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

Hakim AZIZI 1, *Bahram KAZEMI 2,3, Mojgan BANDEHPOUR 2,3, Mehdi MOHEBALI 4,5, Ali KHAMESIPOUR 5, Mojgan ARYAEIPOUR, *Mohammad Bagher ROKNI 1,4

1. Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran
2. Cellular and Molecular Biology Research Centre, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Biotechnology Department, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran
5. Skin and Leprosy Research Center, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding Authors: Email: roknimoh@tums.ac.ir, Bahram_14@yahoo.com

(Received 21 Jan 2015; accepted 10 May 2015)

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 Hyd1 was amplified by PCR with the specific PCR primers from pQE/Hyd1, 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 (metacoe-
todes) 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, PAR1) Puncture, 
Aspiration, Injection and Re-aspiration), and 
chemotherapy. Each of these modalities has limit-
tations depending on the specific case. Chemo-
therapy 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 sur-
gery 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 educa-
tion, 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). Vaccina-
tion has been considered as one of the ways to 
prevent hydatidosis in recent decades. DNA vac-
cines, third generation vaccines, are plasmid that 
has been genetically engineered to produce specif-
ic 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 ac-
quired immunity (CTLs, Abs, T helper) and the 
in innate immune response, strong and lasting im-
mune response, at room temperature resistant, 
easy storage and transport (17, 18).

Antigen B (AgB) in hydatid cyst fluid of E. granu-
losus 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 va-
riants that could be grouped into five clades, cor-
responding 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 contain-
ing the subunit 8-kDa antigen B (HydI) of E. gra-
nulosus (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 (HydI), 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 TTT 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 an 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 pcHydI). 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 pcHydI into eu-karyotic 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 (3x10⁵ cell/well of a 12-well plate) the day before transfection and were transfected at 50-70% confluency with 50 ng of pcHydI 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 Brillant 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 (APEXAE, 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 diaminobenzidine/H₂O₂ 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).

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. 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

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 pcHyd1 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.

**Acknowledgements**

This paper was extracted form Hakim Azizi’s PhD thesis and was supported financially by a grant from Tehran University of Medical Sciences No. 21780. The authors declare that there is no conflict of interests.

**References**

1. Moro P, Schantz PM (2009). Echinococcosis: a review. *Int J Infect Dis*, 13:125-133.
2. Siracusano A, Teggi A, Ortona A (2009). Human Cystic Echinococcosis: Old Problems and New Perspectives. *Interdiscip Perspect Infect Dis*, 2009:1-7.
3. Seimenis A (2003). Overview of the epidemiological situation on echinococcosis in the Mediterranean region. *Acta Tropica*, 85:191-195.
4. Cardona GA, Carmenab D (2013). A review of the global prevalence, molecular epidemiology
and economics of cystic echinococcosis in production animals. Vet Parasitol, 192:10-32.

5. Shahnazia M, Hejazi H, Salehi M, Andalib AR (2011). Molecular characterization of human and animal Echinococcus granulosus isolates in Isfahan, Iran. Acta Tropica, 117:47-50.

6. Torgerson PR, de Silva DS, Fevriere EM, Kasuga F, Rokni MB, Zhou XN, Sripa B, Gargouri N, Willingham AL, Stein L (2014). The global burden of foodborne parasitic diseases: an update. Trends Parasitol, 30:20-26.

7. Jenkinsa DJ, Romigb T, Thompson RCA (2005). Emergence/re-emergence of Echinococcus spp.—a global update. Int J Parasitol, 35:1205-1219.

8. Lewall DB (1998). Hydatid Disease: Biology, Pathology, Imaging and Classification. Clin Radiol, 53:863-874.

9. Thompson RCA (2008). The taxonomy, phylogeny and transmission of Echinococcus. Exp Parasitol, 119:439–446.

10. Craig PS, Larrieu E (2006). Control of Cystic Echinococcosis/Hydatidosis: 1863–2002. Adv Parasitol, 61:1–67.

11. Craig PS, McManus DP, Lightowlers MW, Chabalgoity JA, Garcia HH, Gavidia CM, Gilman RH, Gonzalez AE, Lorca M, Naquira C, Nieto A, Schantz PM (2007). Prevention and control of cystic echinococcosis. Lancet Infect Dis 7:385–394.

12. Bygott JM, Chiodinia PL (2009). Praziquantel: Neglected drug? Ineffective treatment? Or therapeutic choice in cystic hydatid disease? Acta Tropica, 111:95–101.

13. Stamatakos M, Sargedi C, Stefanaki C, Safioleas C, Matthaiopoulou I, Safioleas M (2009). Anthelmintic treatment: An adjuvant therapeutic strategy against Echinococcus granulosus. Parasitol Int, 58:115-120.

