Iranian J Parasitol

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Original Article

Subcloning and Expression of Recombinant *Echinococcus granulosus* Antigen B, in Pqe-30 Expression Vector

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(Received 21 May 2009; accepted 15 Nov 2009)

Abstract

Background: Echinococcosis or hydatid disease is a zoonotic infection caused by larval (metacestode) stages of cestodes belonging to the genus *Echinococcus,* family *Taeniidae*. We aimed to subclone antigen B gene in pQE-30 plasmid, its expression, and purification.

Methods: We subcloned HI gene into pQE-30 expression vector. The recombinant vector was transformed into *E. coli*, M15 and mass cultured. The subcloned gene was expressed by IPTG. Subcloning of gene was confirmed by both PCR and enzyme digestion.

Results: Production of recombinant protein was confirmed by SDS-PAGE. Western blot analysis was carried out by both His-Tag monoclonal Ab and human serum to estimate the expressed protein in *E. coli* cells. Recombinant protein was purified and its specificity was proved by Western blotting.

Conclusion: Production of this recombinant protein can increase sensitivity and specificity in serological test (ELISA).

Keywords : *Cystic echinococcosis, Recombinant antigen B, HI gene*

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Introduction

Cystic echinococcosis (CE) is a zoonotic parasitic disease throughout the world and causes considerable economic losses and public health problems in many countries (1). CE is a disease of livestock and humans that arises from eating infective eggs of the cestode *Echinococcus granulosus*. Dogs are the primary definitive hosts for this parasite, with livestock acting as intermediate hosts and humans as aberrant intermediate hosts. The outcome of infection in livestock and humans is cyst development in liver, lungs, or other organ system (2). The disease is well known in Asia, Africa, South and Central America, the Mediterranean, and Eastern Europe, with some foci in the United Kingdom (3, 4). It is an importance public health not only in areas of endemicity but also in countries or regions without endemicity due to the migration of infected people and livestock exchange (5). In man, early detection of the infection can provide substantial improvements in the quality of the management and treatment of the disease.

Concerning the procedure for diagnosing human CE, imaging methods for detecting space occupying lesions (US, CT, MRI, X-ray, etc.) are commonly the primary approaches (6,7). Immunodiagnostic procedures for serum antibody detection are used for the etiological confirmation of imaging structures suggestive for CE or for diagnosis or differential diagnosis in cases of uncharacteristic imaging findings (6,7).

Classically, tests such as double diffusion, immunoelectrophoresis, indirect hemagglutination were used as references for human CE diagnosis (8,9). Nowadays, these tests are being replaced by more sensitive assays such as ELISA, immunofluorescence and immuonoblotting. Because of its high sensitivity, ELISA is strongly recommended to be used for the detection of specific antibodies in human CE cases (10).

The efficiency of any serodiagnostic test for human CE depends on the specificity of the parasite antigens utilized in the assay. *E. granulosus* hydatid cyst fluid (HCF) is the antigenic source of reference for immunodiagnosis of human hydatidosis. However, there are difficulties associated with the currently available tests, related to their lack of sensitivity and specificity, and problems with the standardization of their use (11). Antibody cross-reactivity with antigens from other parasites, notably other taeniid cestodes, is a major problem when using HCF antigen in CE immunodiagnosis.

It has been suggested that serodiagnosis of CE may be improved by the use of recombinant proteins, combining several defined antigens (including synthetic peptides) and the design of new *E. granulosus*-specific peptides that react with otherwise false-negative sera (12). In the last few years, efforts have been made to prepare recombinant proteins (13). Recombinant antigen B, in contrast to biological products, is easily standardized and can be readily produced in large amounts. Pazoki et al., cloned two published sequence of antigen B, with the aim of production of recombinant subunits of antigen B (14).

We aimed to subclone antigen B gene in pQE-30 plasmid, its expression, and purification. This recombinant protein can be used in hydatidosis serological diagnostic tests after serologic evaluation.

