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Original Article

Genetic Variability of Antigen B2 of Human, Sheep, Goats, Camel and Cattle Isolates of *Echinococcus granulosus* in Iran

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Abstract

Background: Antigen B (AgB) is frequently used for immuno-diagnosis of human cystic echinococcosis (CE). *Echinococcus granulosus* AgBs show a high degree of genetic variability in different hosts or in different CE endemic areas. The present study aimed to evaluate the genetic polymorphisms of encoding antigen B2 gene (AgB2) among different Iranian isolates of *E. granulosus*.

Methods: A total of 50 CE isolates were collected from human, sheep, cattle, goat and camels, 10 isolates from each intermediate host of *E. granulosus*. Total genomic DNA from either protoscolices or germinal layer was extracted from each cyst and PCR-RFLP followed by DNA sequencing was used to evaluate sequence variation and polymorphism of AgB2 in the isolates.

Results: After the PCR amplification, using AgB2 primers, an almost 400 bp band was amplified in all of the isolates. The PCR products were digested with Alu1 restriction endonuclease. After restriction enzyme digestion with Alu1, sheep and human isolates gave a similar pattern of RFLP with the gene size of approximately 140 and 240bp and camel and goat isolates gave a similar pattern, but different from sheep and human, with the gene size of approximately 150 and 250bp. Sequence analysis showed the most genetic similarity of AgB2 between human and sheep isolates.

Conclusion: Findings of this study revealed the differences in the sequences of AgB2 within and between the Iranian isolates of *E. granulosus*. These differences may affect the performance of any diagnostic test which uses AgB.

Introduction

Cystic echinococcosis (CE) is a zoonotic infection of human and domestic animals caused by the larval stages of the cestode *Echinococcus granulosus*. Cases of human CE have been reported from all of the provinces in Iran. This helminthic zoonosis is one of the most important diseases for human and livestock in the country (1-3).

Larvae of *E. granulosus* produce an antigen, known as antigen B, which has extensively been used for immuno-diagnosis of CE (4). The biological function of this antigen is not yet fully understood although it has been suggested that AgB of *E. granulosus* plays a key role in the interaction between parasite and host. It also behaves as an immune modulator that skews Th1/Th2 cytokine ratios to Th2 polarized responses, mechanism that may benefit the parasite's survival within the host (4-5). AgB is a polymeric lipoprotein with a molecular mass of 160 kDa. It comprises polymers of 8-kDa subunits and under reduced condition on SDS-PAGE, the complex are separated to their subunits of 8 or 12, 16, and 24 kDa (4). Antigen B is one of the most abundant parasite antigens in hydatid cyst fluid and is frequently used in immunodiagnosis of human CE (6-10). It is well known that the nature and quality of AgBs are variable among the host species and this may be one of the reasons why different laboratories obtained different results using AgB prepared from different host species (4, 9). Moreover, different strains of the parasite may produce this antigen with different quality or quantity (11).

AgB of *E. granulosus* is comprised of a gene family comprises at least 10 unique genes of five subfamilies (EgAgB1 to EgAgB5) (12). It has been shown that EgAgB3 is expressed in all stages of *E. granulosus* while the rest of AgB genes may be expressed in different stages (12). Furthermore, recent studies demonstrated a similarly high quantity of AgB8/1 subunit in human and bovine isolates and various amount of AgB8/3 subunit between bovine and human cysts (11).

So far, no study has been performed about the genetic variation of AgB coding genes in Iranian isolates of *E. granulosus*. Hence, the aim of the current study was to determine the extent of AgB variation in *E. granulosus* isolates collected from different intermediate hosts, by PCR-RFLP and DNA sequence analysis.