14. Vuitton DM (2003). The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Tropica, 85:119-132.

15. Torgerson PR (2003). The use of mathematical models to simulate control options for echinococcosis. Acta Tropica, 85:211-221.

16. Zhang W, McManus DP (2008). Vaccination of dogs against Echinococcus granulosus: a means to control hydatid disease? Trends Parasitol, 24:419-424.

17. Saha R, Killian S, Donofrio RS (2011). DNA Vaccines: A Mini Review. Recent Pat DNA Gene Seq, 5:1-5.

18. LIU MA (2003). DNA vaccines: a review. J Intern Med, 253:402–410.

19. Mamuti W, Sako Y, Nakao M, Xiao N, Nakaya K, Ishikawa Y, Yamasaki H, Lightowlers MW, Ito A (2007). Molecular characterization of a novel gene encoding an 8-kDa subunit of antigen B from Echinococcus granulosus genotypes 1 and 6. Parasitol Int, 56:313–316.

20. Mamuti W, Sako Y, Nakao M, Xiao N, Nakaya K, Ishikawa Y, Yamasaki H, Lightowlers MW, Ito A (2006). Recent advances in characterization of Echinococcus antigen B. Parasitol Int, 55:57–62.

21. Monteiro KM, Zaha A, Ferreira HB (2008). Recombinant subunits as tools for the structural and functional characterization of Echinococcus granulosus antigen B. Exp Parasitol, 119:490–498.

22. Arend AC, Zaha A, Ayala FJ, Haag KL (2004). The Echinococcus granulosus antigen B shows a high degree of genetic variability. Exp Parasitol, 108:76-80.

23. Abdi J, Kazemi B, Mohebali M, Bandehpour M, T. RM, Rokni MB (2010). Gene cloning, expression and serological evaluation of the 12-kDa antigen-B subunit from Echinococcus granulosus. Ann Trop Med Parasitol, 104:399–407.

24. Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual. 3rd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

25. Laemmli UK (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

26. Fasihi HM, Budke CM, Rostami S (2012). The Molecular Burden of Cystic Echinococcosis in Iran. PLoS Negl Trop Dis, 6:10.

27. Gemmell MA (1966). Immunological responses of the mammalian host against tapeworm infections IV. Species specificity of hexacanth embryo in protecting sheep against Echinococcus granulosus. Immunology, 11:325-335.

28. Marshall WL, Michael DR, Rhonda DH (1986). Serum antibody response following parenteral immunization with hydatid cyst fluid in sheep infected with Echinococcus granulosus. Am J Trop Med Hyg, 35:811-823.

Available at: http://ijph.tums.ac.ir
29. Wadood EA (2007). Immunization Trial Of Sheep Against Hydatid Cyst Infection. *Bat J Vet Res*, 6:64-69.

30. Al-Qaoud AK, S.K. A-H (2005). Humoral and cytokine response during protection of mice against secondary hydatidosis caused by *Echinococcus granulosus*. *Parasitol Res*, 98:54–60.

31. Hashemitabar GR, Razmi GR, Naghibi A (2005). Trials to induce protective immunity in mice and sheep by application of protoscolex and hydatid fluid antigen or whole body antigen of *Echinococcus granulosus*. *J Vet Med B Infect Dis Vet Public Health*, 52:243-245.

32. Abdi J, Kazemi B, Karimfar MH, Rokni MB (2012). Evaluation of rabbit antibody response against 8 and 16 kDa recombinant subunits of antigen B from *Echinococcus granulosus*. *Asian Pac J Trop Med*, 3:355-357.

33. Shi Z, Wang Y, Li Z, Li Z, Bo Y, Ma R, Zhao W (2009). Cloning, expression, and protective immunity in mice of a gene encoding the diagnostic antigen P-29 of *Echinococcus granulosus*. *Acta Biochim Biophys Sin.*, 41:79–85.

34. Sadjjadi SM, Abidi H, Sarkari B, Izadpanah A, Kazemian S (2007). Evaluation of Enzyme Linked Immunosorbent Assay, Utilizing Native Antigen B for Serodiagnosis of Human Hydatidosis. *Iran J Immunol*, 4:167-173.

35. Boutennoune H, Qaqish A, Al-Aghbar M, Abdel-Hafez S, Al-Qaoud Km (2012). Induction of helper 1 response by immunization of balb/c mice with the gene encoding the second subunit of *Echinococcus granulosus* Antigen B (EGGB/2). *Parasite*, 19:183-188.

36. Sarvi S, Dalimi A, Ghafarifar F (2012). Molecular Cloning and Expression of EG95 Gene of Iranian Isolates of *Echinococcus granulosus*. *Iran J Parasitol*, 7:1-7.