Parasitol Res (2010) 106:847–851
DOI 10.1007/s00436-010-1726-0

ORIGINAL PAPER

Application of recombinant *Echinococcus granulosus* antigen B to ELISA kits for diagnosing hydatidosis

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Received: 31 October 2009 / Accepted: 4 January 2010 / Published online: 9 February 2010

Abstract *Echinococcus granulosus* causes human cystic echinococcosis as an important public health problem in many regions of the world. There are some problems in primary diagnosis such as cross-reaction with sera from patients with other parasitic disease in serological tests. The use of an appropriate source of antigenic material is a very important and crucial point in the improvement of the serodiagnostic features such as enzyme-linked immunosorbent assay (ELISA) method. We expressed and purified recombinant AgB of *Echinococcus granulosus* and used as antigen in ELISA method. Serum samples were given from 36 cystic hydatid disease patients that have been confirmed by surgical operation as well as 36 healthy individuals sera were tested by ELISA method using recombinant AgB and compared with commercial kit (Euroimmun) for specificity and sensitivities value. The sensitivity of 91.66% and specificity of 97.22% were determined by homemade kit.

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Introduction

Echinococcus granulosus is a species of hydatid tapeworm causing human cystic echinococcosis, which is an important public health problem worldwide.

Cystic hydatid disease (CHD) is detectable clinically through various imaging techniques such as ultrasonography or radiology. The primary diagnosis must be confirmed by more specific testing, such as serological tests based on the discovery of antibodies against the organismal antigens in the patient's serum (Grimm et al. 1998). Ordinary serological tests such as immunoelectrophoresis, double diffusion in agar, or indirect hemagglutination are being replaced by more sensitive assay methods such as enzyme-linked immunosorbent assay (ELISA), immunoblot (IB), and indirect immunofluorescent antibody test (IFA) (Virginio et al. 2003). ELISA is a high-sensitivity test that is strongly recommended for the detection of specific antibodies in human CHD cases (Parija 1998; Paul and Stefaniak 2001).

The main problems in the serodiagnosis of echinococcal diseases are the often unsatisfactory performance of the available tests and the difficulties associated with the standardization of antigenic preparations and techniques. To overcome them, highly sensitive and specific antigens and antigenic components derived from different developmental stages of *E. granulosus* must be available. The use of an appropriate source of antigenic material is crucial in the serodiagnostic tests (Carmena et al. 2006).

One of the major antigenic components of hydatid cystic fluid is *E. granulosus* antigen B (EgAgB). It is a polymorphic protein of 120–160 kDa that dissociates under reducing conditions into 8/12, 16, and 20/24 kDa subunits (Fernbndez et al. 1996), suggesting that it is composed of multimers of 8 kDa subunits (Carmena et al. 2006; Lightowlers et al. 1998). Rott et al. (2000) have recognized specific antibodies against two subunits of the antigen (AgB8/1 and AgB8/2) by western blotting, and peptide sequencing revealed that both subunits are components of the smallest subunit of native EgAgB (González et al. 1996).

The quality of the parasitic antigens and the characteristics of the serum panel obtained from patients can affect the efficiency (sensitivity and specificity) of any immunodiagnostic test for human CHD. EgAgB is considered to have high sensitivity and specificity in serodiagnostic tests (Chemale et al. 2001). To obtain specific antigens, the EgAg-encoding genes can be cloned and expressed in heterologous systems. Because of the ease in producing and purifying specific recombinant antigens, they may be less prone to cross-reactivity with other parasitic antigens (Virginio et al. 2003). Consequently, *E. granulosus* genes encoding two different AgB subunits have been cloned and expressed in *Escherichia coli*. The recombinant antigens were tested by total IgG ELISA with a panel of sera from patients (McVie et al. 1997; Virginio et al. 2003). A 165-bp deoxyribonucleic acid (DNA) fragment derived from the 12 kDa subunit of EgAgB was cloned in expression vector pMal-c2. A 52 kDa maltose-binding AgB fusion protein (rAgB. MBP) was expressed and purified on an amylase–Sepharose 6B column and used as an antigen in IB, ELISA, and dot-ELISA (McVie et al. 1997). Martin et al. (1996) have determined the presence of specific serum antibodies against a recombinant *E. granulosus* antigen, designated myophilin, by Western blotting and ELISA. Pazoki et al. (2006) amplified two different sequences of EgAgB by reverse transcription-polymerase chain reaction; the amplified fragments (HydI and HydΠ) were cloned in PTz57R and subcloned in expression vector pGEMEX-1. The recombinant plasmids (HydI/pGEMEX and HydΠ/pGEMEX) led to the production of fusion proteins of 32–36 kDa. A recombinant 8-kDa subunit of antigen B from *Echinococcus multilocularis* (EmAgB8/1) was compared with an 8-kDa subunit of EgAgB (EgAgB8/1) by western blotting and ELISA (Mamuti et al. 2004).