Materials and Methods

A total of 50 isolates were collected from human, sheep, cattle, goat and camels, 10 isolates from each intermediate host of *E. granulosus*. The human isolates were from patients who underwent liver or lung surgery due to hydatid cyst and their *E. graunlosus* infection were pathologically confirmed after the surgery. The animal isolates were collected from Shiraz or Yasuj (South and Southwest of Iran) slaughterhouses. Hydatid cyst fluid from each cyst was aseptically collected and the protoscolices were rinsed several times with saline and stored in 70% ethanol at -20 ºC until use. Those cysts which had no protoscolices (mainly cattle cysts), germinal layer were removed from the cysts for extracting the DNA.

Total genomic DNA from either protoscolices or germinal layer was extracted using a manual procedure. Briefly, the protoscolices (30 µl) were rinsed several times with distilled water to remove the ethanol. After three cycles of freezing and thawing, 15 µl of proteinase K was added to each sample and incubated overnight at 37ºC. Then, 300 µl of phenol:chloroform:isoamyl alcohol was added and centrifuged at 4000 g for 10 minutes. Aqueous top phase was removed and absolute alcohol was added to precipitate the DNA. For extracting the DNA from germinal layer, the tissues were homogenized properly before extracting the DNA as explained for the protoscolices. Amplification of AgB2 gene was performed as originally described by Fernan-

dez et al. (1996), using each of two primers, F5' GGATCCTTCGTGGCCGTCGTTCAAGC3' and R5' TCGACAAATCATGTGTCCCGACGCA3' (13). PCR reaction was performed using mixtures of 0.3 µl of Taq polymerase with 2.5 µl of 10X PCR buffer, 17.5 µl of deionized distilled water, 0.6 µl of $MgCl_2$, 0.7 µl (10 mM) of dNTP, 25 pmol of each primer and 2 µl of DNA template, in a total volume of 25 µl.

PCR was carried out by one cycle of initial denaturation at 95ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing temperature at 55ºC for 1 min, extension temperature at 72ºC for 1.5 min, and final extension at 72ºC for 10 min. PCR products were separated by electrophoresis in 1.5% agarose gel and stained with ethidium bromide.

RFLP

RFLP analysis was performed by digesting 8.5 µl (150 ng/ µl) of PCR products, using 1.5 µl (10 U) of Alu1 (Fermentas, Lithuania) in a total volume of 50 µl, for 8 hours at 37ºC. Restriction fragments were separated on 2% agarose gel in TBE (Tris Base/Boric Acid/EDTA) buffer.

Sequencing

PCR products of two isolates from each group were excised from 1.5% agarose gel and purified with a DNA Gel Extraction Kit (Bioneer's AccuPrep Gel Purification Kit), according to the manufacturer's instructions. Purified PCR products were sequenced, using the same primers as described for the amplification process. Sequence chromatograms were analyzed with the Chromas program (Applied Biosystems). BLAST analysis was used to compare the sequences with the sequences of Gene Bank and finally Clustal W algorithm was used to align sequences of the isolates and to find out any possible differences in the sequences of AgB2 from each isolates.

Results

After PCR amplification, in all of the isolates (human, cattle, goat, sheep and camel) an approximately 400 bp PCR product representing a fragment of the AgB2 gene was amplified (Fig.1).

