Immunodiagnostic Value of *Echinococcus granulosus* Recombinant B8/1 Subunit of Antigen B

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ABSTRACT

Background: Cystic echinococcosis (CE), as a chronic parasitic disease, is a major health problem in many countries. The performance of the currently available serodiagnostic tests for the diagnosis of CE is unsatisfactory. **Objective:** The current study aimed at sub-cloning a gene, encoding the B8/1 subunit of antigen B (AgB) from *Echinococcus granulosus*, using gene optimization for the immunodiagnosis of human CE. **Methods:** The coding sequence for AgB8/1 subunit of *Echinococcus granulosus* was selected from GenBank and was gene-optimized. The sequence was synthesized and inserted into pGEX-4T-1 vector. Purification was performed with GST tag affinity column. Diagnostic performance of the produced recombinant antigen, native antigen B and a commercial ELISA kit were further evaluated in an ELISA system, using a panel of sera from CE patients and controls. **Results:** SDS-PAGE demonstrated that the protein of interest had a high expression level and purity after GST tag affinity purification. Western blotting verified the immunoreactivity of the produced recombinant antigen with the sera of CE patients. In an ELISA system, the sensitivity and specificity (for human CE diagnosis) of the recombinant antigen, native antigen B and commercial kit were respectively 93% and 92%, 87% and 90% and 97% and 95%. **Conclusion:** The produced recombinant antigen showed a high diagnostic value which can be recommended for serodiagnosis of CE in Iran and other CE-endemic areas. Utilizing the combination of other subunits of AgB8 would improve the performance value of the introduced ELISA system.

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INTRODUCTION

Echinococcus granulosus, in its larval stage, is the causative agent of cystic echinococcosis (CE), a zoonotic infection affecting both human and livestock (1) and a major health problem in different endemic areas of the world, including Iran (2-5), entailing heavy economic losses in the affected areas (3,6). Nearly 1.2 million people are affected by CE and the annual costs pertaining to the disease are estimated to be around 3 billion US$ for patients' management and damages to livestock productiveness (7). An early and accurate diagnosis of CE is conducive both to its management and treatment. Diagnosis of CE is mainly based on a combination of serological tests, along with imaging methods (6,8). Immunodiagnosis of CE has been considerably improved over the past two decades, improvements that are mainly due to the advancement in defining and synthesizing such immunodominant peptides as recombinant antigens. However, the immunodiagnosis of CE is still problematic and the performances of the available serological tests are not yet satisfactory (8-10).

Purified recombinant antigens and synthetic peptides, mainly derived from two parasite molecules, antigen B (AgB) and antigen 5 (Ag5), have been applied for the serological diagnosis of CE with various accuracy performances where the 8 kDa subunit of AgB has proven the most valuable antigen in native, recombinant or synthetic forms (8,11,12). During the last decades, recombinant DNA technology has been used for the heterologous expression of recombinant antigens in *E. coli* and their application to the immunodiagnosis of infectious diseases (13-15). Owing to their low cost and reproducibility, recombinant proteins have drawn significant attention. However, a few drawbacks such as the differences in codon usage between species may result in the reduction of protein's yield. Several studies have indicated that gene optimization increase the yield of protein expression (16,17). In the current study, the gene encoding the B8/1 subunit of antigen B from *E. granulosus* genotypes 1 was sub-cloned in *E. coli*, using gene optimization for high-throughput protein expression. The produced recombinant antigen was evaluated in an ELISA system for the immunodiagnosis of human hydatid disease.

