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Original Article

Molecular Cloning and Expression an 8-kDa Subunit of Antigen B from G1 strain of *Echinococcus granulosus*

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Abstract

Background: Echinococcosis or hydatidosis is a chronic, zoonotic worldwide infection caused by the larval stage of the dog taeniid tapeworm *Echinococcus granulosus*. Vaccination has been considered as one of the ways to prevent of hydatidosis in recent decades. The aim of this study was to construct a pcDNA3.1 eukaryotic expression vector containing the subunit 8-kDa antigen B (Hyd1) of *E. granulosus* (G1 strain) and investigate its capability to induce protein expression in mammalian cell line, as a basis toward developing a DNA vaccine against hydatidosis.

Methods: The coding sequence of HydI was amplified by PCR with the specific PCR primers from pQE/HydI, and then was sub-cloned into pcDNA3.1 plasmid as expression vector. The pcHyd1 plasmid was digested by restriction enzymes and amplified with the specific PCR primers to confirm cloning of this gene in pcDNA3 plasmid. In last step, the sub-cloned gene was expressed in mammalian cell line (NIH 3T3 cells).

Result: The subunit 8-kDa antigen B (Hyd1) was successfully sub-cloned in pcDNA3.1 and Hyd1 protein was expressed in eukaryotic cell confirmed by SDS-PAGE and Western blot.

Conclusion: Recombinant plasmid of pcDNA3.1 was successfully constructed and express of recombinant Hyd1 protein was confirmed. That is promising step for forthcoming measures on providing vaccine against human and animal hydatidosis.

Keywords: *Echinococcus granulosus*, Hydatidosis, Antigen, Helminths

Introduction

Hydatidosis or cystic echinococcosis, caused by the larval stage of the dog taeniid tapeworm *Echinococcus granulosus*, is a chronic, prevalent and classic zoonotic disease of important public health problem. This disease has a worldwide distribution, especially in countries with a common livestock industry, such as Mediterranean and Middle Eastern countries. It is endemic in some parts of Iran (1-6).

In life cycle of *E. granulosus*, with an indirect two-host, carnivores (mostly dogs) are definitive hosts and wide range of mammals including humans act as intermediate hosts. Hydatidosis in intermediate hosts results from accidental ingestion of tapeworm eggs passed into the environment with fae-

ces from definitive hosts. Hydatid cysts (metacestodes) can be established in any internal organs, mainly liver and/or lungs, of intermediate hosts (7-9).

There are currently three treatment options for cystic echinococcosis: surgery, PAIR) Puncture, Aspiration, Injection and Re-aspiration), and chemotherapy. Each of these modalities has limitations depending on the specific case. Chemotherapy constitutes a non-invasive treatment but has limitations to use in patients with chronic liver diseases, with bone marrow depression and during pregnancy. Further, chemotherapy is ineffective in 40% of cases (10-13).

Surgery is the main treatment, and is only way to completely remove the hydatid cyst. Nevertheless, for many reasons such as operative mortality, complications (anaphylaxis), relapse, temporary and permanent contraindications to surgery such as difficulty to reach the lesion, poor status of the patient, refusal of certain patients to undergo surgery poor and lack medical facilities/structures, is not always feasible (2, 11, 13).

Because of the difficulty of diagnosis due to the long incubation period, the difficulty of treatment and the risks and complications of the disease, control and prevention is important (14). Hydatid control programs have been successful in Iceland and New Zealand, mostly based on health education, control or elimination of home slaughter of sheep and in countries like Argentina, Chile and Uruguay, control programs have been reduced infections in cattle, dogs and humans. Hydatid control programs have been successful only at the local level and therefor, the global distribution and the important public health problem of hydatid cyst has not changed seriously (10, 11). In many endemic areas, effective control of the disease is not available or not applicable. In addition, failure or cessation this programs in endemic are as can turn it into a hyper endemic areas (10).

Mathematical models show that the most effective way of combating hydatid cyst is a combination of vaccination intermediate host and anti-helminthic treatment of definitive host (13, 15, 16). Vaccination has been considered as one of the ways to prevent hydatidosis in recent decades. DNA vaccines, third generation vaccines, are plasmid that has been genetically engineered to produce specific protein/proteins (antigens) from a pathogen, that after inoculation are expressed by cellular machinery. Among the key features of these vaccines can be noted to elicit each the three arms of acquired immunity (CTLs, Abs, T helper) and the innate immune response, strong and lasting immune response, at room temperature resistant, easy storage and transport (17, 18).

