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Genetic Variation of Antigen B among *Echinococcus granulosus* Isolates in Tabriz, East Azerbaijan, North West of Iran

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The aim of the present study is to find the polymorphism of the *Antigen B* among Human and animal isolates in Tabriz, East Azerbaijan, North-West of Iran. 10 hepatic and pulmonary human *hydatid cysts* and 40 animal hydatid isolates of liver and lung cysts (20 sheep, 20 cattle) were prepared. All analysed isolates within two clusters (human and sheep) gave similar patterns of PCR-RFLP after digestion with AluI (approximately 120 bp and 270 bp) and no digestion occur after incubation with EcoRI. The results of RFLP pattern showed high degree of genetic likeness between human and sheep isolates. Amplification to the DNA samples extracted from germinal layer of cattle isolates not completed by using Eg AgB2 specific primer suggesting suggesting substantial level of inter-strain variation in AgB2 related genes.

Key words: *Echinococcus granulosus*, *Cystic Hydatid*, PCR, Iran, Tabriz.

Cystic hydatid disease (CHD) is recognized as one of the most important diseases in the world and posses the second rank in importance of helminthes diseases significance[1]. CHD is caused principally by the larval stage of the tapeworm *Echinococcus granulosus sesu lato* (*E. granulosus s. l.*) [1]. *E. granulosus* has a cosmopolitan distribution, Iran is an important endemic focus of CHD and high prevalence of disease has been reported from different hospitals throughout the country[3, 4, 5]. *E. granulosus s. l.* has been divided in to strains (G1-G10), which identified by molecular analysis based on mitochondrial and nuclear genetic markers. These strains differ in biological characters and named according to their most commonly found intermediate host[2]. Only five strains were known to infect humans; sheep (G1), Tasmanian sheep (G2), cattle (G5), camel (G6), and pig (G7) strains[6]. So far, two distinct genotypes, namely, G1 and G6, have been found from different domestic animals and human in Iran[3, 4, 7, 8]. Some *E. granulosus* antigenic molecules have been identified and characterized. Among them, antigen B (AgB) has received much importance because of its use in serological methods to detect CHD[9]. AgB, a polymeric thermo stable lipoprotein of 120-160 KDa, is synthesized in the tegumental cells of the Protoscolices and in the laminated and germinal layer of the brood capsules[10]. This Antigen is encoded by a gene family of at least five gene loci consisting of AgB1[11], AgB2[12] and AgB3 [13] and recently 2 additional gene loci related to AgB2 and AgB3 gene named respectively AgB4 and AgB5

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have been reported[14]. AgB seems to be involved in the parasite evasion of the host immune response due to its ability to inhibit elastase activity and neutrophil recruitment[10]. The comparative evaluation of the diagnostic performance of antigens encoded by these genes showed that the recombinant antigen AgB2 had the best diagnostic performance[15]. In the ELISA, methods (as screening and diagnosing method) used in order to detect specific antibodies of class IgG, sheep cyst fluid earned from local slaughterhouses, dialyzed and filtered then stored until use in ELISA test as antigen[16]. So far, no study has been carried out for the analysis of the genetic variation of AgB coding genes among human and animal isolates in Iran. In the present study, the genetic variability of AgB2 among different *E. granulosus* isolates collected from sheep, cattle and human analysed using PCR-RFLP. The result of this genetic variability is necessary and important for evaluation, application, and standardization of diagnostic tests using AgB.