Materials and Methods

HI gene (antigen B) was amplified via RT-PCR reaction and amplified fragment was cloned in pTZ57R plasmid by T/A cloning as reported previously (14). The recombinant plasmid (pTZ57R/ HI) was transformed into *E. coli*, XL1blue strain com-

petent cell. The recombinant plasmid was extracted (15) and digested by *Sac*I and *Hind*III restriction enzymes. The released fragment DNA (HI gene) was ligated into *Sac*I and *Hind*III digested pQE-30 expression vector and was transformed in *E. coli*, M15. Recombinant plasmid was confirmed by both colony PCR and enzyme digestion methods (16). PCR reaction was done by specific primers for pQE vector (17).

Expression of recombinant protein

The *E. coli* strain M15 was transformed with the rpQE-30 and spread on Luria Bertani agar containing 100 µg/ml of ampicillin. The transformant was inoculated into 3 ml culture tube containing modified YT medium (18) and allowed to grow at 37°C in a shaker at 160 rpm, overnight. The day after, it was subcultured into a 50 ml flasks containing YT medium and incubated at 37°C in a shaker, at 200 rpm.

The culture in the logarithmic phase (at OD_{600} = 0.6) was induced for 5h and overnight with 1 mM IPTG. Sampling was done and analyzed on 12% (v/v) SDS-PAGE, parallel with uninduced bacterial culture (19). The gel was stained with Coomassie brilliant blue R-250 (20).

Protein purification

Bacteria were grown and followed by IPTG (1mM) induction for 5h then overnight at 37 °C. The cells were centrifuged at 6500 g for 10 min, the pellet was suspended in equilibration buffer (50 mM Tris, 0.5 M NaCl) containing protease inhibitor cocktail (18). The cells suspension were sonicated (5 x 20 S) on ice and harvested by centrifugation at 4000 g for 15 min, then resuspended in 5 ml of ice-cold buffer containing 6M urea and incubated on ice for one hour, centrifuged for removal of insoluble. The recombinant protein was purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography according to the manufacturer's instructions.

Double diffusion

The test was done in 1% purified agar gel (sigma) in PBS (1x) buffer on plate. After pouring the gel, it was allowed to set for 10 minutes. Patterns were cut so that the central well was surrounded by five peripheral wells. The pellet of transformed cell, which were collected 5h after induction, were placed in the center well. This lysate contained the ideal recombinant antigen. The patient's serum was then placed in one (or more) of the outer wells and the plate left in a humidity chamber for 72 hours to develop.

Western blot analysis

Proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane. The membranes were incubated in TBS (Tris-Buffered Saline containing 3% BSA (Bovine Serum Albumin) and then washed several times. The strips were reacted with 1:1000, 1:800 dilution of His-Tag monoclonal Ab and human serum respectively for 1 h at 37°C. The membranes were washed several times with TBS and TBST(TBS -Tween 20) and subsequently treated with horseradish peroxidase (HRP) conjugated Sheep anti mouse Ig and rabbit anti human Ig at a 1 : 500 dilution for 1 hour at 37°C. The strips were visualized for color after development in Di Amino Benzidine/H_2O_2 substrate solution for 15 min at room temperature. The reaction was stopped by washing four times in distilled H_2O.

Result

Recombinant pTZ57R/ HI was digested by *Sac*I and *Hind*III restriction enzymes and released expected DNA band recovered by P.C.I (phenol/chloroform/isoamyl alcohol) extraction method (Fig.1), then ligated in to *Sac*I and *Hind*III digested pQE-30 (Fig. 2). Fig, 3 demonstrates digestion of recombinant expression vector pQE-30/ HI by *Sac*I and *Hind*III restriction enzymes. Fig. 4 shows PCR product of recombinant

plasmid. The recombinant pQE-30/HI must be encoded with fusion protein with approximately 12-24 kDa molecular weights, after induction. Fig. 5 shows the SDS-PAGE. Recombinant proteins were expressed as NH_2-terminally polyhistidine-tagged fusion proteins and purified from the M15, *E. coli* cell extracts by Ni-NTA affinity chromatography. The 6xHis affinity tag facilitates binding to Ni-NTA.