Antigen B (AgB) in hydatid cyst fluid of *E. granulosus* is a polymeric lipoprotein of 160 kDa and a highly immunogenic major antigen. The antigen is comprised of a group of subunit monomers of approximately 8 kDa in molecular size. Molecular studies have demonstrated that AgB is encoded by a multigene family having at least five gene loci (B1–B5), each one consisting of several minor variants that could be grouped into five clades, corresponding to the genes EgAgB8/1, EgAgB8/2, EgAgB8/3, EgAgB8/4 and EgAgB8/5 (19-22).

The aim of the present study was to construct a pcDNA3.1 eukaryotic expression vector containing the subunit 8-kDa antigen B (Hyd1)of *E. granulosus* (G1 strain) and investigate its capability to induce protein expression in mammalian cell line (NIH 3T3 cells, mouse embryonic fibroblast cell line), as a basis toward developing a DNA vaccine against hydatidosis.

Materials and Methods

In a previous study, for the preparation of the 8-kDa antigen-B subunit (Hyd1), total RNA was extracted from *E. granulosus* (Iranian G1 strain) protoscoleces so that HydI complementary DNA could be synthesised, HydI cDNA was utilized by PCR with the specific PCR primers based on the nucleotide sequence from HydI was available at GenBank (accession number HydI:DQ835667). The purified fragment (HydI:276bp) was cloned into pQE using T4 DNA ligase (23).

Amplification and Preparation HydI

To sub-clone HydI into pcDNA3.1 plasmid, the coding sequence of HydI was PCR-amplified with

the specific PCR primers HydIF:(ATA TAT ATA AGC TTG CTC ATA TGA GGA C) and HydIR:(ATA TAT ATC TCG AGC TAC TTT GAA TCA TC) from pQE/HydI. The upstream primer contained a HindIII site and the downstream primer contained a XhoI restriction site for HydI to facilitate subsequence cloning.

The 30-ml reaction mixture for the PCR contained 10 pg of pQE/HydI plasmid, 20 pmol of each primer, 1.25 U Taq DNA polymerase (Cinna Gen, Tehran, Iran) and 0.5 ml of a solution containing 10 mM of each dNTP. PCR amplification was performed using the following conditions: 1 cycle of 94 °C for 5 min then 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 30 sec and primer extension was extended to 5 min at 72 °C . PCR product was analyzed by electrophoresis on 3% agarose gel.

Cloning of HydI

The PCR product was digested by HindIII and XhoI restriction enzymes (Fermentas) was then cloned into HindIII/XhoI digested pcDNA3.1 plasmid using T4 DNA ligase (Roche-Pharma)(named pcHyd1). Competent *E. coli* (TOP10) cells were transformed with the ligation mixture by the heat shock method (24) and were cultured on LB agar plate containing 100 μg/ml ampicillin and were incubated for 16 h. Colony-PCR was then used to confirm the gene cloning. In addition, recombinant plasmid was digested with HindIII and XhoI restriction enzymes.

Transfection of recombinant pcHyd1 into eukaryotic cell

NIH 3T3 cells, mouse embryonic fibroblast cell line, were grown at 37 °C with 5% CO2 in culture flasks in Dulbeccos Modified Eagle Medium(DMEM) containing 50 μg/ml penicillin, 0.25 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum. These cells were consistently passaged 2–3 times/wk. Cells were plated ($3x10^5$ cell/well of a 12-well plate) the day before transfection and were transfected at 50-70% confluency with 50 ng of pcHyd1 or pcDNA3.1 using lipofectamine 2000 reagent (GIBCO-BRL,USA) according to the manufacture's instruction. The transfected and untransfected (as positive control) cells, were incubated for 16 h before replacing the lipofectamine-containing medium with of fresh serum-supplement DMEM medium. After 48 and 72 h, cell monolayers were scraped into microtubes. Cells were then recovered by centrifugation at 10,000 x g for 10 min and stored at – 80 °C (24).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis

Transfected and untransfected cell were cultured for either 48 h or 72 following respectively, and were harvested and were centrifuged. The pellet were resuspended in 50 μL of protein lysis buffer at 37 °C for 1 h, added loading protein and heated for 10 min at 80 °C, the protein mixture was separated using 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (25); following electrophoresis the gel was stained with Coomassie Briliant Blue G250.