As evident from the recent literature, recombinant proteins and synthetic peptides are more reliable for serodiagnostic purposes than native antigens and their purified subunit fractions. The aim of this study was to examine the application of recombinant EgAgB (HydI) as an antigen in ELISA for the diagnosis of hydatidosis in Iranian patients.

Materials and methods

Recombinant plasmid pQE-30 (Taghipour et al. 2009) containing the *HydI* gene (accession no. DQ835667) was transformed in *E. coli* Bl21-competent cells. The recombinant plasmid was extracted and confirmed by restriction digestion using *Sac*I and *Hind*ΙΙΙ.

Protein purification

A colony containing the recombinant plasmid was preinoculated in Luria–Bertani (LB) medium and subcultured the next day in culture media until an optical density at 600 nm (OD_{600}) of 0.7 was observed, followed by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h at 37°C. The bacterial samples were given off before and after the induction and analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For protein purification, a colony containing the recombinant plasmid was preinoculated in LB medium and subcultured the next day in 50 ml culture media until an OD_{600} of 0.7 was observed, followed by induction with 1 mM IPTG for 5 h at 37°C. The cells were harvested by centrifugation (8,000 rpm for 10 min at 5°C), and the resultant pellet was resuspended in 5 ml equilibration buffer (0.4 M urea, 50 mM Tris, and 0.5 M NaCl) and sonicated (5×10 s) on ice. The resultant cell lysate was centrifuged at 10,000 rpm for 5 min at 5°C, suspended in 2 ml equilibration buffer, and then incubated overnight at 5°C. It was centrifuged again the next day at 10,000 rpm for 5 min at 5°C. The clear supernatant was collected and used for protein purification (Bandehpour et al. 2006). The recombinant protein was purified by affinity chromatography, based on its *N*-terminal His_6 tag. The cell lysate was applied to the column at a flow rate of 15 drops/min and allowed to bind. The bound protein was eluted with elution buffer (4 mM urea, 50 mM Tris, 0.5 M NaCl, and 1 mM imidazole).

Western blot analysis

The recombinant protein was transferred by 12% SDS-PAGE onto a nitrocellulose membrane and detected by the His-tag monoclonal antibody. Western blotting was performed as described previously (Campbell et al. 2001). In brief, the membrane was incubated with the His-tag monoclonal antibody as the primary antibody (1:500) and goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate as the secondary antibody (1:1,000) and

Table 1 Summary of the results of specificity with sera from non-CHD patients obtained in total IgG ELISA against the recombinant antigen B

Non-CHD sera	Number	Positive reaction	Specificity (%)
Tuberculosis	5	2	60
Kala azar	5	2	60
Toxoplasmosis	3	1	66
Hepatitis B (Hbs Ag)	6	0	100
Hepatitis C(Hcv Ag)	6	1	66
Total	25	6	80.6

Specificity for home made kit=(25/25+6)×100=80.6

detected by colorimetry with diaminobenzoic acid (DAB) and hydrogen peroxide (H_2O_2).

ELISA

Serum samples were obtained from 36 patients with CHD, confirmed surgically. Five samples from patients with tuberculosis (*Mycobacterium tuberculosis* infection), five samples from patients with kala azar (*Leishmania donovani* infection), three samples from patients with toxoplasmosis (*Toxoplasma gondii* infection), six samples from hepatitis B surface antigen (HbsAg)-positive patients, and six samples from hepatitis C virus-positive patients, confirmed by serodiagnostic assay or clinically by other research centers, were selected. We examined all the samples with ELISA to confirm that they were negative for CHD. Sera from 36 healthy individuals were also included in this study as negative controls; the donors had no recent history of parasitic disease. ELISA was performed with recombinant EgAgB and a standard kit (Euroimmun) for all the samples to compare the specificity and sensitivity values.

Microplates were coated with 2 μg/well of each recombinant antigen (in 100 μL volume) diluted in 0.1 M carbonate/bicarbonate buffer (pH9.6) for 13 h at 4°C (Bora et al. 2002). After three washes, the microplates were coated with a blocking solution (1% bovine serum albumin, 300 μL) diluted with phosphate-buffered saline (PBS) for 1 h and 30 min at room temperature. After three washes, the serum samples were coated, diluted (1:400) in PBS containing 0.1% Tween-20, and vibrated for 2 h at room temperature. They were then coated with anti-human conjugate at a dilution of 1:10,000 as the second antibody for an hour on the vibrator in the dark. *O*-phenilendiamine dihydrochloride (0.4 mg/ml) in 0.1 M phosphate/citrate buffer (pH5) and H_2O_2 were added to deionized water and used to visualize the antigen–antibody reaction. The OD was registered at 490 nm (OD_{490}) after the addition of a stop solution (1 N H_2SO_4). The mean±2 SD of the absorbance reading of the 36 healthy individuals at 0.47 was considered as the cut-off for positive reactions. The sensitivity and specificity were expressed as

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{number of false negatives}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{number of false positives}}$$

Results

Recombinant EgAgB (24 kDa) was expressed in the PQE-30 plasmid and purified. The purified protein was coated onto ELISA microplates and tested in an anti-IgG ELISA with sera from patients with or without CHD and healthy individuals.