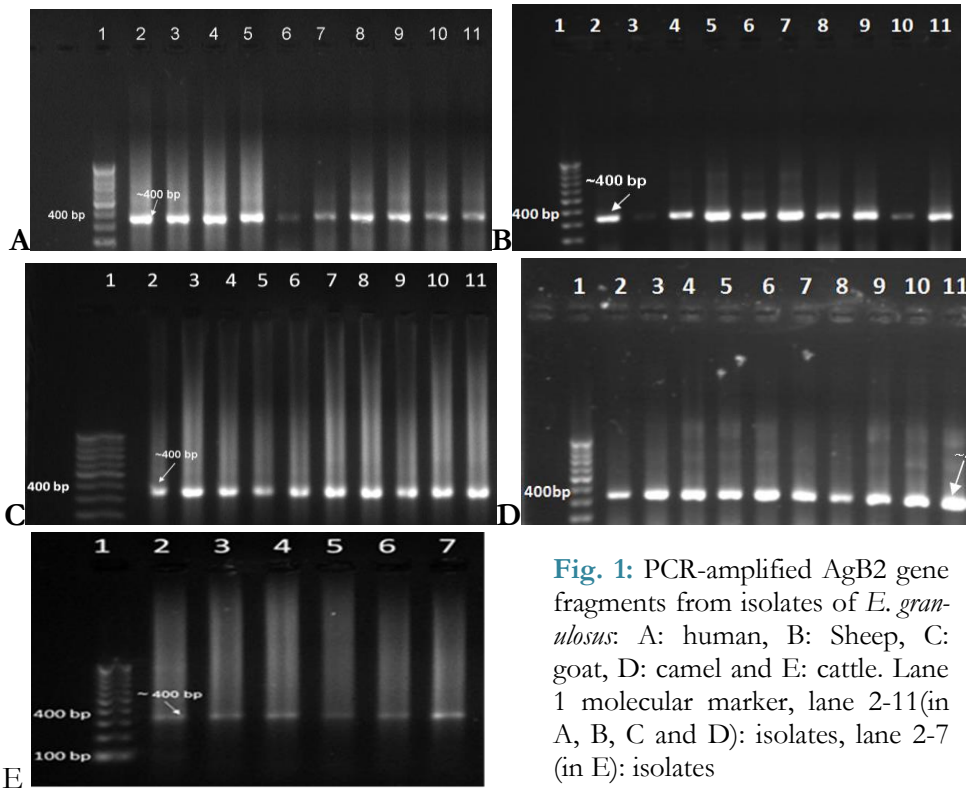

Fig. 1: PCR-amplified AgB2 gene fragments from isolates of *E. granulosus*: A: human, B: Sheep, C: goat, D: camel and E: cattle. Lane 1 molecular marker, lane 2-11(in A, B, C and D): isolates, lane 2-7 (in E): isolates

The 400 bp amplified fragment was digested by Alu1 restriction enzymes and produced a distinct pattern of digestion in the isolates. Isolates from human and sheep had a similar pattern with a 140 and 240 bp bands. The digestion patterns in camel and goats were different from those observed by human and sheep. These isolates produced a 150 and a 250 bp bands. All isolates within each group produced a similar pattern of RFLP-PCR. For cattle isolates, since the PCR products of this isolate was not satisfactory, therefore no detectable bands were seen in this isolate when digested with Alu1. The RFLP-PCR patterns of isolates are depicted in Fig. 2. Results of PCR-RFLP with enzyme AluI revealed that human isolates have the highest similarity patterns with sheep isolates.