An additional SDS-PAGE was performed for Western blotting, and protein were transferred to nitrocellulose membrane (Whatman, Germany) via electrophoresis, carried at 10 v and 200 mA for 1h, using transfer system (APLEXE, France). The membrane was blocked with 3% fat-free dried milk in TBS buffer (Sigma, USA) for overnight, and washed 2 times with TBS buffer containing 0.05% Tween 20 (TBST) and one time with TBS, and Nitrocellulose membrane was incubated for 2 h at room temperature with positive human serum diluted 1:500 in TBS.

Then the membrane was washed with TBST (2 times) and TBS (one time) and then incubated with anti-human antibody horseradish peroxidase (HRP) conjugate antibody (Sigma, USA)(diluted at 1/1000 In TBST) for 2 h at room temperature, after three times of washing, the membrane was treated using diaminobenzidene/H_2O_2 as substrate, and placed in darkness until the appearance of the protein band.

Results

The coding sequence of 8 kDa subunit of antigen B (HydI) was amplified by PCR with the specific

PCR primers HydIF:(ATA TAT ATA AGC TTG CTC ATA TGA GGA C) and HydIR:(ATA TAT ATC TCG AGC TAC TTT GAA TCA TC) from pQE/HydI. These primers contained HindIII and Xho1 restriction site for HydI to facilitate subsequence cloning (Fig. 1).

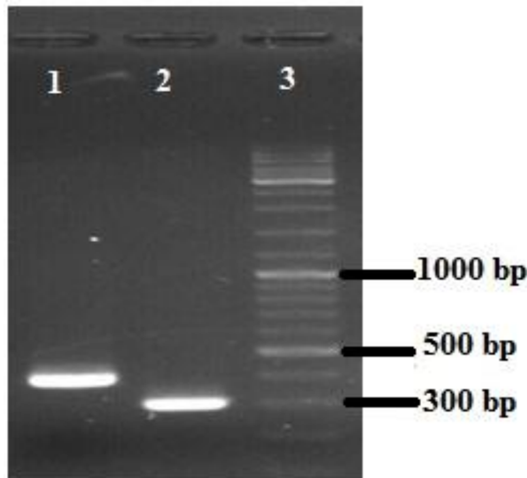

Fig.1: Fig.1: PCR amplification and gel electrophoresis of PCR product. Lane1: 400 bp representing the PCR product from pQE/HydI with universal-primer. Lane2: ~ 276 bp fragment PCR product from pQE/HydI with specific-primer. Lane3: DNA marker

After sub-cloning of HydI into pcDNA3.1 expression vector the recombinant plasmid was confirmed by restriction analysis and amplified with the specific PCR primers (Fig. 2).

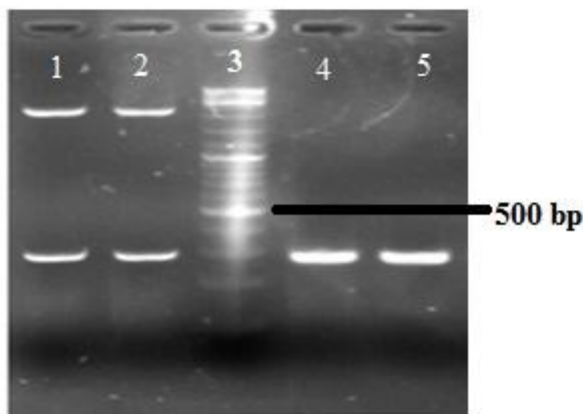

Fig. 2: Agarose gel electrophoresis of digested pcHyd1. Lanes 1&2: ~276 bp fragment released from recombinant plasmid pcHyd1, Lane3: DNA marker. Lanes 4&5: The results of the PCR from the recombinant plasmid

Recombinant expression vector was transfected into NIH. NIH samples were lysed and the protein of HydI was analyzed by Western Blot (Fig. 3).

