Cloning and Expression of Two Genes Encoding Subunits of Echinococcus granulosus Antigen B.

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Abstract: Two different sequences of Echinococcus granulosus Antigen B, (A major hydatid cyst fluid antigen), acquired from Gene Bank and amplified via RT-PCR reaction. The amplified fragments (HI, HII) cloned into pTZ57R vector by T/A cloning and subeloned into pGEMEX-1 expression vector. The subcloned genes were expressed by IPTG. To confirm the expression of subeloned genes, the SDS-PAGE performed and production of about 35 KDa recombinant fusion proteins were confirmed for either two cloned genes. The immunoreactivity of the recombinant fusion proteins were tested using double diffusion and immunoblotting. Both recombinant fusion proteins derived from lystate of transformed bacteria, were reactive for antibodies in serum of cystic hydatid patient.

Key words: Antigen B, recombinant protein, cloning, hydatidosis, Echinococcus

INTRODUCTION

Cystic Hydatid Disease (CHD) is a zoonosis caused by the infection with the metacestode stage of Echinococcus granulosus (Cestoda, Taeniidae). In humans, the metacestodes develop as large cysts, especially in the liver and lungs, causing unspecific symptoms that depend on the size and localization of the lesions (Rigano et al., 2001). Most human CHD cases are initially discovered by clinical examinations using different imaging techniques, such as radiology or ultrasonography (Blaev et al., 2001). However, this preliminary diagnosis must be complemented by more specific tests, such as immunological assays based on the detection of anti parasite circulating antibodies in patients’ sera.

The efficiency (sensitivity and specificity) of any immunodiagnostic test for human CHD depends on the quality of the parasite antigens utilized in the assay. (Ortona et al., 2000). Antigen B is one of the specific antigens in hydatid cyst fluid. It is a complex 120 KDa lipoprotein (Oriol and Oriol, 1975). Native antigen B, when resolved on sodium dodecylsulphat polyacrylamid gel electrophoresis (SDS-PAGE), shows three major immunogenic bands at approximately 8-12, 16 and 23-24 Kda (Lightowlers et al., 1989). Immunoblot detection of smallest subunit (8 KDa) of antigen B provides a good diagnosis tool for cystic hydatid disease (Leggate and Memanu, 1994). Echinococcus 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 (Rott et al., 2000; Ortona et al., 2000; Virgini et al., 2003).

This study intended to amplify, clone and express of two published sequences of antigen B, with the aim of production of recombinant subunits of antigen B, which can be use in hydatidosis serological diagnosis tests.

MATERIALS AND METHODS

This study was carried out from April 2004 to July 2005 in Cellular and Molecular Biology Researches Center, Shaheed Beheshti University of Medical Sciences, Tehran, Islamic Republic of Iran.

Two sequences of gene encoding subunits of antigen B (HI and HII), containing 273 and 246 bp, respectively, obtained from gene bank and designed specific primers for amplifying of them. BamHI and Sacl restriction sites were achieved at 5 ends of forward and reverse primers respectively.
Echinococcus granulosus protozoa were collected from liver of infected sheep and total RNA was extracted by previously described method (Chomczynski et al., 1997). mRNA was reversetranscribed using MMLV Reverse Transcriptase (Fermentase®) and specific reverse primers.

PCR mix contained 1 × PCR buffer, 0.1 mM dNTP, 1.5 mM MgCl₂, 1 μg cDNA, 20 pmol each of primer and 1 unit TaqDNA polymerase in 50 μl volume.

**PCR reaction was done by following parameters:**

Denaturation at 94°C for 30 sec., annealing at 63°C (in case of HI) and 68°C (in case of HII) for 30 sec and extension at 72°C for 30 sec, repeated 30x and 10 min for final post extension.

Purified Poly A ended PCR products ligated to pTZ57R plasmid via T/A cloning method recombinant pTZ57R plasmids (pTZ57R HI and pTZ57R HII) were transformed into E. coli, DH5α strain competent cells (Fermentase®). Bacterial colonies containing recombinant plasmids were screened by X-gal- IPTG. Plasmids were extracted from corresponding cells and subjected to restriction analysis. Plasmids were digested by sacI and BamHI and inserted genes were separated via gel electrophoresis on 2% LMP agarose gel, purified and subcloned in pGEMEX expression vector (Promega®).

