

## Original Article

# Application of Western Blotting Using Native Antigen B for Serodiagnosis of Human Cystic Echinococcosis

B Sarkari<sup>1</sup>, \* SM Sadjjadi<sup>1</sup>, H Abidi<sup>1</sup>, A Izadpanah<sup>2</sup>, S Kazemian<sup>1</sup>, A Rafati

<sup>1</sup>Dept. of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>2</sup>Dept. of Surgery, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

(Received 2 May 2007; accepted 30 Jun 2007)

### Abstract

**Background:** Hydatidosis is one of the most important helminthic diseases causing serious health and economic problems in many countries including Iran. Currently available diagnostic approaches for diagnosis of cystic echinococcosis (CE) are not satisfactory since they have not achieved a reasonable validity in diagnosis of CE. This study aimed to assess the performances of Western blotting (WB), using native antigen B, for serological diagnosis of human hydatidosis.

**Methods:** Antigen B was prepared from sheep hydatid cyst fluid. Serum samples obtained from 40 surgically proven cases of hydatidosis along with serum samples from non-hydatidosis patients and samples from healthy persons were tested by immunoblotting, using native antigen B.

**Results:** From 40 sera of hydatidosis patients, 32 cases (80%) detected 8 kDa subunit, 29 cases (72.5%) recognized the 16 kDa component and 29 cases (72.5%) detected 24 kDa subunit of antigen B. The highest sensitivity (80%) was achieved with 8 kDa subunit of antigen B while the other components (16 and 24 kDa) showed a lower sensitivity (72.5%). Samples from healthy controls and non-hydatidosis patients did not yield any detectable band in the Western blotting assay. Accordingly, specificity of the system was found to be 100%.

**Conclusion:** Considering the use of native antigen B, application of western blotting, based on 8 kDa subunit of antigen B, is highly recommended for the confirmatory diagnosis of hydatidosis.

**Key words:** Western blotting, Antigen B, Serodiagnosis, Hydatid cyst

### Introduction

Cystic echinococcosis (CE) caused by *Echinococcus granulosus*, is a cosmopolitan parasitic diseases. It is one of the most important helminthic diseases in the Middle East including Iran (1). The clinical symptoms of hydatidosis are unclear in some circumstances; therefore suitable tests are needed for accurate diagnosis of CE. Diagnosis of CE is mainly based on serological approaches as well as various imaging techniques (2). Among the serological methods, countercurrent immunoelectrophoresis (CCIEP), using crude hydatid cyst fluid and ELISA have been used in different laboratories. In spite of the development of sensitive and specific techniques,

such as ELISA, the immunodiagnosis of CE in clinical practice remains problematic. The main difficulty is that most available screening tests give a high percentage of false negative or false positive results (up to 25%) (3). Using crude hydatid cyst fluid (HCF) as antigen in diagnostic assays reduces the specificity of the tests since HCF contains various metabolites of host and parasite. To circumvent this problem component of HCF including antigen B (AgB), obtained from various host species in different endemic areas, has been used in different diagnostic system for serodiagnosis of hydatidosis (4-8). Nevertheless, the nature and quality of the AgB in hydatid cyst fluid is variable among the host species and this is one of the reasons that different laboratories

obtain different results using AgB prepared from different host species. Antigen B has been used in ELISA system for diagnosis of CE with reasonable sensitivity and specificity (9, 10). However immunoblotting system seems to be a more accurate and confirmatory approach for diagnosis of CE since it detects the antibody produce against any or all components of antigen B.

Here we prepared native antigen B from the HCF obtained from infected sheep liver at a local slaughterhouse in Shiraz, Fars Province, and evaluated its efficacy for diagnosis of surgically proven cases of hydatidosis in an immunoblotting system.