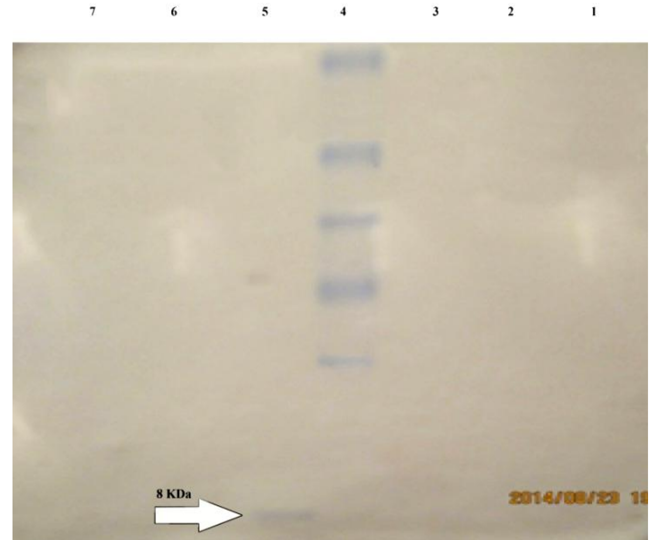

Fig. 3: The results of western blotting with positive human serum. Lines 1&7: NIH lysate without plasmid, lanes 2&6: NIH lysate containing plasmid without recombinant protein, lanes 3&5: NIH lysate containing recombinant pcHyd1. Lanes 1,2 and 3 after 48 h. Lines 5,6 and 7 after 72 h. Lane 4: protein molecular weight marker

Discussion

In this study, in order to access an appropriate DNA vaccine against hydatid cyst, the coding sequence of 8-kDa subunit of antigen B (Hyd1) was amplified by PCR with the specific PCR primers from pQE/HydI and was then cloned into pcDNA3.1 mammalian expression vector. In the next step, expression of HydI antigen in NIH cells transfected with pcHydI was analyzed to ensure the ability recombinant plasmid to express and produce HydI antigen.

Hydatidosis or cystic echinococcosis, is a global public health-economic problem with annual estimated cost around three billion US dollars and

around 5-20% mortality (1) and in Iran was estimated more than US$ 230 million per year (26).
Prevention programs including public education campaigns, anti-helminthic treatment of the definitive host and finally vaccination of intermediate hosts is currently being evaluated as an additional intervention (2, 10, 13).
Early attempt for vaccination the sheep against *E. granulosus* had been performed by Gemmell, where he used oncosphere of the parasite as a crude antigen (27). Marshal et al. and Wadood showed that hydatid cyst fluid has potent to prevent hydatid cyst (28, 29). Among three hydatid antigen sources (AgB, crude sheep hydatid fluid CSHF and protoscoleces homogenate PSH), the greatest reduction in cyst load was achieved in mice immunized with the genus-specific AgB, known to be a highly immunogenic lipoprotein (30). According to Hashemitabar et al. "protective immunity was induced in mice with protoscolex protein and with hydatid fluid, and in sheep with whole-body homogenate of *E. granulosus* and the levels of protection afforded were found to be 72.1, 82.6 and 90.9% respectively" (31).
In recent years, several genes from different stages of *E. granulosus* were cloned and expressed by many researchers around the world, with different goals such as finding an effective vaccine (both recombinant proteins and DNA vaccines) or recombinant antigen for serological diagnosis (23, 32-34). Boutennoune et al. constructed recombinant plasmid pDRIVE-EgAgB8/2 and showed that EgAgB8 DNA vaccine induce a Th1 response (35). Abdi et al. in a study indicated that 12 and 16 kDa recombinants proteins of antigen B can induce the immune response in rabbits (32). Sarvi et al. cloned EG95 fragment (Iranian strain) in pcDNA3 as eukaryotic expression vector to produce protein for DNA vaccine, recombinant EG95 protein confirmed by SDS-PAGE and Western blot (36).
In comparison with traditional vaccines, DNA vaccines have some distinguishing properties, for example: easy production, the final cost-down, heat stable, safer and the ability to induce more powerful and long-lasting cellular and humoral immune response (17, 18). They also have potential to increase immunogenicity through modification of the vector or incorporation of adjuvant-like cytokine gene. Accordingly, we want the ability of pcHyd1 to induce protective immune response well be investigated in mouse models.

Conclusion

We cloned the Hyd1 gene into pcDNA3.1 and expressed in eukaryotic cells successfully, which might be used as a candidate antigen for DNA vaccine against hydatid cyst disease. In the next study, the ability of pcHyd1to induce protective immune response will be investigated in mouse models.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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