Recombinant pGEMEX plasmids (pGEMEX HI and pGEMEX HII), transformed to E. coli, JM109 strain competent cells (promega®) and transformed cell colonies were screened by ampicillin.

pGEMEX HI and pGEMEX HII were sequenced and compared the sequences of inserted fragments with their Gene Bank origin, to confirm the recombinant plasmids containing the interested genes.

Transformed E. coli, JM109 cells cultured in LB medium containing 100 μg mL⁻¹ ampicillin. Plasmid promoters were induced by adding 0.5 mM IPTG. Samples were collected before induction, 4 h and overnight after induction.

Cells lysate obtained by SDS lysis procedure (www.promega.com) and loaded onto 12% polyacrylamid gel with protein molecular weight size marker (SDS-PAGE).

Gel diffusion was carried out using cell pellets as antigen against serum sample of an infected person, on 1% agarose gel. The agarose gels then stained with coomassie brilliant blue dye, to visualize antigen-antibody complex precipitation arcs.

To confirm that the expressed proteins are those of interests, lysate of cells collected after induction and cells without plasmid and cells with intact pGEMEX plasmid, were electrophoresed on a 12% polyacrylamid gel and protein bands transferred into nitrocellulose membrane. Primary antibody (Human cystic disease sera) diluted 1: 1000 and secondary antibody (Rabbit anti-human immunoglobulin IgG conjugated to horseradish peroxidase) diluted 1: 4000 were applied on the nitrocellulose and finally Di Amino Benzidine (DAB)/H₂O₂ were used to visualize the antigen-antibody reaction.

**RESULTS**

**PCR reaction:** Figure 1 showed 2% agaros gel electrophoresis of PCR products and 100 bp DNA ladder.

**Gene cloning:** The PCR products were electrophoresed onto LMP agarose (Fig. 2a) and subjected band was scissored by scalpel blade and purified by phenol chloroform. Figure 2b showed 2% agarose gel electrophoresis containing purified PCR product.

Figure 3 showed digestion of recombinant T vectors (pTZ57R HI, pTZ57R HII) by BamHI and SacI restriction enzymes. The fragments separated from these vectors, were used as inserts in subcloning to pGEMEX expression vector, after purification.

Figure 4 showed digestion of recombinant expression vectors (pGEMEX HI, pGEMEX HII) by BamHI and SacI restriction enzymes.

![Fig. 1: Electrophoresis of PCP products. M= Marker](image1)

![Fig. 2: a: Electrophoresed PCR products onto LMP agarose b: HI and HII after purification. M = Marker](image2)
Fig. 3: 2% Agarose gel electrophoresis of digested recombinant pTZ57R plasmids
a: pTZ57R HI b: pTZ57R HI M: 100 bp DNA marker (White arrows indicate separated fragments after digestion)

Fig. 4: 2% Agarose gel electrophoresis of digested recombinant pGEMEX plasmids M: 100 bp DNA marker. (White arrows indicate separated fragments after digestion)

**SDS-PAGE:** The intact PGE-MEX encodes protein 10 containing 260 amino acid with molecular weight of 26 KDa, after induction by IPTG (Romero et al., 2001) and subunits of *Echinococcus granulosus* antigen B have 8-12 KDa molecular weight (Leggate and Memaranus, 1994; Gonzalez et al., 1996). So the recombinant PGE-MEX HI and PGE-MEX HII must be encodes fusion protein with approximately 34-38 KDa molecular weight, after induction.

Figure 5 showed the SDS-PAGE of lysate of transformed JM109 cells and JM109 cells without plasmid and it seems to be difference in pattern of protein bands, at about 35 KDa, in lysate of cells containing both recombinant pGEMEX plasmids, before and after induction.