## Materials and Methods

### *Serum samples*

A total of 150 sera samples from surgically and pathologically proven CE patients (40 samples), healthy controls (70 samples) and samples from non-CE patients (consisting of 9 fasciolosis, 2 taeniasis, 11 toxocariasis (kindly provided by Prof. A Dalimi) and 20 malignancies) were the subjects of this study. Serum from patients with malignancies was collected from those patients who underwent surgery because of malignancies in Shahid Faghihi Hospitals in Shiraz. Healthy control samples were collected from people with non infectious diseases referred to health centers in Shiraz. The proposal was approved by Ethical Committee of our institution and informed consent was taken from subjects before collecting samples.

### *Preparation of antigen B*

Hydatid cyst fluid was obtained from a local slaughterhouse in Shiraz, Fars Province, from infected sheep liver. Antigen B was prepared from HCF as earlier described (11). Briefly; 100 ml of HCF was dialyzed overnight against 5 mM acetate buffer (pH 5) at 4°C. Sample was centrifuged (50,000 g for 30 min), supernatant was removed and the pellet was dissolved in 0.2 M phosphate buffer (pH 8). Saturated ammonium sulfate was used to remove the globu-

lin from the sample. Finally the sample was boiled in a water bath for 15 min and centrifuged at 50, 000 g for 60 min to separate heat stable antigen B from other components. The sample was checked for protein content, using Bradford protein assay (12) and stored at -20 °C until use.

### **SDS-PAGE and Western blotting**

Antigen B (20 µg) was subjected to discontinuous reducing SDS- PAGE, using 12.5% gel in a Bio-Rad apparatus at 50 mA/gel for 1 hour. The antigen was transferred from the gel into nitrocellulose membrane (Schleicher and Schuell). Efficacy of transfer was checked by staining the membrane with Ponceau S stain (0.001 g/ml in 3% trichloro acetic acid). The membranes with blotted antigen were cut into strips and blocked with 5% (w/v) of skimmed milk in washing buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20; pH 7.4) for 2 hours. The strips were incubated with test sera (1/100 dilution in washing buffer with 1% bovine serum albumin (BSA)) for 2 hours at room temperature. After 3 washes (each 15 min), the strips were incubated with horseradish peroxidase conjugated anti-human Ig (Sigma) at a dilution of 1/2000 (in washing buffer+1% BSA) for 2 h at room temperature. After 3 washes as before, bound antigens was developed using diaminobanzidine (DAB) substrate (0.1% H<sub>2</sub>O<sub>2</sub>+ mg/ml DAB in 50 mM Tris-HCl, pH 7.6). Low molecular weight marker (Sigma, 6.5- 66 kDa) was used in this study.

## Results

Different subunits of antigens were separated by SDS-PAGE (Fig.1). Detection of antigen B subunits by sera from CE and non-CE patients and also healthy controls is presented in Table 1. Fig. 2 shows the different subunits of antigen B which have been recognized by sera of CE patients. From 40 sera of hydatidosis patients, 80% detected 8 kDa subunit, 72.5% recognized the 16 kDa component and 72.5% detected 24

kDa subunit of antigen B. The highest sensitivity was achieved with 8 kDa subunit of antigen B while the other components (16 and 24 kDa) showed a lower sensitivity (72.5%). Sample from healthy controls and non-CE patients did not yield any detectable band in the Western blotting assay. Accordingly, specificity of the

system was found to be 100%. Positive predictive value of the assay was found to be 100 with any of the subunits while the best negative predictive value was seen with 8 kDa component. Table 2 summarizes the features of immunoblotting assay in diagnosis of human hydatidosis.