MATERIALS AND METHODS

Gene optimization, synthesis and cloning. Sequence data was obtained from NCBI GenBank with the accession number DQ372074. Gene optimization of the AgB8/1, without signal sequence for heterologous expression in *E. coli*, was performed using Optimum Gene™ Algorithm (Genscript, New Jersey, USA). Gene synthesis was ordered from Biomatik Company (Biomatik, Ontario, Canada). The optimized AgB8/1 gene was cloned in pBluescript II SK(+) (Biomatik, Ontario, Canada) vector and excised by restriction enzymes EcoRI and XhoI according to the manufacturer's instructions. So as to confirm the size of the fragments, the product was further checked with agarose gel electrophoresis. Subsequently, the band corresponding to the AgB8/1 coding sequence was excised from the gel and purified through the use of gel extraction kit (Bioneer, Korea). Next, the purified fragment was cloned into pGEX-4T-1 (GE Healthcare, Illinois, US) expression vector. Plasmids transformation into the DH5α cells was performed using standard $CaCl_2$ method followed by plasmid extraction, employing QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's

guideline. After that, a new round of transformation was performed to transfer the plasmids into BL21 (DE3)pLysS cells for expression under the T7 promoter. CE AgB8/1 was expressed in fusion with N-terminus GST tag in BL21(DE3) pLysS *E. coli* strain by 0.5 mM IPTG (Invitrogen, USA) induction for 4 hours.

Purification of the recombinant protein. The produced *Echinococcus granulosus* recombinant AgB8/1 antigen (rEgAgB8/1) was purified via immunoaffinity column for GST-tag (Sigma-Aldrich, Germany), according to the manufacturer's instructions as to the native purification procedure. Digestion with thrombin was made for the removal of the tag. Purified protein was desalted overnight using a dialysis membrane in PBS (pH: 7.5) (14). Native AgB was purified as previously described (18).

SDS-PAGE and western blotting analysis of the recombinant protein. We carried out the SDS-PAGE of the cleaved and uncleaved recombinant proteins and the native protein (native antigen B purified from hydatid cyst fluid) in 18% (w/v) polyacrylamide gel containing 0.1% SDS. Protein samples (with and without GST tag) were mixed, with reducing sample buffers, and boiled in a water bath, for 5 minutes (14). Gel was run at a constant voltage of 200 V and transferred to nitrocellulose membranes (GE Healthcare) using a Mini Trans-blot Cell (Bio-Rad, Berkeley, California). The recombinant proteins were evaluated by use of the pooled sera of CE patients at 1:1000 dilution. Secondary horseradish peroxidase conjugated anti-human IgG (Sigma, USA), was diluted at 1:4000 dilution. Bound proteins were developed utilizing diaminobanzidine (DAB) substrate.

Serum samples. Serum samples of 30 pathologically-confirmed CE patients were obtained from university affiliated hospitals in Shiraz, southern Iran. The cysts locations in the patients were lungs, liver, lungs along liver, spleen (one case), or bladder along pelvis (one case). Samples were also collected from 32 patients with parasitic diseases other than hydatid cyst, including giardiasis (n=3), hymenolepiasis (n=3), toxocariasis (n=3), toxoplasmosis (n=3), fascioliasis (n=3), malaria (n=2), cryptosporidiasis (n=2), trichostrongyliasis (n=1), and patients with autoimmune diseases (n=9, including 3 with vitiligo, 3 with Behçet's disease and 3 with systemic lupus erythematosus). Laboratory diagnosis of the parasitic diseases was based on the microscopic findings, with the exception of toxocariasis and toxoplasmosis which were based on serology, using *Toxocara* IgG ELISA (IBL International Gmbh, Hamburg, Germany) and *Toxoplasma* IgG ELISA (Yekta Tajhiz, Iran). Moreover, sera were collected from 30 healthy controls with no sign, symptoms or history of CE. Based on the tests, the healthy controls were all negative for anti-hydatid cyst antibodies. The study was approved by Ethics Committee of Shiraz University of Medical Sciences and informed consent was taken from the subjects prior to collecting the samples.

