

EVALUATION AND COMPARISON OF ANTIGEN B-ELISA AND ANTIGEN B-IMMUNOBLOTTING IN IMMUNODIAGNOSIS OF CYSTIC HYDATID DISEASE

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ABSTRACT

Objective: Evaluation and comparison of the diagnostic value of ELISA using partial purified antigen B (AgB-ELISA) and antigen B subunits immunoblotting in immunodiagnosis of cystic hydatid disease (CHD).

Design: Antigen B was obtained and partially purified from sheep hydatid cyst fluid. Serum samples were collected from surgically confirmed CHD patients, other parasitic disease, patients with malignancies and normal individuals. Sera were analyzed by ELISA using antigen B and immunoblotting based on antigen B subunits.

Setting: School of Public Health serum blood bank, Tehran University of Medical Sciences, volunteers and selected CHD patients from different local hospitals.

Subject: Serum samples were obtained from 64 surgically confirmed CHD patients, 55 from individuals infected with toxocarasis or fascioliasis, 50 from patients with malignancies and 73 from normal individuals.

Main Outcome Measures: The sensitivity, specificity and cross-reaction of comparative tests evaluated.

Result: The sensitivity of AgB-ELISA was 89%, while that of 8/12-, 16-kDa immunoblot was 80%. Specificity of AgB-ELISA was 98% and that of 8/12-, 16-kDa immunoblot was 100%. The sensitivity and specificity of ELISA using crude hydatid fluid antigen (CHFAg-ELISA) were 94% and 83%, respectively.

Conclusion: AgB-ELISA as well as 8/12-, 16-kDa immunoblot can be convenient in specific and confirmatory diagnosis of CHD.

KEY WORDS: *Hydatidosis, Immunodiagnosis, Immunoblotting, AgB-ELISA*

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INTRODUCTION

Serology of hydatidosis or cystic hydatid disease (CHD) offers a useful adjunct to imaging and may provide confirmatory diagnostic information. It may also be an important element to control, surveillance and early diagnosis.¹ The strategy for immunodiagnosis of CHD currently recommended is to rely upon a diagnostically sensitive test such as ELISA employing hydatid fluid antigen. Then positive test results, dependent upon the geographical origin of the patient and the implied problems of cross-reactivity due to potential infection with other parasite species, have subsequently to be confirmed by a specific test such as arc-5 immunodiffusion or immunoblot assay.²

Hydatid cyst fluid contains two major lipoprotein antigens, namely antigen 5 (Ag5) and antigen B (AgB) besides the other components.² Resolution of hydatid fluid by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting resulted in the identification of the Ag5 (arc-5) subunits, including two subunits as 37/38-kDa and 20/22/24-kDa, respectively². The second major parasite antigen in hydatid fluid, AgB, is a thermo stable lipoprotein which resolves as three bands of 8/12-, 16- and 23/24-kDa by SDS-PAGE and immunoblotting^{3, 4, 5}. In many studies, subunits of AgB by immunoblotting show high specificity and less cross-reaction with other parasitic infections^{4,6,7}. This assay, although having shown higher specificity, but is highly expensive, time consuming and troublesome. Therefore, it is difficult to carry out in many poor equipped laboratories, especially in developing countries and there is a need for immunoassays, which should be relatively simple to carry out. Thus, it may preferable to carry out the ELISA using more specific semi-purified preparation of AgB as described previously by Oriol et al.⁸. In addition to this, serologic evaluation of these antigens has given some different results. These differences can not only be depending on nature and purity of antigens, but also in some cases are related to quality and quantity of host immune responses in different patient groups. Also strain variations in the parasite may significantly affect the serological responses. Thus, such tests must be evaluated in various endemic regions and countries according to local facilities and using endemic antigens.

In the current study, we assessed and compared the diagnostic value of AgB-ELISA and immunoblotting using endemic antigen in diagnosis of CHD.

MATERIALS AND METHODS

Human sera: Serum samples were collected from surgically confirmed CHD patients (n=64), patients infected with toxocariasis (n=22), fascioliasis (n=33), histologically confirmed malignancies (n=50) including breast

cancer, 25 cases; pancreatic and liver neoplasm, 7; lymphoma, 6; colon adenocarcinoma and other neoplasm, 12. All of them were collected from different local hospitals and Tehran School of Public Health serum blood bank. Control serum samples were obtained from 73 volunteers at Tehran University of Medical Sciences, Iran. These sera were checked as having no parasitic infection. The human's ethics committee at the Tehran School of Public Health approved the study.

Preparation of CHFAG: Hydatid fluid was aspirated from hydatid cysts obtained from livers and lungs of sheep slaughtered at the local abattoir. The hydatid fluid was centrifuged at 1000g for 15 min and supernatant was dialyzed against extensive distilled water overnight at 4°C. The dialysate was then filtered (by Millipore, 0.2 µm), lyophilized and stored at -20°C until use.