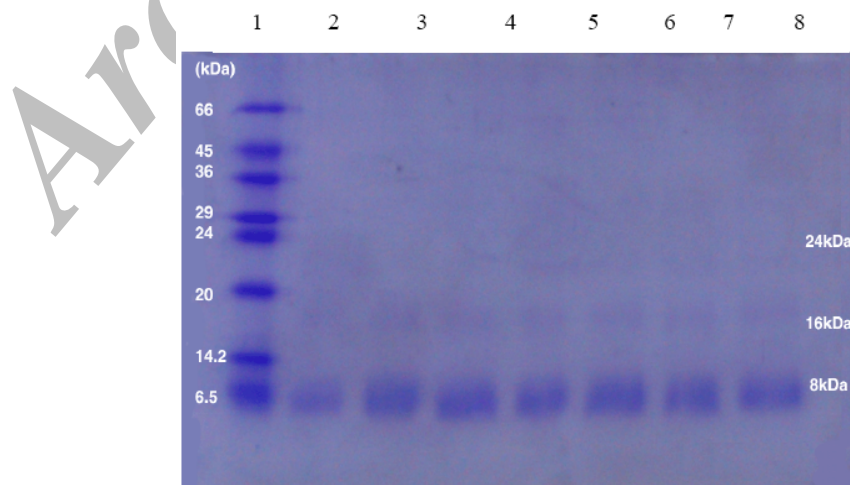
**Table 1:** Detection of antigen B subunits by sera from CE and non-CE patients and healthy subjects

Tested sera	No.	Detection of antigen B subunits		
		8 kDa	16 kDa	24 kDa
Hydatidosis	40	32 (80%)	29 (72.5%)	29 (72.5%)
Fasciolosis	9	0	0	0
Toxocariasis	11	0	0	0
Taeniasis	2	0	0	0
Malignancies	20	0	0	0
Healthy controls	70	0	0	0

**Table 2:** Features of immunoblotting, using native antigen B, in diagnosis of human hydatidosis

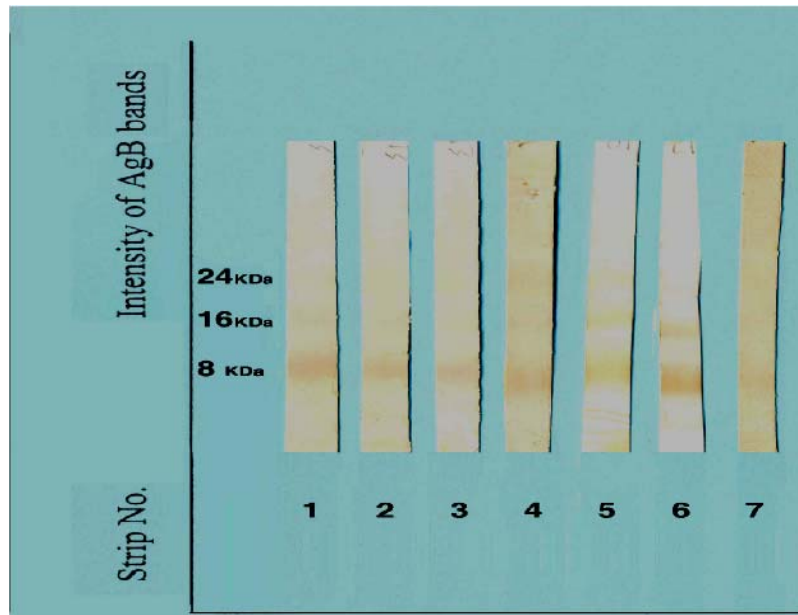
Antigen B subunits	False positive	False negative	Sensitivity (%)	Specificity (%)	PPV <sup>1</sup> (%)	NPV <sup>2</sup> (%)
8 kDa	0	8	80	100	100	93.2
16 kDa	0	11	72.5	100	100	90.9
24 kDa	0	11	72.5	100	100	90.9
8, 16 and 24 kDa	0	11	72.5	100	100	90.9

<sup>1</sup>positive predictive value; <sup>2</sup>negative predictive value.



**Fig. 1:** SDS-PAGE analysis of Antigen B, using polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) under danturing conditions.