MATERIALS AND METHODS

10 hepatic and pulmonary human hydatid cysts and 40 animal hydatid isolates of liver and lung cysts (20 sheep, 20 cattle) were prepared. Animal isolates of *cystic echinococcosis* collected from domestic animals at slaughterhouses of Tabriz while human isolates were obtained from patients who underwent surgery procedure. Fertile cyst of E. granulosus was identified based on the presence of protoscolices and brood capsules after microscopic examination. Hydatid cyst fluids aspirated from fertile cysts. The hydatid fluid centrifuged at 3000g for one minute and the protoscolices were deposited. The protoscolices fixed in 70% (v/v) ethanol and stored at -20°C until needed[8]. The deposited protoscolices were rinsed several times with distilled water to remove the ethanol, and then total gDNA was extracted from the protoscolices. Germinal layer from cattle cysts isolated to extract genomic DNA .In human isolates before DNA extraction, rehydration in 100%, 90%, 80%, and 70% ethanol carried out[17] then, the 70% ethanol was removed and total gDNA was extracted from each individual sample using a commercial tissue DNA isolation kit *AccuPrep® Genomic DNA Extraction kit Cat.No:K-3032*. The primers used were described by Fernandez *et al* [12], which are expected to amplify EgAgB2 variants. The PCR reaction was performed in a final 25 ¼l volume containing sample DNA (50 ng), 10 pmol of each of 2 primers, and 12.5 ¼l of 2x super hot PCR master mix *(Emerald, Takara, Japan)* 8.5 ¼l H_2O. The reaction was carried out in an eppendorf thermal cycler gradient 5331 version under the following conditions: 5 min at 94°C (initial denaturation), 35 cycles of 1 min at 94°C, 1 min at 62°C (annealing temperature) and 1 min at 72°C (extension temperature) and finally, 5 min at 72°C (final extension). The size of the PCR products were assessed by electrophoresis in 1.5% (w/v) agarose gels and stained with ethidium bromide. PCR-RFLP bands were defined by their molecular weights estimated from the size standards. The specific band approximately 390 bp was cut from the gel and purified by gel purification kit (Jena Bioscience) according to the manufacturer's instructions. The PCR products were digested with two separate restriction endonucleases; used individually (ALUI and EcoRI) as described previously (18) using the buffers recommended by the manufacturer (Jena Bioscience). To ensure the complete digestion, incubation period at optimal temperature (37°C) last for 1 hr. The digestion reaction was inactivated by heating to 65°C for 20 min. Fragments were separated on 2% agarose gel, stained with ethidium bromide and photographed under UV light.

RESULTS

In the present study, we analyzed, by PCR-RFLP, 50 isolates from the *EgAgB2* encoding genes from three *E. granulosus* strains. Two different *EgAgB2* sequences (approximately 390, 500 bp) were observed in the analysis of 27 hydatid cysts (7 human, 20 sheep), but only one of them, EgAgB2 (Fig. 1, 2), was identical with an already reported sequence (Rott *et al*., 2000**).** Amplification process to the DNA samples extracted from cattle isolates was not completed by using Eg AgB2 specific primer. The PCR products of 390 bp of the AgB2 gene were digested with (AluI & Eco RI) restriction enzymes (Fig. 3). All analysed isolates within two cluster (human and sheep) present very similar patterns of PCR-RFLP after digestion with AluI (approximately 120 bp and 270 bp) and no digestion

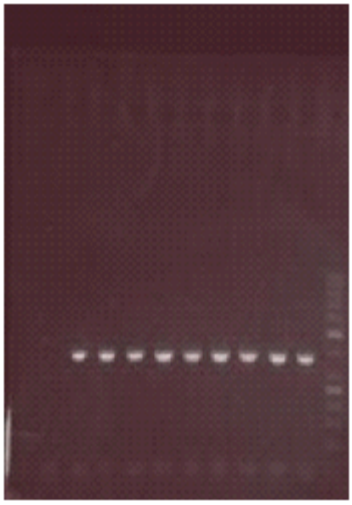

Fig. 1. PCR-amplified Ag B2gene fragments from human (H) isolates of *E. granulosus.* M = marker Arrow points to specific bands

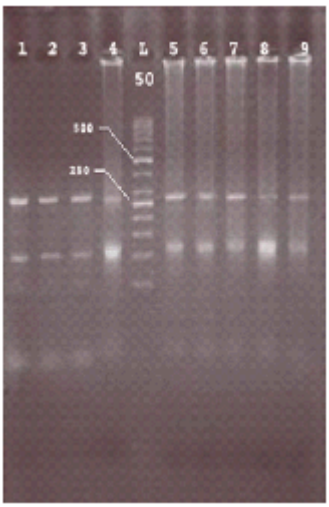

Fig. 2. PCR-RFLP patterns of AgB2 genes of *E. granulosus* isolates using restriction endonucleases enzymes AluI human (H), sheep (S), M = marker

occur after incubation with EcoRI. The results of RFLP pattern showed high degree of genetic similarity between human and sheep isolates. All human isolates presented identical RFLP pattern indicating high genetic similarity among all isolates of human hydatid cysts. In the case of sheep isolates, RFLP pattern of all isolates was it and this finding follow the previous data showing genetic similarity in each strain. DNA samples extracted from germinal layer of cattle isolates did not amplified by using Eg AgB2 specific primer suggesting substantial level of inter-strain variation in AgB2 related genes.