ELISA with native and recombinant antigens. ELISA was performed in flat-bottom 96-well microplates (Guangzhou Jet Bio-Filtration, Guangzhou, China). The microplate were coated with 100 µl/well of either rEgAgB8/1 or native antigen B (nAgB) with a concentration of 5 µg/mL in 0.1 M carbonate/bicarbonate (pH 9.6) buffer through an overnight incubation at 4˚C. Plates were blocked with 300 µl of 5% non-fat skimmed milk in phosphate-buffered saline with 0.1% (V/V) Tween 20 for 2 hours at 37˚C. The wells were then washed 5 times with 300 µl of the washing buffer (PBST; PBS containing 0.1% Tween 20). Serum samples were diluted (1:100) in PBST, applied to the plates and incubated at 37˚C over a period of 2 hours. The plate was washed as before and 100 µl aliquots of HRP-conjugated goat anti-human IgG (Sigma, USA) at a 1:4000 dilution in PBST were added to the plates and incubated for 1 hour at room

temperature. After washing, the plates were incubated with substrate (100 µl/well of 0.4 mg/ml OPD, 0.3% H_2O_2 in 0.1 M citrate buffer, pH 5) for 20 minutes and the reaction was stopped by 1 N H_2SO_4. The absorbance at 450 nm was monitored with a microplate reader (Bio-Tek, ELx800). The cutoff point was set at 2SD from the mean of control samples. The ELISA was also performed with tagless and also GST tag-containing rEgAgB8/1.

ELISA with commercial kit. All the sera samples were further tested by a commercial ELISA kit (Euroimmune, Germany) for the detection of anti-hydatid cyst antibodies based on the manufacturer's instructions.

Statistical analysis. The diagnostic accuracy of the native antigen B, rEgAgB/1 and commercial kit were assessed by the area under the receiver operating characteristic (ROC) curve (AUC) using SPSS software (ver. 18). Cutoff values were set for the test according to Youden's Index. Cohen's kappa statistic was used to find out the extent of agreement among the ELISA systems, using different antigens and the commercial kit.

RESULTS

In the present study, the gene encoding a B8/1 subunit of antigen B from *E. granulosus* was sub-cloned in *E. coli*, with high-throughput protein expression. The produced recombinant antigen was evaluated in an ELISA system for the serological diagnosis of human CE.

Expression and purification of rAgB8/1.

A fragment of *E. granulosus* AgB8/1, without the signal sequence, was synthesized and cloned into pGEX-4T-1 expression vector, under the control of T7 promoter, and transformed into the BL21 (DE3) pLysS expression host. The sequencing of the vector confirmed the correct reading frame and the insertion of the coding sequence in the GST-tag frame. REgAgB8/1 protein was expressed in LB as the culture medium. Furthermore, SDS-PAGE analysis confirmed the expression of the recombinant protein with the expected molecular weight of 34 kDa (Figure 1).

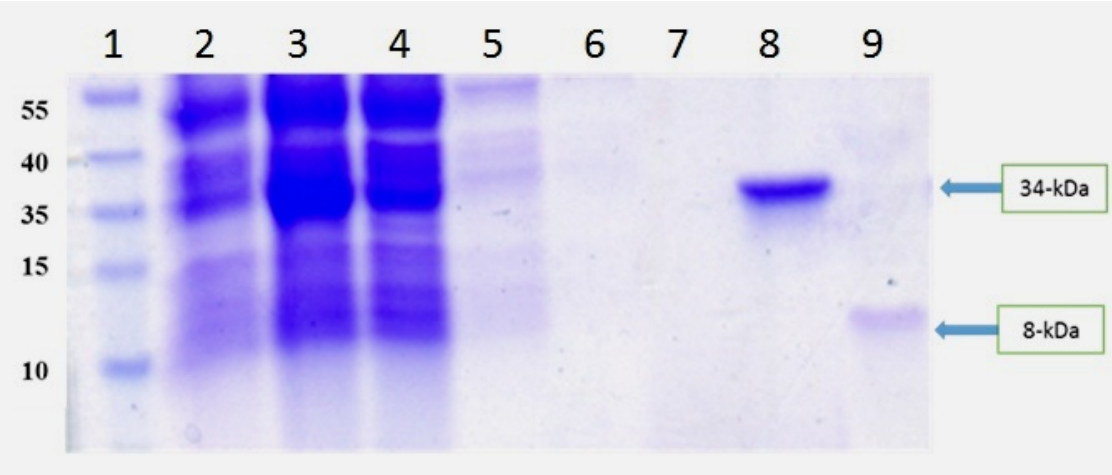

Figure 1. SDS-PAGE analysis of the rEgAgB8/1 protein expression and purification. Line 1 Protein weight marker; Line 2 and 3, uninduced and induced cultures of *E. coli* by IPTG; Line 4 prewash; Line 5, 6 and 7 consecutive washes; Line 8 final elution (GST-AgB8/1); Line 9 cleaved.