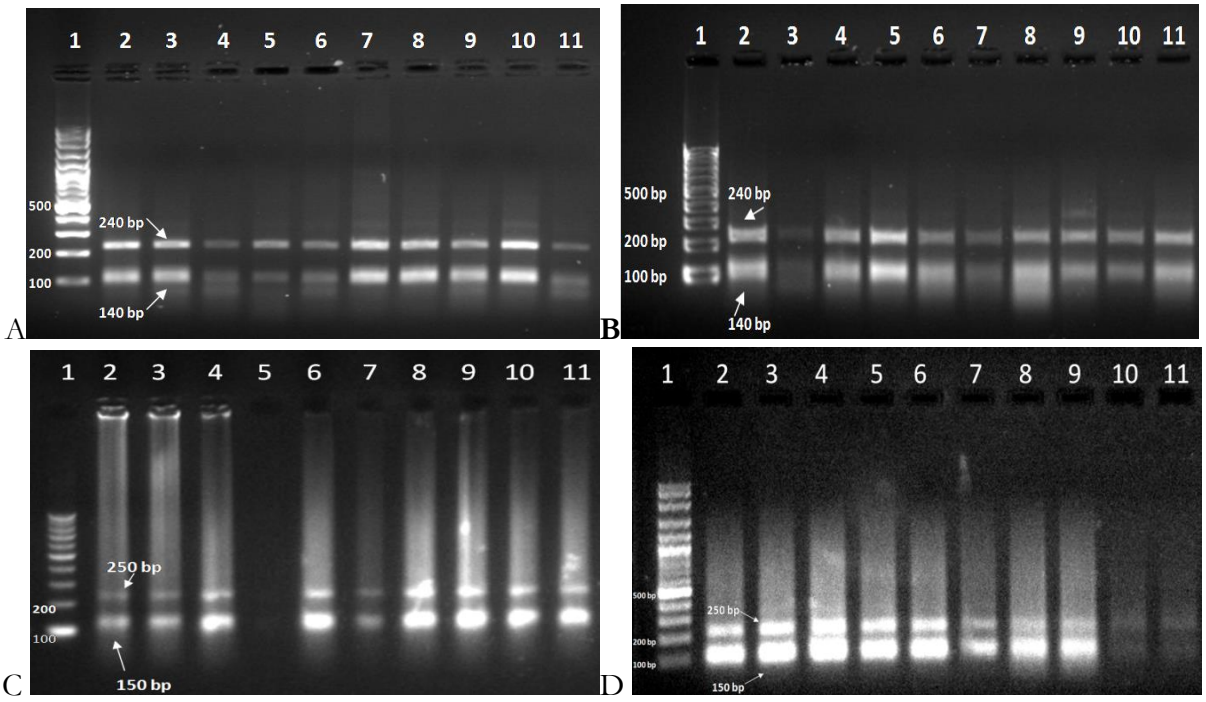

Fig. 2: PCR-RFLP patterns of AgB2 genes of *E. granulosus* isolates, using restriction endonuclease enzymes AluI. A: human, B: sheep, C: goat and D: camel. Lane 1: molecular marker, lane 2-11: isolates

DNA sequence analysis of AgB2

The sequence analysis demonstrated that human isolates have 91% identity to sheep strain. These rates of identities were 85 and 77 for camel and goat respectively. Considering the alignments of AgB2 sequences, nucleotide substitution or deletion in few positions were detected in human isolates in comparison with sheep, the most relevant isolates (Fig. 3).

Discussion

Antigen B is a lipoprotein secreted by the *E. granulosus* metacestode and is involved in key host-parasite interactions during infection (4). The antigen is comprised of a group of subunit monomers of approximately 8 kDa in molecular size (11).

Antigen B is an important diagnostic antigen of *Echinococcus* which has extensively been used for serodiagnosis of human cystic echinococcosis due to its encouraging performance. In a study conducted by Mohammadzadeh et al., (2012), two types of antigen B prepared from Iran and China, and one recombinant antigen B8/1 (RAgB) along with three native antigens were evaluated by ELISA, using serum samples from Iran, Turkey, China and Japan.