During our studies using double diffusion gel precipitation, we noticed a strong precipitation arc that was produced in the reaction of lysate of M15 cell containing recombinant PQE30, collected 5h after induction with the serum of cystic hydatid patient. This immune complex precipitates in the gel to give a thin white line as shown in Fig. 6.

Western blotting was used to test the sensitivity of pQE-30/HI, affinity-purified pQE-30/HI , pQE-30 , M15 against patient sera and monoclonal anti His-tag. Western blots of pQE-30 HI, affinity-purified pQE-30/HI demonstrated a fusion protein that reacted with patient's serum and monoclonal anti His-tag (Fig.7 and 8).

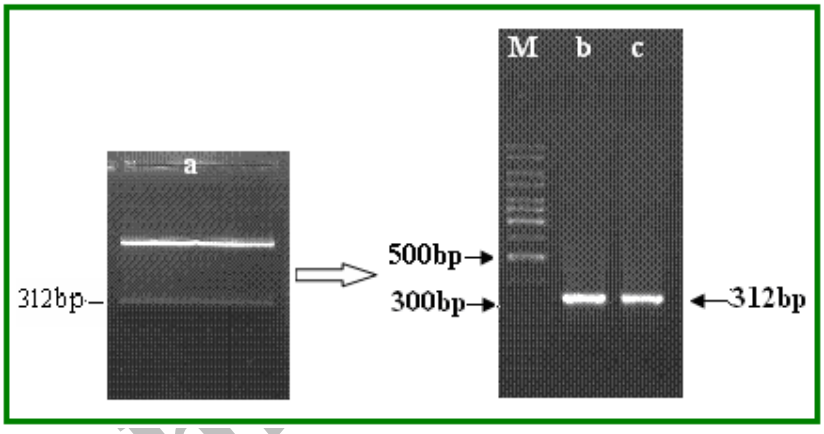

Fig.1:
a : Digested recombinant plasmid (312bp)
b , c : Purified band by using a P.C.I method.
M: Ladder marker100bp DNA

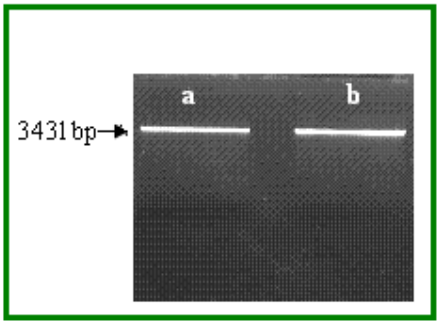

Fig.2:
a ,b : Digested intact pQE-30 vector by *Sac*I and *Hind*III

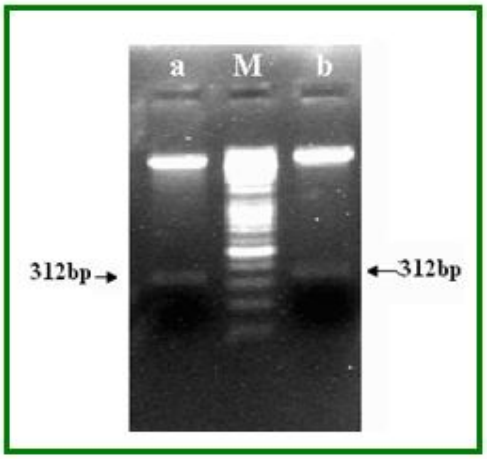

Fig.3: a , b :Digested recombinant pQE30/HI by *Sac*I and *Hind*III
M: 100bp DNA ladder marker

