extraction kit (Vivantis DNA Recovery Kit, Malaysia), and sequenced.

The sequences were edited using Geneious software (www.geneious.com) and the consensus sequences were compared with each other and reference sequences available in GenBank. The phylogenetic tree was constructed and pairwise comparisons were made of the level of sequence differences within species using MEGA 7.0 software and by the maximum likelihood method. Bootstrap analyses (using 1,000 replicates) were performed to determine the robustness of the finding.

Results

The PCR of 12S rRNA in all 28 samples amplified a 280 bp fragment representing the G1–G3 genotype of *E. granulosus* (sensu stricto).

PCR of *EgAgB8/1* in all the isolates amplified an approximately 315 bp fragment corresponding to *E. granulosus EgAgB8/1* (Fig. 1). PCR products were purified from the gel and sequenced.

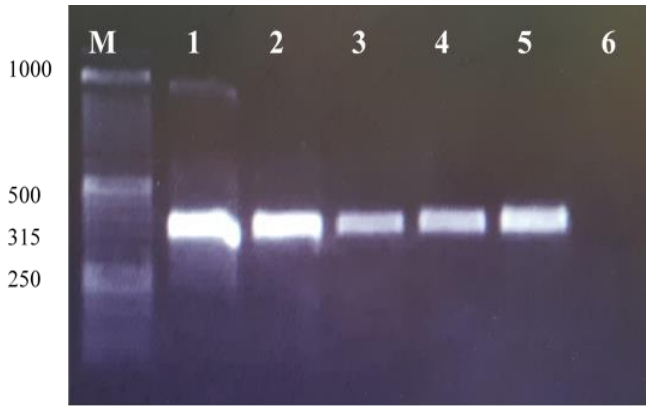

Fig. 1. PCR-amplified *EgAgB8/1* fragments from *E. granulosus* isolates. (M) 50 bp DNA ladder, (1) cattle isolate 1and (2) cattle isolate 2, (3) human isolate, (4) sheep isolate, (5) positive control, DNA extracted from human isolate of hydatid cyst, (6) negative control.

The *EgAgB8/1* sequences obtained in this study were deposited in the GenBank database with accession numbers KY709266-KY709293.

The phylogenetic tree analysis revealed that the isolates from human, sheep, and cattle all clustered in one group and are homologous to the *EgAgB8/1* M1 allele described earlier (AY773091, DQ137827, DQ137833, DQ137834, AF143813 and FJ810076) (Fig. 2). Nucleotide sequences from all the isolates in this study were identical.

The polymorphism variation between the isolates was 0.0, while intra-species variation between our isolates and related *EgAgB* in GenBank was estimated to be 0.5-1%.

Pairwise comparisons of the sequences found no differences in nucleotide sequences between isolates collected in this study and the *E. granulosus* isolates collected from bubaline, sheep, and humans in India, Argentina, and Brazil (AF143813, AY773091, DQ137827, DQ137833, DQ137834, and FJ810076). However, differences of 0.5-1% were found between the *EgAgB8/1* isolates in this study and some isolates from Argentina and Brazil (AY871011, AY871013, AY871017, AY871018, AY871019, AY773092, DQ137829, DQ137828, DQ137835, and DQ1378290).

Discussion

The aim of this study was to identify potential *EgAgB8/1* variations in isolates from humans, sheep, and cattle, the three main host species in Iran, where CE is a major health problem. Antigen B is the most-evaluated antigen of *E. granulosus* and is used to diagnose human CE. Of the different EgAgB subunits, EgAgB8/1 displays the highest diagnostic sensitivity and specificity (15, 16, 20-23).

Jiang et al. (2012) analyzed the reactivity of the five AgB subunits in serodiagnosis of CE and found the highest diagnostic sensitivity for EgAgB8/1 (83.06%), followed by the EgAgB4 (75.81%) and EgAgB2 (62.90%) subunits (24).

Mohammadzadeh et al. (2012) evaluated the diagnostic accuracy of antigen B, prepared from the isolates of hydatid cysts from Iran and China, along with a recombinant antigen AgB8/1 (RAgB), using serum samples from Iran, Turkey, China, and Japan. It was found that RAgB has higher specificity than native antigens (20).

Recent studies demonstrated that the *E. granulosus* AgB has a high degree of genetic variability (25, 26). Sequence variations in the

genes encoding the five AgB subunits in different *E. granulosus* isolates may affect the diagnostic performance of the related subunit proteins. Thus, the knowledge of similarities in AgB genes could help in the design and application of AgB-based diagnostic assays.

Molecular evaluation of *E. granulosus* isolates has shown differences in *EgAgB* nucleotide sequences resulting in amino acid sequence variations (27). Considering the satisfactory diagnostic performance of the EgAgB8/1 subunit, we tried to identify potential polymorphisms in the gene encoding this antigen in different *E. granulosus* isolates. We found all the *EgAgB8/1* sequences from human, sheep, and cattle isolates to be identical in our study.