Western blot analysis of rEgAgB8/1 expression in *E. coli.*

Western blotting analysis confirmed the reactivity of the produced recombinant antigen with the sera of CE patients (Figure 2) in both the tagged and tagless types.

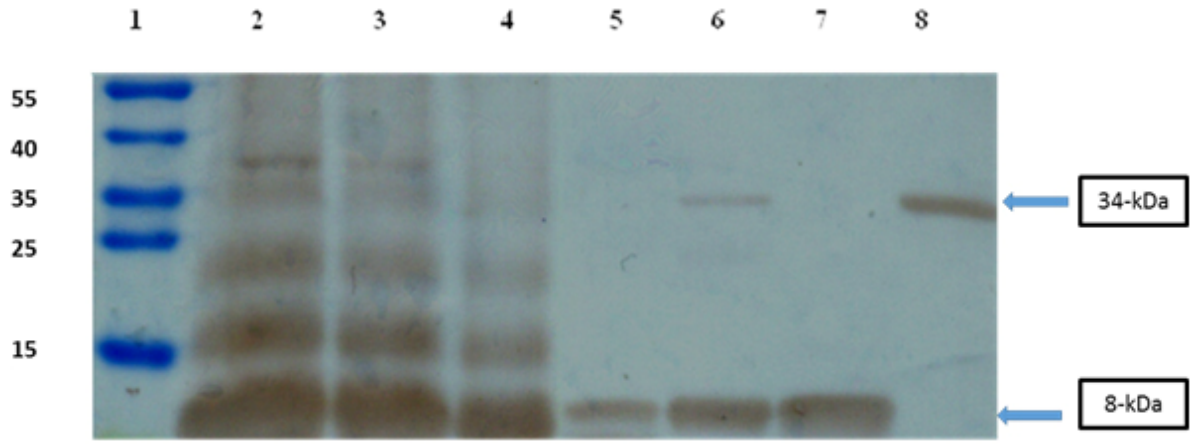

Figure 2. Western blot analysis of rEgAgB8/1 expression in *E. coli.* Line 1: molecular weight marker; Lines 2, 3 and 4 native AgB with 20, 10 and 5 µg/well respectively; Lines 5, 6 and 7 rEgAgB8/1; Line 8 rEGAgB8/1 fused with GST tag.

Purification of rEgAgB8/1 by anti-GST-tag immunoaffinity column.

The concentration of the purified rEgAgB8/1 prior to the cleavage of tag with thrombin was 3 mg/mL; following the cleavage, on the other hand, the concentration was 1 mg/mL. Appraising the purity of the purified proteins with SDS-PAGE, it became clear that no unwanted proteins were present in the elutes, (Figure 1).

Evaluation of rEgAgB8/1 for the diagnosis of human CE.

Both pure rEgAgB8/1 (without tag) and GST-containing rEgAgB8/1 were employed in the ELISA system for the detection of anti-hydatid cyst antibodies in the sera of CE patients and controls. Using ELISA checkerboard titration method, the optimal concentration of the rEgAgB8/1 antigen (both the tagless and GST-containing forms), serum and HRP-conjugated goat anti-human IgG were found to be 5 µg/ml, 1:100 and 1:4000, respectively. Using rEgAgB8/1 in ELISA system, 28 out of 30 CE patients (93.3%) were found to be positive and in the controls, 5 cases (8.1%) were false positive. Therefore, the sensitivity and specificity of the produced rEgAgB8/1 antigen for the diagnosis of human CE were 93% and 92%, respectively. Table 1 demonstrates the performance of rEgAgB8/1, in the diagnosis of human CE.

Table 1. Performance of ELISA, using recombinant Ag B8/1, in serodiagnosis of CE.