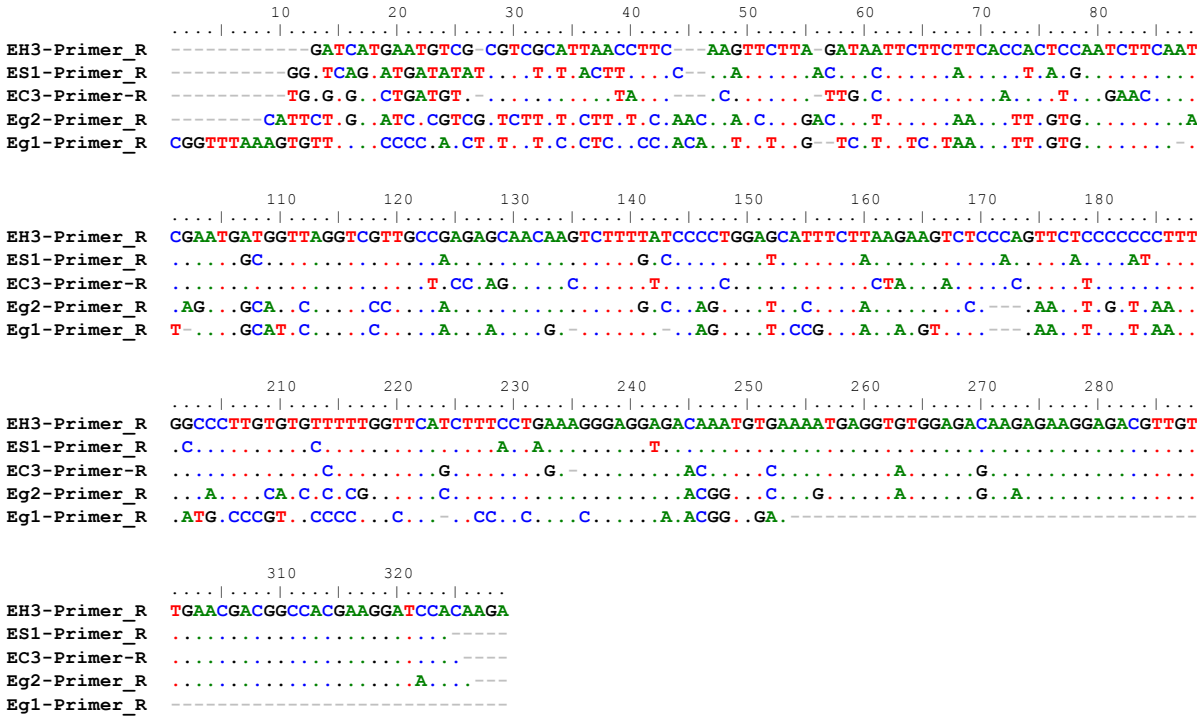

Fig. 3: Alignment of sequences of AgB2 of human, sheep, goat and camel isolates. EH: human, ES: sheep, EC: camel and Eg: goat isolates

Both the Iranian native antigen B and RAgB had high sensitivity, but RAgB showed higher specificity in comparison with native antigens (6). Recent studies have revealed that the *E. granulosus* AgB shows a high degree of genetic variability (4, 14-15).

The present study has defined combined use of PCR-RFLP and DNA sequencing to elucidate polymorphism of AgB2 in human, sheep, cattle, camel and goat isolates in Iran. The high genetic similarity between human and sheep isolates of AgB2 was confirmed in our study. This is consistent, with some minor differences, with the results obtained in other studies which found close similarities between human and sheep isolates of AgB (12, 14).

In Tawfeek et al. study on Egyptian isolates of AgB2, the highest identity was found between human and sheep isolates whilst the lowest identity was observed between human and pig (14). Findings of our study along with those of previously reported studies reconfirm that the human and sheep isolates of *E. granulosus* are the closest relative's isolates amongst the different intermediate hosts of *E. granulosus*.

Moreover, a relatively high similarity (97%) was found between AgB2 which were isolated from different goats.

BLAST similarity search revealed that the human isolate of AgB2 in our study has the highest similarity (88%) with those of sheep isolates reported from other CE-endemic areas (16).

The genetic variability in different host and/or different level of host immune responses against AgB may be relevant factors

in the variability that is observed in the immunogenicity of AgB among different CE patients or CE intermediate hosts (4). It has been shown that antigen B is composed of different subunits within a single cyst, and that subunits have different abundances within and between bovine and human cysts (11).

Polymorphism of AgB genes among different isolates may affect its diagnostic performance. The knowledge of variation in AgB genes could help in proper designing and application of diagnostic tests. It seems that the closest AgB to human isolates may have the best performance in diagnosis of human CE. Support from this idea comes from Rahimi et al. study who reported that the highest sensitivity (97.8%) for diagnosis of CE can be seen with AgB prepared from human cysts followed by AgB isolated from sheep liver and lung cysts, while the lowest sensitivity can be seen with AgB prepared from cattle and camel (7).

Conclusion

Findings of this study revealed the differences in the sequences of AgB2 within and between the Iranian isolates of *E. granulosus*. These differences may affect the performance of any diagnostic test which uses AgB. Whether such differences would be present in other subunits of AgB in the area remains to be explored.

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