

Introduction of An Indirect Haemagglutination Test as a Rapid Diagnostic Method in Comparison With ELISA Using Antigen B for Diagnosis of Human Hydatid Disease

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Abstract

Early diagnosis of human hydatid disease by detecting the specific antibodies in patients' sera is considered as an important step in treatment of infection. But the diagnostic efficiencies of assays greatly depend on the characteristics of antigen that is used and various conditions in performance. In present study, we tried to standardize an indirect haemagglutination test, using antigen B for diagnosis of hydatid disease. Sera from 80 patients with surgically confirmed hydatidosis and 40 sera from healthy donors were examined. To detect the cross-reactant antibodies, 53 sera from patients with other parasitic infectious and diseases were applied in this study. IHA was performed with sheep RBC that was sensitized by various concentrations of crude antigen and antigen B. The best results were obtained by IHA with applying antigen B (10µg/ml) for 40 min at 37°C or 60 min at room temperature. Diagnostic value of antigen B (sensitivity 93.75%, specificity 100% and efficiency 97.12%) was significantly higher than related value of crude antigen (sensitivity 65%, specificity 100% and efficiency 83.81%) in IHA under the optimum condition. Sensitivity and specificity of ELISA using crude antigen (10µg/ml) were obtained 80% and 94.62%, respectively. Corresponding values of ELISA using antigen B were also obtained as 72.5% and 98.92%, respectively. It is suggested that the IHA, as a serological assay, is a valuable method with high diagnostic efficiency for serodiagnosis of hydatid disease, when is performed by purified antigen B. It is a rapid diagnostic assay with any needs neither for expensive instruments nor expert personnel so is useful for seroepidemiological studies and field trial in endemic areas.

Keywords: *Hydatid disease, Hydatid cyst, Hydatid antigen, IHA, ELISA, Hydatid antigen, B, Iran*

Introduction

Cystic hydatidosis, caused by larval stage of *Echinococcus granulosus*, affects both human and domestic animals and is recognized as one of the world's major zoonoses (1). It has public health importance not only in areas of endemicity but also in countries or region without endemicity due to the migration of infected people and livestock exchange. Today, the clinical diagnosis of hydatid disease is feasible by means of imaging techniques to give essen-

tial information on the location of cyst (2). Clinical symptoms do not appear until the larval cyst structure of parasite has reached a certain size, which normally requires years after the primary infection. Therefore, the clinical diagnosis is based only on assumption and needs specific confirmation, for example by immunodiagnosis, to differentiate hydatidosis from other cystic lesions and tumors (2). The presence of raised specific antibody titers in patients with hydatid disease has been assayed by

various techniques such as indirect haemagglutination (IHA) or latex agglutination, immunoelectrophoresis, complement fixation, indirect fluorescent antibody test and enzyme-linked immunosorbent assay (2). The sensitivity and specificity of current immunological techniques based on specific serum IgG rely on the way antigens are purified (2). The most widely used antigen for routine serological testing in hydatidosis is fluid taken from the cyst. This fluid is, however, a complex mixture of substances and contains several protein and carbohydrate fractions as well as end products of carbohydrate and protein metabolism. Variations in potency of different cyst fluid antigens have been reported. Some investigators have associated the variability from cyst to cyst as from host to host (3), have attributed the variations to the fertility and development of the cyst (4). According to the results, the highest concentration of antigen has been found in cyst fluid from the liver of sheep (5). Several techniques using crude antigen of hydatid cyst, have been used for serodiagnosis of hydatid disease (6), but all of them show some lack of sensitivity and specificity. Therefore, identification of discrete parasite antigens that could provide a more sensitive and specific serology has become a relevant research aim (7). One of the diagnostically relevant antigens in hydatid cyst fluid is a 160 kDa thermostable lipoprotein complex named antigen B (AgB) (8). It has been proved that lipoprotein in complete form or its subunits show a noticeable immunological reaction with hydatid patients' sera (9, 10, 11). Qualitative AgB-immunoblotting assessment of IgG isotype responses identified IgG4 as the predominant isotype, which is the immunoglobulin isotype most clearly associated with the progression of the disease. In addition, peripheral blood mononuclear cell (PBMC) proliferative assay revealed a response to AgB in 100% of patients' PBMC (9). These findings confirm AgB as a good diagnostic molecule as well as a possible antigenic marker associated with the clinical stage of hydatid disease (9, 11,

12). Final results show that it is needed to standardize techniques and antigen preparations and to improve the performance of immunodiagnosis by designing the reliable, available and cost-effective method that is useful in medical laboratory diagnosis and screening studies.