Fig. 5: 15% SDS-PAGE
Lane 1: Lysate cells of JM109 alone.
Lane 2: Protein molecular size marker
Lane 3: Lysate of JM109 cells containing pGEMEX HI, collected overnight after induction.
Lane 4: Lysate of JM109 cells containing pGEMEX HI, collected 4 h after induction.
Lane 5: Lysate of JM109 cells containing pGEMEX HI, collected before induction.
Lane 6: Lysate of JM109 cells containing pGEMEX HII, collected before induction
Lane 7: Lysate of JM109 cells containing pGEMEX HII, collected 4 h after induction.
Lane 8: Lysate of JM109 cells containing pGEMEX HII, collected overnight after induction.

Fig. 6: Double diffusion test for confirming of immunogenicity of both HI (a) and HII (b) recombinant proteins. (1) Wells filled with lysate of JM109 cells without plasmid. (2) Wells filled with lysate of JM109 cell containing intact PGEMEX, collected overnight after induction. (3) Wells filled with lysate of JM109 cells containing recombinant PGEMEX, collected 4 h after induction. (4) Wells filled with lysate of JM109 cells containing recombinant PGEMEX collected overnight after induction. (a) Wells filled with serum of cystic hydatid patient. (Gray arrows indicate the precipitation of antigen and antibody complex)

**Double diffusion:** As shown in Fig. 6 the pellet of transformed cells which were collected 4 h and overnight after induction, reacts with patient’s serum antibody.

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Fig. 7: Immunoblot of Cell lysates without plasmid (1). Lysate of transformed bacteria containing intact pGEMEX, collected overnight after induction (2). Lysate of bacteria containing pGEMEX H1, collected overnight after induction (3). Lysate of bacteria containing pGEMEX H1, collected 4h after induction (4). Lysate of bacteria containing pGEMEX H1, collected overnight after induction (5). Lysate of bacteria containing pGEMEX H1, collected 4h after induction (6). (White arrows indicate visualized complex of antigen and antibody)

antigen-antibody precipitation arcs were seen in case of expressed genes, while this reaction were not seen with pellet of non transformed cells, or transformed cells with intact plasmid, as controls.

**Western blotting:** Figure 7 shows reactivity of recombinant proteins by immunoblotting, probed with patient’s serum.

**DISCUSSION**

Serology is an important tool for diagnosis of human cystic Echinococcosis and is primarily used to confirm a clinical diagnosis based on imaging methods. ELISA test with crude hydatid cyst fluid antigen are widely use for antibody detection and have also been applied to serological screening for asymptomatic cystic Echinococcus in endemic area (Craigie, 1993). Currently, there is no single standardized test used for the serological diagnosis of human CE even though hydatid cyst fluid antigens have been reasonably well characterized of which antigen 5 and antigen B are the most abundant lipoproteins in hydatid fluid (Leggate and Memanu, 1994). The subunits of Ag B show much greater specificity than antigen 5 (Ag5) for antibody binding in immunoblotting using native crude hydatid cyst fluid (Gottstein, 1995).

Recombinant subunits of Ag B cloned and expressed by many investigators and immunodiagnostic properties of these recombinant proteins were evaluated by them. McVie et al. (1997) cloned and expressed a 165 bp fragment derived from 12 KDa subunit of Echinococcus granulosus antigen B, purified the produced maltose binding fusion protein and evaluated that by ELISA. Rott et al. (2000) expressed two different subunits of Ag B from Echinococcus granulosus and compared their sequences together and evaluated their immunogenicity by ELISA. Ortina et al. (2000) compared and evaluated native and recombinant antigen B in the immunodiagnosis of human cystic Echinococcosis by ELISA, immunoelectrophoresis (IE) and Immunoblotting (IB).

Virginio et al. (2003) cloned and expressed a set of recombinant proteins of Echinococcus granulosus. Their purified recombinant proteins were tested in Enzyme-linked Immunosorbent Assay (ELISA) for specific IgG. Among of them, the recombinant antigen B subunit presented the highest sensitivity (93.1%), in reaction with sera from patients with cystic hydatid disease surgurally confirmed and specificity (99.5%).

In this study we successfully cloned and expressed two subunits of Echinococcus granulosus antigen B, which can be used in hydatidosis immunodiagnostic tests, after purification and serologic evaluation. This will promote the detection accuracy and efficiency, since the antigens are provided from parasite strains prevalent in our region and this will be beneficial, compared to ready to use kits, imported from other countries.

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**REFERENCES**