Line 1: Molecular marker, Line 2-8: antigen B



**Fig. 2:** Different subunits of antigen B detected by sera of cystic echinococcosis patients.

## Discussion

Common laboratory diagnosis of CE relies on detection of anti hydatid cyst antibody. A variety of techniques have been used for diagnosis of CE including complement fixation test, indirect haemagglutination test, latex agglutination test and enzyme linked immunosorbent assay. Different antigens have been used for diagnosis of CE among them the lipoproteins antigen B and antigen 5 (Ag5), the major components of hydatid cyst fluid, have received the most attention (10). Antigen 5 is a lipoprotein of high molecular weight (about 400 kDa) comprising of 60-70 subunits while AgB is a polymeric lipoprotein with a molecular mass of 120 kDa. It appears ladder-like under reduced condition on SDS-PAGE, with three bands with molecular sizes of approximately 8 or 12, 16, and 24 kDa, suggesting that it comprises polymers of 8-kDa subunits (13).

Our findings showed that using the components of antigen B, a sensitivity of 80% (with 8 kDa subunit) and 72.5% (with 16 or 24 kDa subunits) and specificity of 100% (with all subunits of Ag B) can be achieved for diagnosis of CE in

immunoblotting assay. This is consistent with those studies which evaluated the efficacy of antigen B for diagnosis of CE (14-16).

A sensitivity of 60 to 100 percent has been reported for 8 kDa component and 50-100 percent for the second component, 16 kDa, of antigen B in diagnosis of CE (14-16). In our study the 16 kDa component was detected by 72.5 percent of the sera from CE patients.

Haniloo *et al.*, reported a sensitivity of 80% and specificity of 100% for 8, 12, and 16 kDa subunits of antigen B. The sensitivity of the test was increased to 90.6% when the reaction with one of the 8, 12, 16 or 24-kDa component was considered while the specificity was decreased to 95.4% (17).

Kharebov *et al.*, reported a sensitivity of 50% for the 22-24 kDa subunit of antigen B (18) while a sensitivity of 63% was reported for this component (19). Maddison *et al.*, found that the antibodies in about 80% of sera from 70 CE patients and all of 4 patients infected with *E. vogeli* reacted with the 8 kDa component of the hydatid cyst fluid (20). However, none of 205 sera from patients with other parasitic diseases, including 26 cysticercosis, cases reacted with

the 8 kDa antigenic component. Specificity of antigen B subunits in diagnosis of CE is more than 95% in most of studies (14-17). This is consistent with our findings where we found a specificity of 100% for all subunits of antigen B. The 8 kDa component of antigen B was detectable by 80 percent of the sera from CE patients in our study. This is again in agreement with other studies which suggest that the smallest subunit of antigen B has the highest sensitivity in diagnosis of CE (10). It is well known that the nature and quality of antigen B in hydatid cyst fluid are variable among the host species and this may be one of the reasons why different laboratories obtained different results using AgB prepared from different host species (10). Moreover method of antigen preparation, differences in strain of parasite, location of the cyst, clinical status of the host and type of the cyst might be accountable for differences in results of different studies, using antigen B for serodiagnosis of CE. These differences support the idea that any CE-endemic area might try to prepare and use the native antigen for routine diagnosis of CE.

In conclusion, our findings showed that Western blotting assay, using native antigen B, is a reliable method for confirmatory diagnosis of CE.

### Acknowledgements

This study was supported by a grant (No. 2094) from Office of Vice Chancellor for Research, Shiraz University of Medical Sciences, Shiraz, Iran.