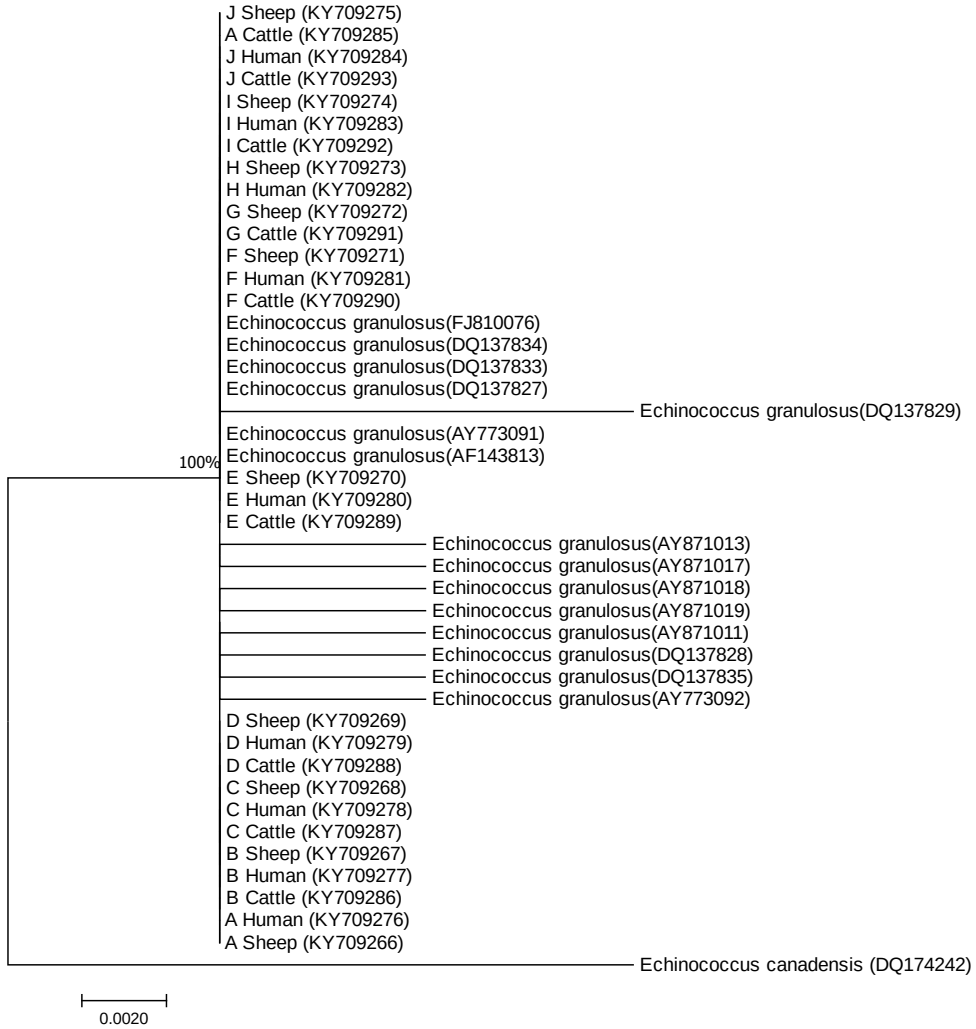

Fig. 2. Phylogenetic relationship of *EgAgB8/1* sequences of *E. granulosus* isolates from Iran and reference sequences available in GenBank, using the maximum likelihood method with *E. canadensis* (AN: DQ174242) as the out-group.

Very close similarities, with some minor differences, between human and sheep isolates of other antigen B subunits, including AgB2, have been reported in other studies (28, 29).

Sismek et al. evaluated the *E. granulosus EgAgB8/1* polymorphism from sheep, cattle, and human isolates from Turkey and showed that cattle isolates have two nucleotide substitutions at nucleotides 7 and 8 (GA to AG) and a deletion at nucleotide 12 (A), while in the human isolate a T to C substitution was identified at nucleotide 44. No polymorphisms were identified in the sheep isolates in their study (30).

Pan et al. analyzed *E. granulosus EgAgB8/1* by PCR-single strand conformation polymorphism (SSCP). The phylogenetic tree showed that all the cattle and buffalo isolates clustered in one group while the sheep and goat isolates clustered in

another (31). Muzulin et al. reported 19 *EgAgB8/1* polymorphic sites (32). In a study by Tawfeek et al. of *E. granulosus* AgB2 isolates from Egypt, the highest similarity occurred between sheep and human isolates, while the lowest similarity occurred between human and pig isolates (28).

To further understand the differences in amino acid sequences of the AgB subunits, it has been shown that *E. granulosus* strains differ in the type and abundance of some of the AgB subunits (33). While all the AgB subunits are detectable in HCF from cattle, AgB8/2 is not available in *E. canadensis* (G6 and G7 genotypes). It was concluded that AgB2 might be a pseudogene in *E. canadensis* (18).

Kamenetzky et al. showed that the expression of different *AgB* isoforms is related to the host species and most variation occurred in the N-terminal region of *AgB*. Therefore it would be useful to understand the performance of each AgB subunits in the immunodiagnosis of human CE (25).

We found no differences in the *EgAgB8/1* sequences within and between the *E. granulosus* isolates in Fars province, south Iran. This may imply that *EgAgB8/1* of any sheep, cattle, or human intermediate host may have a similar performance in the diagnosis of CE. However, differences were found between the *EgAgB8/1* sequences in the current study and those reported from other CEendemic areas. Whether such similarities are present in other AgB subunits in the area necessitates further studies.

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