The main aim of the present study was standardization a rapid diagnostic method for human hydatidosis which can detect the serum antibodies against the major antigen of hydatid cyst fluid, AgB, in patients' sera.

Materials and Methods

Human sera sample collection Sera from 80 patients with surgically confirmed hydatid disease were collected. All of them had records of cyst location, which were as follow: liver (n=52), lungs (n=23) and multiple sites (n=5). In order to evaluate the specificity of the test, 53 sera from patients with the following diseases were included in the study: *Taenia saginata*, taeniasis (n=7), toxocarasis (n=5), fascioliasis (n=5), toxoplasmosis (n=12), rheumatoid arthritis (n=11), cirrhosis (n=10), hepatic cancer (n=3). Negative controls comprised 40 sera from healthy volunteer donors.

Crude antigen Sheep hydatid cyst fluid (SHCF) was obtained through a local abattoir in Ziaran (Qazvin, Iran) from infected sheep livers. Freshly aspirated SHCF containing proto-scolecocytes and the other cyst materials was incubated overnight at 4°C, adding EDTA 5mM and NaN₃ 500 mg/l, and then was homogenized with a glass homogenizer (OMNI-OCI Instrument International). The procedure was followed by ultrasonication at 60 cycle/s in maximum tune during 60s, for four times in ice bath (2). The suspension was centrifuged in cool at 10,000g for 30 min and supernatant was filtered by 0.45 µm pore size micro-filter (Millipore Co. Bedford, Massachusetts) and kept in -70°C before use.

Antigen B SHCF was collected from fertile cysts, clarified by centrifugation at 10,000g for 60 min and supernatant was collected and fil-

tered by XM-100 ultra-filtration membrane (Amicon-Holland). One hundred and fifty ml polyethylene glycol 50% (w/v in distilled water) was added to a 100ml protein solution while stirring gently and mixture was centrifuged 10 min at 10,000g. The supernatant was decanted and the pellet was resuspended in two pellet volumes of Tris buffer, pH 8.3 (13). Suspension was loaded to a 2.5 x 5 cm column of sephacryl S-200 (S-200-HR, Sigma) equilibrated in the application buffer (20mM sodium phosphate, 0.20M NaCl, pH 7.4), flow rate 3ml/min, 4°C. Buffer was changed to 20mM sodium phosphate, 0.45M NaCl, pH 7.4, after washing with 5 column volumes and the eluted material was collected (14). Partially purified fraction which was obtained by the procedure as explained was loaded to a single well of a 1.5mm-thick 12% polyacrylamide gel. Gel was run at 30mA at 4°C in Tris buffer containing 192 mM glycine, pH 8.3. The part of gel below the 25kDa marker was then excised and stained with coomassi blue to localize the protein bands. Protein was eluted from polyacrylamide strips with a model PROT0030 Electro-Eluter kit (ProteoPLUS tm, USA) at 10mA /tube for 3h at 4°C as described by Ioppolo et al 1996 (9).

IHA Fresh sheep blood was collected aseptically by bleeding into a flask containing equal volume of Alsvær's solution that was prepared by some modification of the method described earlier (15), and the flask was gently shaken. The suspension was kept at least 3 d at 4°C. RBC was then washed 5 times with 3 volumes of phosphate buffer saline (PBS) 15mol, pH 7.2; and centrifuged in cold at 1000g for 10min. After the last washing, the packed cells were resuspended in 19 volumes of tannic acid solution (1:20,000 v/v with distilled water) and incubated for 10 min at 37°C. Then the suspension was centrifuged at 1000g for 10min. The supernatant was removed and the packed RBC was washed at least 5 times with 15 volumes of PBS 15mol, pH 7.2. Packed tanned RBC was also sensitized adding 1:10 v/v of 5, 10, 15, 20µg/ml protein concentration of crude antigen

and AgB, separately. The mixture was suspended in PBS, pH 7.2, and kept overnight at 4°C. Sensitized RBCs were packed and washed 5 times with PBS and adjusted to a 2-2.5 v/v suspension with normal rabbit serum 1% (1:100 with PBS 15 mol, pH 7.2). All of the described process was applied for normal and tanned sheep RBC.