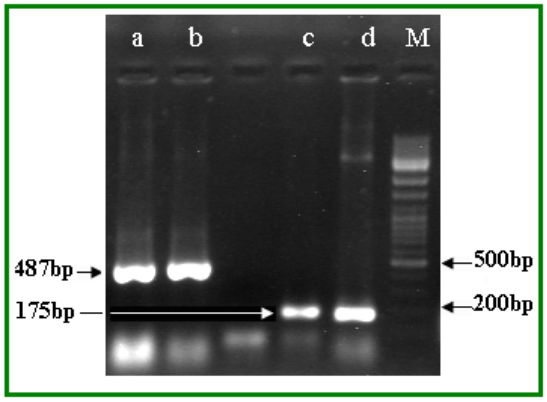

Fig. 4:
a , b : PCR product of recombinant plasmid (487bp)
c , d : PCR product of intact plasmid (175bp)
M : 100bp DNA ladder marker

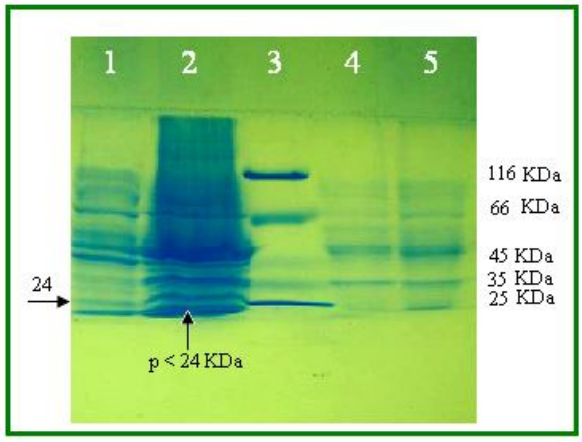

Fig. 5:
1: lysate of M15 cell containing recombinant PQE30, collected 3h after induction
2: lysate of M15 cell containing recombinant PQE30, collected 5h after induction
3: Protein molecular size marker
4: lysate of M15 cell, collected 5h after induction
5: lysate of M15 cell containing intact PQE30, collected 5h after induction

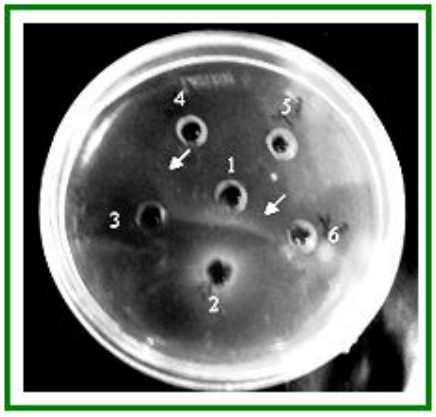

Fig. 6:
1: lysate of M15 cell containing recombinant PQE30, collected 5h after induction
2: Serum of patient with cystic hydatid
3: 1:10 titer of serum
4,5,6: respectively,1:100,1:1000,1:10000 titer of serum

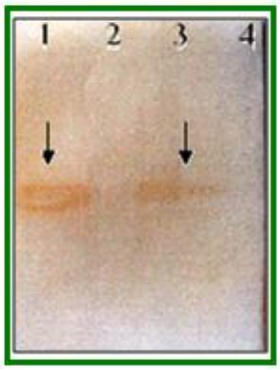

Fig.7:
1:Lysate of bacteria containing pQE30/ HI
2:Lysate of bacteria containing intact pQE30
3: Purified 6×His-HI of recombinant pQE30
4: Cell lysate without plasmid

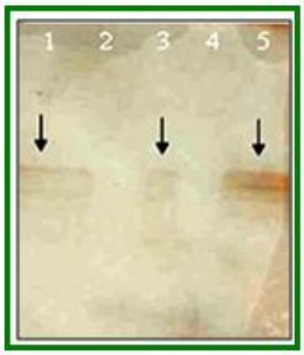

Fig. 8:
1,3: Purified 6×His-HI of rpQE30
2: Lysate of cell containing intact pQE30
4: Cell lysate without plasmid
5 :lysate of M15 cell containing rpQE30

Discussion

Hydatid disease, a zoonotic infection caused by the cystic stage of *E. granulosus* is a major health and economic problem in most areas of the world where livestock are kept. It is prevalent in different parts of Iran (21, 22).