Type of serum	Number	Number of positive cases in rEgAgB8/1- ELISA	
		Number	Percentage (%)
CE patients	30	28	93
Vitiligo	3	0	0
Behcet's disease	3	0	0
Lupus	3	0	0
Giardiasis	3	0	0
Hymenolepiasis	3	0	0
Toxocariasis	3	0	0
Toxoplasmosis	3	1	33
Fascioliasis	3	1	33
FUO	3	0	0
Malaria	2	0	0
Cryptosporidiasis	2	0	0
Trichostrongyliasis	1	0	0
Normal	30	3	10

Evaluation of native antigen B for the diagnosis of human CE.
Using native AgB, 26 out of 30 CE patients (86.7%) were found to be positive and 6 cases (9.7%) in the controls were false positive. Accordingly, the sensitivity and specificity of this antigen were 87 and 90%, respectively. Table 2 illustrates the performance of native AgB regarding the diagnosis of human CE.

Table 2. Performance of ELISA, using native antigen B, in serodiagnosis of CE.

Type of serum	Number	Number of positive cases in native antigen B - ELISA	
		Number	Percentage (%)
CE patients	30	26	87
Vitiligo	3	0	0
Behcet's disease	3	0	0
Lupus	3	0	0
Giardiasis	3	1	33
Hymenolepiasis	3	0	0
Toxocariasis	3	0	0
Toxoplasmosis	3	1	33
Fascioliasis	3	1	33
FUO	3	1	33
Malaria	2	0	0
Cryptosporidiasis	2	0	0
Trichostrongyliasis	1	0	0
Normal	30	2	7

Evaluation of commercial ELISA kit for the diagnosis of human CE.
Using commercial ELISA kit, 29 out of 30 CE patients (96.7%) were found to be positive and 3 control cases (4.8%) were false positive. In this regard, the sensitivity and specificity of the commercial kit were respectively 97% and 95%. Table 3 shows the performance of commercial ELISA kit and Table 4 compares the performances of rEgAgB8/1, native AgB and commercial ELISA in the diagnosis of human CE.

Table 3. Performance of commercial ELISA kit in serodiagnosis of CE.

Type of serum	Number	Number of positive cases in commercial ELISA kit	
		Number	Percentage (%)
CE patients	30	29	97
Vitiligo	3	0	0
Behcet's disease	3	0	0
Lupus	3	0	0
Giardiasis	3	0	0
Hymenolepiasis	3	0	0
Toxocariasis	3	1	33
Toxoplasmosis	3	0	0
Fascioliasis	3	0	0
FUO	3	0	0
Malaria (p.vivax)	2	1	33
Cryptosporidiasis	2	0	0
Trichostrongyliasis	1	0	0
Normal	30	1	3

Based on the statistical analysis of the data, there existed a good agreement (kappa=0.74) between the three systems using native AgB, rEgAgB8/1 and commercial kit (kappa=0.88 between rEgAgB8/1 and native AgB, kappa=0.76 between native AgB and commercial kit, and kappa=0.83 between rEgAgB8/1 and commercial kit).

Table 4. Comparative performances of recombinant AgB8/1, native AgB and commercial kit in the serodiagnosis of CE.

Type of antigens	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Recombinant AgB8/1	93(76-98)	92(81-96)	85(67-94)	96(87-99)
Native AgB	87(68-95)	90(79-96)	81(63-92)	93(83-98)
Commercial kit	97(81-100)	95(85-99)	90(74-97)	98(90-100)