### References

1. Sadjjadi SM. Present situation of echinococcosis in the Middle East and Arabic North Africa. *Parasitol Int.* 2006; 55(S): 197-202.
2. Sadjjadi SM, Ardehali S, Noman-Pour B, Kumar V, Izadpanah A. Diagnosis of cystic echinococcosis: ultrasound imaging or counter-current immunoelectrophoresis? *East Mediterr Health J.* 2001; 7(6):907-11.
3. Ortona E, Rigano R, Buttari B, Delunardo F, Ioppolo S, Margutti P, Profumo E, Teggi A, Vaccari S, Siracusano A. An update on immunodiagnosis of cystic echinococcosis. *Acta Trop.* 2003; 85(2):165-71.
4. Barbieri M, Fern´andez V, Gonz´alez G, Mart´inez V, Nieto A. Diagnostic evaluation of a synthetic peptide derived from a novel antigen B subunit as related to other available peptides and native antigens used for serology of cystic hydatidosis. *Parasite Immunol.* 1998; 20:51–61.
5. Ortona E, Rigano R, Margutti P, Notargiacomo S, Ioppolo S, Vaccari S, Barca S, Buttari B, Profumo E, Teggi A, Siracusano A. Native and recombinant antigens in the immunodiagnosis of human cystic echinococcosis. *Parasite Immunol.* 2000; 22:553–59.
6. McVie A, Ersfeld K, Rogan M.T, Craig PS. Expression and immunological characterisation of *Echinococcus granulosus* recombinant antigen B for IgG4 subclass detection in human cystic echinococcosis. *Acta Trop.* 1997; 67:19–35.
7. Williams JF, Perez Esandi MV, Oriol R. Evaluation of purified lipoprotein antigens of *Echinococcus granulosus* in the immunodiagnosis of human infection. *Am J Trop Med Hyg.* 1971; 20(4):575-9.
8. Lightowers MW, Liu D, Haralambous A, Rickard MD. Subunit composition and specificity of the major cyst fluid antigens of *Echinococcus granulosus*. *Mol Biochem Parasitol.* 1989; 37: 171–182.
9. Sadjjadi SM, Abidi H, Sarkari B, Izadpanah A, Kazemian S. Evaluation of enzyme linked immunosorbent assay, utilizing native antigen B for serodiagnosis of human hydatidosis. *Iran J Immunol.* 2007; 4(3):162-72.
10. Carmena D, Benito A, Eraso E. Antigens for the immunodiagnosis of *Echinococcus granulosus* infection: An update. *Acta Trop.* 2006; 98(1):74-86.

11. Oriol R, Williams IF, Pervez-Esandi MV, Oriol C. Purification of lipoprotein antigens of *Echinococcus granulosus* from sheep hydatid fluid. *Am J Trop Med Hyg.* 1971; 20:569-74.
12. Bradford M. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248-254.
13. Mamuti W, Sako Y, Nakao M, Xiao N, Nakaya K, Ishikawa Y, Yamasaki H, Lightowers MW, Ito A. Recent advances in characterization of *Echinococcus* antigen B. *Parasitol Int.* 2006; 55(S):57-62.
14. Kanwar JR, Kanwar RK, Grewal AS, Vinayak VK. Significance of detection of immune-complexed 8 kDa hydatid-specific antigen for immunodiagnosis of hydatidosis. *FEMS Immunol Med Microbiol.* 1994; 9:231-236.
15. Leggatt GR, Yang W, McManus DP. Serological evaluation of the 12 kDa subunit of antigen B in *Echinococcus granulosus* cyst fluid by immunoblot analysis. *Trans R Soc Trop Med Hyg.* 1992; 86(2):189-92.
16. Profumo E, Ortona, Rigano R. Detection of antibodies against *Echinococcus granulosus* major antigens and their subunits by immunoblotting. *Trans R Soc Trop Med Hyg.* 1991; 85(2):239-43.
17. Haniloo A, Massoud J, Rokni MB. Evaluation and comparison of antigen B-ELISA and antigen B-immunoblotting in immunodiagnosis of cystic hydatid disease. *Pak J Med Sci.* 2005; 21(3):352-56.
18. Kharebov A, Nahmias J, El-On J. Cellular and humoral immune responses of hydatidosis patients to *Echinococcus granulosus* purified antigens. *Am J Trop Med Hyg.* 1997; 57(5):619-25.
19. Siracusano A, Ioppolo S, Notargiacomo S, Ortona E, Rigano R, Teggi A, De Rosa F, Vicari G. Detection of antibodies against *Echinococcus granulosus* major antigens and their subunits by immunoblotting. *Trans R Soc Trop Med Hyg.* 1991; 85(2): 239-43.
20. Maddison SE, Slemenda SB, Schantz PM, Tsang VCW. A specific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 kDa. *Am J Trop Med Hyg.* 1989; 40:377-83.