Preparation of AgB: AgB was prepared from sheep hydatid cyst fluid as described previously⁹, based on the methods of Oriol et al.⁸ and Williams et al.¹⁰. Briefly, 100 ml of sheep hydatid fluid was dialyzed against 0.005 M acetate buffer, pH 5 overnight and centrifuged at 50000g for 30 min. The precipitate was dissolved in 10 ml of 0.2 M phosphate buffer, pH 8 and treated with ammonium sulfate to 40% saturation for 60 min. The supernatant was then boiled in a water bath for 15 min and centrifuged at 50000g for 60 min. The pellet was discarded and the supernatant, containing AgB was filtered by Millipore 0.2 µm. Finally, sodium azide (NaN₃, 0.02% W/V) was added and aliquots of 1 ml vials stored at -20°C until use. The antigen was assayed for protein content by the Brad-ford method protein assay¹¹.

SDS-PAGE and immunoblotting: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the discontinuous buffer system of Laemmli¹² using 12.5% slab gels under reducing conditions adopted from Bollag and Edelstien¹¹. Briefly, the mixture of four volumes CHFAG (1 mg protein/ml) and one volume sample buffer (4x) were boiled and loaded (20 µg protein/lane).

Low molecular weight marker (SigmaMarker™, No. M-3913) was used as a standard. After running gel, the separated proteins transferred to nitrocellulose membrane (GibcoBRL, No. 11467-032) overnight at 35 mA¹³. Membrane was blocked 90 min in Tris-buffered saline containing 3% gelatin powder (TBS-3%G) at room temperature. Sera were added at 1:100 dilutions in TBS-1%G for 90 min. After washing with TBS, the membrane was incubated in Antihuman IgG-HRP conjugates (BioGen, NO. BA114) diluted 1:1500 in TBS-1%G for 90 min. The membrane was washed with TBS and developed in 3.3'-Diaminobenzidine (DAB) substrate (5 mg DAB, 50 ml of 10 mM Tris-HCl pH 7.4, 20 ¼ 30% H₂O₂).

CHFAG-ELISA and AgB-ELISA: The ELISA was performed in 96 well microplates (Nunc, Denmark) as previously described by Craig et al.⁹ with some modifications. Microplate wells were coated overnight at 4°C with 100 µl CHFAG or AgB (5µg/ml) in 0.05 M bicarbonate buffer, pH 9.6. Wells were washed 3 times in PBS plus 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 1% BSA for 30 min at 37° C. Sera were added at 1:200 dilutions in PBS-T, incubated at 37° C for 1 hour then washed as before. Antihuman IgG-HRP conjugates were added at 1:8000 dilutions in PBS-T and the microplate incubated and washed as before. This was then developed in OPD substrate (5 mg 1,2-phenylenediamine, 12.5 ml of 0.2 M citrate phosphate buffer pH

5, 10 µl 30% H₂O₂). The absorbance was read at 492 nm after 10 min using an automatic microplate reader (State Fax® 2100, Awareness, USA).

Statistical analysis: Threshold values were defined by adding three standard deviations to the mean absorbance value at 492 nm of negative control sera. The Chi-square test or Fisher's exact test (Epi Info program, Version 5) was applied as appropriate to determine the significant differences between diagnostic sensitivity or specificity of ELISA and immunoblotting.

RESULTS

The CHFAG-ELISA showed a diagnostic sensitivity of 94% with sera obtained from 64 confirmed CHD patients and specificity of 83% with high cross-reactions by non-hydatid sera including fascioliasis, toxocariasis, malignancies and normal sera. Among the non-hydatid sera, patients with fascioliasis exhibited a very high cross-reactivity of 54.5% by CHFAG-ELISA (Table 1). The sensitivity and specificity of AgB-ELISA was 89% and 98%, respectively, only with 3 cross-reaction by non-hydatid sera. The overall cross-reactivity of AgB-ELISA against non-hydatid sera was significantly lower than that of CHFAG-ELISA (1.7% vs. 16.9%, $p < 0.05$).

The CHFAG was effectively separated into a number of distinct protein components under reducing conditions using 12.5% SDS-PAGE. The major parasite bands at approximately 8/12-, 16-, 24-, 27- and 38-kDa, were clearly vis-

Table-I: Serologic reactivity of cystic hydatid disease (CHD) and non-hydatid sera by ELISA and immunoblotting

Sera	No. (%) of positive sera by				
	No. of Sera	CHFAG-ELISA	AgB-ELISA	8/12-, 16-kDa immunoblot	8/12-, 16-, 24-kDa immunoblot
CHD patients	64	60 (94)	57 (89)	51 (80)	58 (90.6)
Fascioliasis	33	18 (54.4)	1	0	7 (21)
Toxocariasis	22	8 (36)	0	0	0
Malignancies	50	1	1	0	0
Normal individuals	73*	3	1	0	0