Setting up the test Human sera samples were incubated at 56°C for 30min to inactivate complement system before use. Sera were diluted to 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 with PBS, pH 7.2, and 25µl of each diluted serum was added to a V shape well of microplate. Twenty-five µl of sensitized RBC was added to each well and incubated separately at 37°C at room temperature. Results were observed after 30, 40, 50 and 60min.

ELISA ELISA was performed essentially as described by Barbieri et al 1998 (16). Briefly, microtitration plates were coated by incubation with 100 µl of both antigens (crude antigen and AgB) solution (10 µg/ml protein concentration) per well and serum samples were diluted 1:256 in phosphate buffer saline 0.05% with Tween 20 (PBS-T), containing 1% bovine serum albumin, then 100 µl was dispensed into each well. One hundred µl of HRP-labeled polyclonal antibodies against human IgG (Organ Teknika, Netherlands) at a 1:1000 dilution in 1% casein was loaded into all the wells and incubated for 1 hour at 37°C for the detection of crude antigen and AgB specific total IgG. O-phenylenediamine dehydrochloride (OPD) 0.01 M in PBS, pH 7.4, was used as a substrate for peroxidase and optical densities at 600 nm were measured with an ELISA reader (ICN Biomedicals, UK). Cut-off was described by mean +2SD of absorbance reading in sero-negative controls. IgG levels in sample sera were determined by standard serum (Bohringer).

Diagnostic parameters analysis The sensitivity, specificity and diagnostic efficiency (DE) were used to calculate the corresponding diagnostic parameters. DE calculated as follows (17): $DE = \frac{\text{true positive} + \text{true negative}}{100}$

(true positive+true negative+false positive+false negative).

The verification of the DE related to the using each antigen, crude antigen and AgB, were run using χ^2 test ($P<0.05$).

Results

IHA with crude antigen and AgB separately showed a different pattern to recognition by almost all of the examined sera. As the results show, the closest time for each two type of antigens for the best result, was 40min at 37°C or 60min at room temperature. Table 1 shows the number of positive reactions of each sample group with definitive concentration of both antigens (crude antigen and AgB) according to different times and temperatures. It was indicated that 75 sera (93.75%) of 80 surgically confirmed hydatidosis sera shown positive reaction while 10µg/ml AgB concentration was used to sensitize RBC and was incubated at 37°C for 40min. At the same condition, 52 sera (65.00%) of 80 hydatidosis sera had positive reaction with crude antigen.

When crude antigen was used as the antigen, immunoreaction with hydatidosis patients and normal donors' pooled serum presented that the titers of greater than or equal to 1/320 was considered positive. This titer limit was also applied for reading results of IHA test when AgB was used for sensitizing the RBC. Using crude antigen also exhibited relatively a level of cross

-reactivity with 1 serum from cirrhosis patients at 37°C in 50min. It was assumed that the specificity and sensitivity of both antigens for IHA correlate to the protein concentration of antigen extract as well as time and temperature to incubate the wells containing antibody and sensitized RBC. Figure 1 shows the differentiation of diagnostic sensitivity that relates to both crude antigen and AgB in various conditions.

The highest diagnostic parameters of IHA test using AgB, were obtained under the following condition: 10µg/ml antigen concentration, 37°C temperature for 40min (Sensitivity = 93.75%, Specificity = 100%, Efficiency = 97.12%). The related values of IHA with crude antigen were 65%, 100% and 83.81% respectively. Normal and tanned RBC without using both crude antigen and AgB in IHA observed no Positive reaction. Table 2 indicates the diagnostic parameters of IHA and ELISA using crude antigen and AgB under the optimum condition.