The cut-off value (0.47%), differentiating positive and negative reactions, sensitivity (91.66%), and specificity (97.22%) were determined by SPSS analysis (SPSS, Inc., Chicago, IL, USA). There were three negative reactions among

Table 2 Summary of the results of specificity with sera from non-CHD patients obtained in total IgG commercial ELISA kit

Non-CHD sera	Number	Positive reaction	Specificity (%)
Tuberculosis	5	0	100
Kala azar	5	0	100
Toxoplasmosis	3	0	100
Hepatitis B (Hbs Ag)	6	0	100
Hepatitis C (Hcv Ag)	6	1	83
Total	25	1	96.1

Specificity for commercial kit=(25/25+1)×100=96.1

the 36 positive sera with recombinant EgAgB. The non-CHD sera, considered for calculating the specificity values, were determined in 60–83% of the cases (Table 1). All the samples were tested with a commercial kit (Euroimmun). The sensitivity was reported to be 100% for the patients with CHD and the control group, and the specificity was calculated for the patients without CHD (Table 2).

$$\text{The specificity of the new kit} = (25/25 + 6) \times 100 = 80.6$$
$$\text{The specificity of the commercial kit} = (25/25 + 1) \times 100 = 96.1$$
$$\text{The specificity of the new kit} = (36/36 + 3) \times 100 = 92.3$$

Discussion

In this study, we used recombinant EgAgB for the detection of circulating specific antibodies in patients with CHD. Recombinant antigens are considered to be diagnostically valuable, with reliable sensitivity between 79% and 91.7% (Ortona et al. 2000; Virginio et al. 2003). Mamuti et al. (2004) have reported that recombinant EgAg8/1 showed positive reactions with 88% (44/50) of the serum samples from patients with CHD. In our study, the antigenic activity of the recombinant antigen in ELISA was positive with 91% (33/36) of the serum samples from the patients with CHD, as seen in previous studies.

Mcvie et al. (1997) reported that rAgB.MBP showed approximately 65% sensitivity by ELISA from patients with CHD, which is less than the sensitivity of our method. Recombinant EgAgB8/1 presented a better-than-discriminatory value in total IgG ELISA in comparison with the native antigen tested (Rott et al. 2000; Virginio et al. 2003). Nevertheless, in our research, native EgAgB from the commercial ELISA kit had better sensitivity (100%) than the fusion protein (92.3%). The lower diagnostic sensitivity of recombinant EgAgB8/1 is probably due to the presence of other subunits (Chemale et al. 2001; González et al. 1996). Differences in the expression of the subunits among different hydatid cysts could also produce antigenic preparations with different diagnostic values (Chemale et al. 2001). Despite these differences, an AgB subunit (8 kDa) has been described as specific for *E. granulosus*, with some cross-reactivity reported by other authors (Ferreira and Zaha 1990; Rott et al. 2000). By extending these previous results, we have shown the high specificity of recombinant EgAgB in relation to normal sera but not to sera from the patients with CHD (80.6%). Among the 36 serum samples utilized in this study, only three showed no reaction in total IgG ELISA with the recombinant protein.

Some authors have suggested that serodiagnosis of CHD can be improved by using various Ig classes and that the problem of low specificity due to cross-reactivity with sera from patients with other parasitic diseases will be reduced by using IgG subclass detection. Because of the association-specific response to antigens of *E. granulosus*, IgG4 would be the subclass of choice for serodiagnostic tests of complementary value in relation to a total IgG assay (Grimm et al. 1998; Ioppolo et al. 1996; McVie et al. 1997; Virginio et al. 2003; Wen and Craig 1994).

According to our results, recombinant EgAgB (HydI) has good performance in total IgG ELISA for the detection of antibodies in sera from patients with CHD. In that regard, we searched among the antigens to elucidate the possibility that the recombinant antigen can show improved sensitivity. Although the potential role of antigens in diagnosis cannot be omitted, it seems that for some patients and particular stages of the disease, the limiting factor is the actual existence of a measurable antibody response (Lorenzo et al. 2005).

In conclusion, the current strategies for immunodiagnosis of CHD need reappraisal. To improve their diagnostic yield, studies must be undertaken to standardize the techniques and antigenic preparations, characterize new antigens, produce recombinant antigens, and detect distinct Ig classes (Ortona et al. 2000).

Acknowledgments The authors thank the Vice Chancellor for Research of Shahid Beheshti University, M.C. for support of this study and the directors of the Cellular and Molecular Biology Research Center, in which this study was performed.

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