DISCUSSION

CE is known as a disease mostly remaining in an asymptomatic state during the primary years of infection (6). Since a late diagnosis entails severe implications with the severity of the outcomes, early diagnosis of the disease is crucial. Diagnosis of CE is mostly made performed through such imaging modalities as ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI) (6,19). Further employed in diagnosing CE are serological tests with specific antigens of the parasite (8,10,20-22). Serological methods can be used for the confirmation of the results of imaging and also to diagnose the disease in subclinical stages. These methods suffer from not being standardized, resulting in poor specificity or sensitivity. Hence the developing a reliable test with reasonable validity for routine laboratory diagnosis of CE is necessary. Recent studies have been directed toward the use of recombinant antigens (11,12,23,24). In order to develop an immunodiagnostic assay in any regions, world health organization has recommended the use of antigens corresponding to the sequences obtained from the same region. Accordingly, the current study was designed to produce a recombinant antigen of *E. granulosus* for serodiagnosis of CE in Iran. It has been reported that AgB8/1 is one of the most appropriate antigens for the development of any immunoassay as far as diagnosing CE is concerned (8,12,19). Owing to the fact that the presence of signal sequence residues of 17 to 81 of AgB8/1 affects the heterologous expression of the protein, interferes with its collection in the cytoplasm and influences the immunoreactivity of the antigen, we removed these parts from the sequence to optimize the heterologous expression of the antigen (24). In the present research, high levels of heterologous AgB8/1 expression were achieved using several approaches. Firstly, gene optimization and gene synthesis were applied so as to surmount issues such as codon usage bias and differences between *E. coli* as a bacterium and *E. granulosus* as a eukaryote. The former problem poses challenges concerning the heterologous expression of recombinant proteins in biotechnology applications. A number of other studies have successfully used this approach to overexpress the protein of interest in a heterologous host (16,17). In the present study, we were able to obtain a relatively high-throughput expression level of 3 mg/mL of purified protein. Without gene optimization, the yield of recombinant protein would be much lower in comparison to what we have achieved in the current study. Secondly, we used GST tag as a fusion partner in the amino tail of the protein which, by itself, may ameliorate the expression of the protein (25). Beyond the use of this fusion partner as a protein purifier, it is always possible that fusing the N-terminus of recombinant proteins with

one of the abundant proteins of the host augment the expression of its gene, thereby accelerating the translation (14). Therefore, it may positively induce the level of protein expression and improve the production yield of the recombinant protein. Furthermore, it conduces to the increase in the solubility of the recombinant protein, reducing the formation of inclusion bodies and, in the process, the toxicity of the recombinant proteins to the host cells. Also noteworthy is the facile purification of the recombinant protein with GST-tag columns. In the current study, we followed the protocol for native purification, conducive to maintaining the native structure of a protein. Native purification pre-empts the denaturing of the protein, an event which may lead to the loss of certain important conformational epitopes of the antigen. The tag was successfully used for the purification of recombinant antigens where we obtained a soluble expression of recombinant proteins with high yield. In this method, the tag can be easily removed through enzymatic cleavage by thrombin enzyme.

Sensitivity and specificity of the rEgAgB8/1-based ELISA in the current study were 93% and 92%, respectively, which implies its dominance, regarding accuracy, comparisons with the commercial kits which, not infrequently, employ a combination of several recombinant antigens. Similar performance have been reported by Mohammadzadeh *et al.* in which sera samples from CE patients in Iran, Turkey, China and Japan were tested by a recombinant antigen B8/1 (12). A study by Kalantari *et al.* reported the production and use of recombinant antigen B for the diagnosis of hydatid cysts in Iran. Using AgB8/4 subunit of antigen B, they observed 91.66% sensitivity and 97.22% specificity (23). There exist, however, a number of differences between their study and ours. Firstly, in the latter, AgB8/1, known as the most appropriate subunit of antigen B for the diagnosis of CE, was produced and used while in the former AgB8/4 subunit was generated and utilized. Secondly, we used gene optimization to enhance the heterologous expression of recombinant protein of interest. Another difference is that, in the present study, we used GST-tag affinity columns for the native purification of the produced recombinant antigen; in the other study, on the other hands, his-tag affinity (a denaturing protocol) was used for the purification (23).

It is concluded that gene optimization enhances the heterologous expression of the rEgAgB8/1 antigen in *E. coli*. GST-Tag fusion is a valuable option for the purification of the produced antigen which in turn contributes to the high expression level of the antigen. The rEgAgB8/1 antigen have a higher diagnostic value, in comparison to the native AgB and can be recommended for the serodiagnosis of CE in Iran and, perhaps, other CE-endemic areas. Combining other subunits of AgB8 with the current recombinant antigen would potentially improve the performance value of such antigen.

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