Diagnosis is currently based on identification of cyst structures by imaging techniques (ultrasonography, computed tomography, X-rays) and confirmation by immunodiagnostic tests, predominantly ELISA and immunoblotting (13).

In most cases, the early stages of infection are asymptomatic; therefore, methods that are both relatively easy to use and cheap are required for large-scale screening of populations at high risk for infection (23).

Early diagnosis of human hydatid disease by detecting of the specific antibodies in patient's sera is considered as an important step in treatment of infection (7).The main problems in the immunodiagnosis of the disease are the often-unsatisfactory performances of the available tests and the difficulties associated with the standardization of antigen preparations and techniques. The diagnostic efficiencies of assays greatly depend on the characteristics of antigen that is used and various conditions in performance.

The most common antigenic sources used for the immunodiagnosis of hydatid disease are hydatid cyst fluid, somatic extracts, and excretory-secretory products from protoscoleces or adults of *E. granulosus* (13, 14).

Serodiagnosis of CE may be improved by use of recombinants proteins or by combining several defined antigens, including synthetic peptides. Nonetheless, to date there is no standard, highly sensitive and specific test available for immunodiagnosis of human CE (12, 13).

E. granulosus antigen encoding genes can be cloned and expressed in heterologous systems, in order to obtain recombinant antigens that can be produced and purified easily, and may be less prone to cross reactivity (24-26).

Recombinant proteins and synthetic peptides are proved more reliable for immunodiagnostic purposes than native antigens

and their purified subunits/ fractions. Recombinant antigens typically evidence higher diagnostic performances than their homologous native proteins and recombinant technology can produce well-defined polypeptides in large quantity, allowing the standardization of the antigen source (13).

Ortona et al. evaluated and compared the sensitivity and specificity of antigen B and the elongation factor-1b/d with several immunologic tests. Antigen B was obtained by PCR of *E. granulosus* genomic DNA using primers derived from the antigen B sequence. After PCR, products were digested with restriction enzymes and cloned in GST pGEX vector (27).

Virginio et al. cloned and expressed 6 recombinant antigens from *E. granulosus*. Each antigen was cloned previously and expressed in *E. coli* strains using plasmid vectors of the pGEX series. Their purified recombinant proteins were tested by ELISA. Among them, the recombinant antigen B showed highest sensitivity and specificity (10).

In this study, the production of recombinant protein with approximately 24-kDa molecular weight was confirmed by using SDS-PAGE. Western blot analysis was carried out by both His-Tag monoclonal Ab and human serum to estimate the expressed protein in *E. coli* cells after induction with 0.8 mM IPTG for 5h .Using pQE vectors the 6xHis affinity tag can be placed at the C- or N-terminus of the protein of interest. It is poorly immunogenic, and at pH 8.0 the tag is small, uncharged, and therefore does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell.

In most cases, the 6xHis tag does not interfere with the structure or function of the purified protein as demonstrated for a wide variety of proteins, including enzymes, transcription factors, and vaccines. Therefore, segregate of 6xHis tag was not necessary but Glutathione S-Transferase (GST) fusion protein in the pGEX series (used in other studies) should be purified by gluthatione Sepharose 4B.

In this survey, we successfully subcloned and expressed HI (recombinant antigen B) in pQE-30, which can be used in hydatidosis immunodiagnostic tests. Tests with a larger number of specific and cross-reactive sera must be performed to confirm its diagnostic value. Such tests are currently under way in our laboratory. This will promote the detection accuracy and efficiency, since the antigens are provided from parasite strains prevalent in Iran and this will be beneficial, compared to ready to use kits, imported from other countries.

Acknowledgments

This study was supported by Shahid Beheshti University, M.C. and was done in Cellular and Molecular Biology Research Center. The authors would like to express special thank to Mrs. L Maghen for her sincere cooperation. The authors declare that they have no conflicts of interest.

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