Minimum anti-hydatid IgG level that was measured by ELISA using crude antigen (0.6 µg/ml) and AgB (0.8 µg/ml) showed a rarely similarity with IgG count in IHA under optimum conditions (0.8 µg/ml with crude antigen and 0.8 µg/ml with AgB). Five out of 40 and 1 out of 40 sera from healthy donors, showed positive reactions by ELISA, when crude antigen and AgB were used, respectively. No cross-reaction was observed with other patients' sera in ELISA using both two antigens.

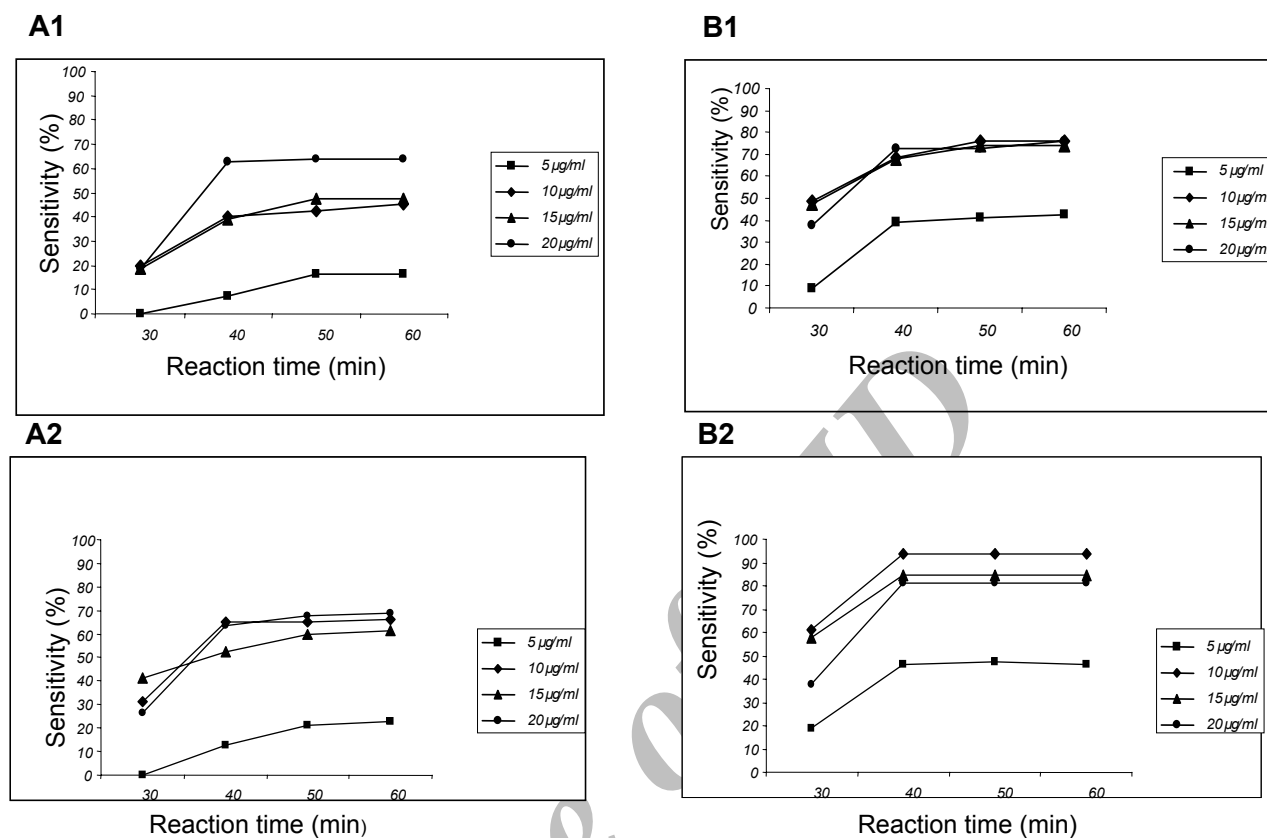


Fig. 1: Evaluation of IHA sensitivity according to the different antigen concentrations, reaction times and incubation temperatures.

A1: IHA applied by crude antigen at room temperature.

A2: IHA applied with crude antigen at 37°C.

B1: IHA applied with AgB at room temperature.