DISCUSSION

The purpose of the current study was to detect and to find the polymorphism of the *Antigen B* among Human and animal isolates in Tabriz, East Azerbaijan, North-West of Iran. Rosenzvit *et al*. (19) mentioned that the contact with the host molecules and cells could cause antigenic variation in AgB proteins, which affect their practical application. Therefore, serologic evaluation of AgB extracted from different E. granulosus hosts in endemic countries must always be considered (20). In 2005, Kamenetzky *et al*. (21) used PCR-SSCP and DNA sequencing to evaluate sequence variation of AgB coding gene in five *E. granulosus* strains. The result of this study showed that AgB2 were present as functional gene exclusively in G1/2 cluster and as nonfunctional gene in G5 and G6/7 cluster and no polymorphism in AgB2 genes was found in each strain but there was a considerable level of inter-strain variation in AgB2 encoding genes and that cysts from the same strain shared more genomic DNA than cysts from different strains. As the previous study, Muzulin *et al*[22] using PCR-SSCP and sequencing, analyzed genomic clones of EgAgB1/B3/B5 gene cluster from five E. granulosus strains. The result of this research showed that all the EgAgB genes were present in G1 and G7 strains except of EgAgB5 that were not detected in G7 strain suggesting inter strain variation of EgAgB. In accordance with other molecular studies, in 2010, Al-Saimary *et al*[23] studied molecular characterization of antigens extracted from germinal layer of human and other intermediate hosts. In all of isolates DNA amplified by related primers but amplification process to the DNA samples extracted from cows and buffaloes hydatid cysts was not completed by using all primers. Another molecular analysis of AgB2 encoding gene performed in Egypt by Tawfeek *et al*.[24]. In this research, genetic polymorphism of antigen B2 gene (AgB2) in *Echinococcus granulosus* studied using PCR-RFLP and DNA sequencing among Egyptian isolates. All examined isolates of each host group(humans, camels, pigs, and sheep) gave very similar patterns of PCR-

RFLP after restriction enzyme digestion with AluI, with the gene size of approximately 140 bp and 240 bp for sheep and human isolates, and approximately 150 bp and 250 bp for pig and camel isolates. No digestion pattern obtained after incubation of all studied isolates with EcoRI. DNA sequence analysis highlighted that human infecting strain showed 100% identity for sheep infecting isolate, 96%, and 99% with pig and camel infecting isolates, respectively. In the present study using PCR-RFLP we studied genetic variability of AgB2 between human and animal isolates and found high degree of genetic similarity between human and sheep isolates and no polymorphism inAgB2 genes was found in each strain but there was a substantial level of inter-strain variation in AgB2 related genes. This finding supported by the previous study using PCR-RFLP, DNA sequencing which also found 100% identity between human, and sheep isolates. This is consistent with the results explained by Zhang *et al*[8] .Who reported close similarities between human and sheep isolates with distinctive PCR-RFLP pattern between them and camel isolate. Comparison of results from our study and previous reports are limited due to dissimilarities in technique. The present study is designed to evaluate the methods use AgB in the diagnosis of suspected clinical cases of hydatid disease. Diagnosis of hydatidosis is still an unresolved problem. Serological tests using crude antigens for diagnosis of *E. granulosus* are sensitive, however their specificity are not satisfactory. Therefore, WHO recommended specific serological methods using specific antigens, especially native AgB for proper detection. In human, early diagnose of the infection can provide considerable improvements in the quality of the management and treatment of the disease. Immunodiagnostic procedures for serum antibody detection are used for the etiological confirmation of imaging methods. Because of its high sensitivity, ELISA using native antigen B is a suitable method and preferable to be used in order to detect specific antibodies in human CE cases[15] but the efficiency of any serologic methods for human CE depends on the specificity of the parasite antigens used in the test. *E. granulosus* hydatid cyst fluid (HCF) is the antigenic source of reference for immunodiagnosis of human hydatidosis. However, there are difficulties related to lack of sensitivity and specificity, and problems with to standardize their use[25].Therefore the results of variation in AgB genes is necessary for the rational design and application of diagnostic tests, Anthelmintics drugs, and immunoprophylaxis reagents. Further work needs to be done to establish whether molecular, investigations on the echinococcosis. It is suggested that the association of these factors is investigated in future studies.

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