* Only 48 sera of 73 normal individual tested by immunoblot

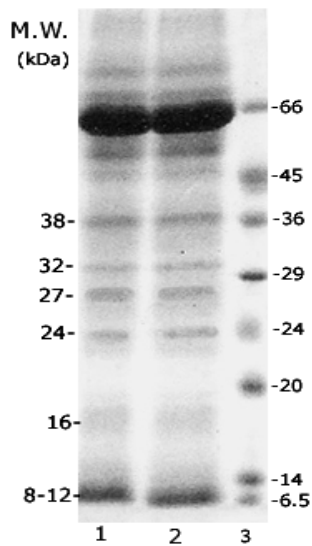


Fig. 1: Crude hydatid fluid antigen (CHFAG) resolved by SDS-PAGE in a 12.5% slab gel under reduced condition. Coomassii blue used for staining. Lan 1 and 2: CHFAG 20 and 25 µg, respectively; lane 3: low molecular weight SigmaMarker™10 µg; M.W. = estimated molecular weight of bands.

ible (Fig. 1). It should be mentioned that owing to use linear slab gel in SDS-PAGE, we could not able precisely to estimate molecular weight of the smallest AgB subunit. Thus, we estimated and assigned that as a range molecular weight of 8/12-kDa. Immunoblot analysis of CHD sera to 8/12- and/or 16-kDa subunits showed a diagnostic sensitivity of 80%. Non-hydatid sera demonstrated no cross-reactivity with these two subunits (Fig. 2). Accordingly, specificity was detected as 100%. The sensitivity of the test based upon reactivity of at least one of the 8/12-, 16- and 24-kDa increased to 90.6%, while specificity decreased to 95.4%. Both 24- and 27-kDa bands showed cross-reaction with 7 sera from fascioliasis. The 38-kDa band revealed cross-reaction not only with sera from toxocariasis, but with some normal sera as well.

DISCUSSION

Immunodiagnosis is an important tool in diagnosis of CHD. It may also be an important element to control, surveillance and early diagnosis of infection. Conventional serology of CHD is based primarily on a sensitive test such

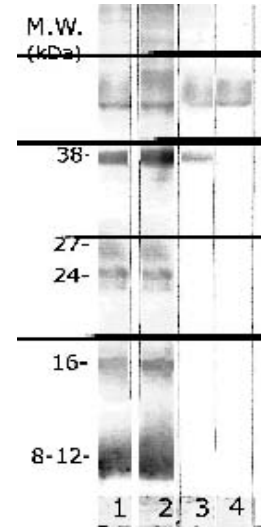


Fig. 2: Immunoblot analysis of crude hydatid cyst fluid Antigen (CHFAG) probed with respective sera. Lane 1 and 2: cystic hydatid disease sera (CHD); lane 3: toxocariasis; lane 4: normal human serum. M.W. = estimated molecular weight of bands.

as an ELISA employing hydatid fluid antigen and a subsequent confirmation test such as arc-5 immunodiffusion or immunoblotting for the small subunit of AgB^{2, 9}.

In this study, primarily, we showed that the sensitivity of CHFAG-ELISA for diagnosis of CHD is significantly higher (94%) than that of AgB-ELISA (89%) and 8/12-, 16-kDa immunoblot (80%) ($P < 0.05$), but specificity of test was low. This was clearly confirmed by the 54.4% cross-reactivity with fascioliasis and 36% with toxocariasis. Since remarkable human cases of these two parasites occur in Iran^{14,15,16} as well as many countries, CHFAG-ELISA can only be useful in primary screening of CHD. AgB especially 8-kDa subunit, however, is now thought to be highly genus-specific for *Echinococcus*¹⁷ and a partial purified preparation of this antigen may be preferable in some situations. To elucidate specificity of the serologic reactions, we assessed AgB-ELISA and 8/12-, 16-kDa immunoblot with the respective non-hydatid sera. In result, the 8/12-kDa immunoblot showed 100% specificity and 70% sensitivity. When both of the 8/12- and/or 16-kDa bands were included as diagnostic

markers, the sensitivity of test increased to 80% without losing specificity. Some investigators, of course, showed a cross-reactivity of small subunit of AgB (i.e. 8/12-kDa) with antibodies in the sera of patients infected with alveolar cyst due to *Echinococcus multilocularis* and *Taenia solium* cysticercosis^{4, 17, 18}. It should be noted that, *E. multilocularis* is much less widespread than *E. granulosus* and is confined to the northern hemisphere and *T. solium* cysticercosis is not endemic in Islamic countries⁹. Thus, the 8/12-, 16-kDa immunoblot shows a high value in confirmatory serodiagnosis of CHD in non-endemic area of *E. multilocularis* and *T. solium*. The AgB-ELISA, also, demonstrated a high diagnostic specificity of 98%, with only 3 cross-reactions by 178 non-hydatid sera and sensitivity of 89% in detecting of CHD sera. Therefore, this test as well as 8/12-, 16-kDa immunoblot can be convenient in confirmatory diagnosis of CHD.

CONCLUSION

We suggest that the CHF_{Ag}-ELISA which exhibit a relatively high diagnostic sensitivity is only convenient for primary screening test and 8/12-, 16-kDa immunoblot having high specificity for subsequent confirmatory test. Since immunoblot is somewhat expensive, troublesome and time consuming, AgB-ELISA can be considered as an alternative test for the diagnosis of human hydatidosis.

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