B2: IHA applied with AgB at 37°C.

Table 1: Outcome of IHA in human hydatidosis and non- hydatidosis sera with crude antigen and AgB in different reaction time, reaction temperature and antigen concentrations.

Type of Sera	Ag Concentration (mg/ml)	Crude Antigen																Antigen B							
		Reaction time(min)		30		40		50		60		30		40		50		60							
		Temperature(C°)		37	RT	37	RT	37	RT	37	RT	37	RT	37	RT	37	RT	37	RT						
Hydatodosis (Liver cyst) N=52	5	0	0	9	6	15	11	16	11	10	7	28	23	28	25	28	25								
	10	15	10	39	24	39	24	39	26	32	27	52	41	52	43	52	43								
	15	12	8	40	15	25	20	25	20	30	28	45	37	45	40	45	40								
	20	13	10	40	38	43	39	43	39	20	20	45	43	45	43	45	43								
Hydatodosis (Lung cyst) N=23	5	0	0	0	0	1	1	1	1	5	0	7	7	8	7	8	7								
	10	10	5	10	5	10	7	11	7	16	11	19	11	19	14	19	14								
	15	19	7	20	15	20	17	21	17	16	10	20	16	20	17	20	17								
	20	7	5	10	11	10	11	11	11	9	9	17	12	17	12	17	15								
Hydatodosis (Multiple site) N=5	5	0	0	1	0	1	1	1	1	0	0	2	1	2	1	2	1								
	10	0	1	3	3	3	3	3	3	1	1	4	3	4	4	4	4								
	15	2	0	2	1	3	1	3	1	0	0	2	2	2	2	2	2								
	20	1	0	1	1	1	1	1	1	1	1	3	3	3	3	3	3								
Cirrhosis N=10	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	20	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0								
Negative donors N=40	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	15	0	0	0	0	2	2	2	2	2	0	0	0	0	0	0	0								
	20	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0								

For the specificity identification, in addition cirrhosis (n=10) and Negative controls (n=40), 43 non- hydatidosis sera [Taeniasis (n=7) Toxocariasis (n=5), Fascioliasis (n=5), Toxocariasis (n=2), Rheumatoid arthritis (n=11) and Cancer (n=3)] were evaluated. Sera reaction with both crude antigen and AgB in the different condition of the IHA method were negative.

Table 2: Diagnostic parameters *of crude antigen and antigen B by IHA and ELISA under the optimum conditions (10µg/ml of each antigen at 37°C for 40 min)

Serum samples	IHA				ELISA			
	Crude antigen		Ag B		Crude antigen		Ag B	
	DE ** %	Sensitivity %	DE %	Sensitivity %	DE %	Sensitivity %	DE %	Sensitivity %
Hyatidosis patients with liver cysts (n= 52)	85.87	75.00	95.65	100.00	90.22	92.31	93.48	90.38
Hydatidosis atients with lung cysts (n=23)	79.37	43.48	93.65	82.61	74.60	52.17	73.02	30.43
Hydatidosis atients with cysts in multiple sites (n=5)	95.56	60.00	97.78	80.00	86.67	80.00	95.56	80.00
Total (n=80)	83.81	65.00	97.12	93.75	87.86	80.00	86.71	72.50

* Specificity of crude antigen and antigen B in IHA test was obtained 100%,. And in ELISA was obtained 94.62% and 98.92%, respectively.

** Diagnostic efficiency.

Discussion

Much of the works on immunology of hydatidosis has so far been devoted to the development of suitable methods for serological diagnosis the slow growing cysts (metacestodes). It may persist for several years after the initial oncospherical infection; and in many cases, appear to be refractory to the immunological responses of the host (18). Then the serodiagnosis of hydatid disease in human relies on the detection of specific antibodies against antigens of the metacestode from *Echinococcus granulosus* (19). Many of scientists (10, 20, 21) reported different diagnostic value of various forms of AgB in serodiagnosis of hydatid disease.

AgB which accounts as 10% of the total content of hydatid fluid is a heteroprotein that produces 3 subunits at 8/12, 16 and 20/24 kDa in sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. Several lines of evidence indicate that sera from patients with cystic echinococcosis contain all antibodies isotypes specific for AgB (11). Many scientists, for serodiagnosis of hydatid disease, have also used Ag5, the other major antigen of hydatid fluid. Although, there are only a few studies that have assessed the clinical value of tests based on the Ag5. Because its serodiagnosis usefulness is limited by cross-reactivity with sera from patients infected with nematodes, cestodes and trematodes (22).

Many of serological techniques have been evaluated for diagnosis of cystic hydatid disease. Each, to varying degrees, has been shown to give false results, with considerable variation between laboratories (23) and purification procedures of antigen (2). Despite the development of sensitive and specific technique, such as immunoblotting, the immunodiagnosis of hydatid disease has two important problems in clinical practice. The first problem is that most available screening tests give a high percentage of false negative results. The other problem includes false positive reactions and the lack of

standardization of the assay that contribute to discrepant results reported by the various laboratories (12). According to these reasons, particularly in endemic areas, there is much needs to design and evaluate a standard serological test that is not only sensitive and specific, but also is easy to operate and cost-effective.

There have been numerous reports of diagnostically relevant crude or purified antigens for diagnosis of human hydatidosis. The reported diagnostic sensitivity and specificity vary greatly among the different reports even for similar antigen preparation. Our results show that purified AgB is more efficient for diagnostic use than crude antigen, but ELISA showed higher sensitivity in compare with IHA when crude antigen was used to perform the tests. Also it was obtained that the IHA is more valuable than ELISA for diagnosis the total anti-hydatid IgG in sera of patients with liver or lung cysts when the tests were performed with AgB ($P<0.05$).

Sensitivity shown by AgB (93.75%) is significantly higher than that of crude antigen (65%) in IHA test at the optimum condition. According to our results, the sensitivity and specificity that have been obtained concerning by crude antigen in ELISA (80% and 94.62%), are not consistent with what have already been reported in ELISA (74%-94%) (24). The diagnostic sensitivity and specificity of the crude antigen by using ELISA was 91% and 82% respectively, according to the study of Poretti et al 1999. But some studies indicate that ELISA lacks specificity when crude antigen is used as the antigen source (25, 26) with most of the cross-reactivity created against the other parasitic diseases. The diagnostic parameters of ELISA by using AgB have been obtained as sensitivity 72.5% and specificity 100%, in present study. Ioppolo et al 1996 (9), found that AgB has 63% sensitivity while using by ELISA. The low sensitivity of crude antigen followed by ELISA (72%) and IHA (54%) has been also reported by Ortona et al 2000 (27). They have mentioned that the recombinant and native AgB yield similar

sensitivity (74%) followed by immunoblotting. The sensitivity that has been observed by IHA test with crude antigen among patients with known liver and lungs cysts, was (75%) and (43.48%), respectively under the optimum condition. The corresponding values of AgB according to our study were (100%) and (82.61%), respectively. Abdel Aal et al 1996 (28), indicated higher sensitivity of crude antigen-IHA in compare with crude antigen-ELISA (83% vs 68%). Azazy and Abdelhamid have reported high sensitivity (89%) and specificity (97%) of IHA in Yemen (29). Sbihi et al 2001 (22), evaluated the sensitivity and specificity of ELISA by using the purified AgB-rich fraction and obtained noticeable results: sensitivity 93.0%, specificity 89.7% and diagnostic efficacy 92.3%. Regarding low sensitivity of ELISA using both crude antigen and AgB for detecting lungs cysts (22), and some difficulties in experience of ELISA (for example needs the advanced technical instruments and educated personnel.), it should be preferably use IHA for epidemiological studies at the field. Due to antibody response to AgB which is IgG subclass markedly IgG4 (30, 31, 32, 33), as an agglutinin antibody, there are good reasons to use IHA in pre-surgical detection and screening studies for hydatid disease. A rarely high sensitivity and specificity of IHA to detect anti-hydatid cyst antibodies have been reported by Azazy and Abdelhamid 2000 (29) (89% and 97%, respectively). Auer et al 1988 (34), used the crude antigen of *Echinococcus granulosus* (Eg) and *Echinococcus multilocularis* (Em) in both, Em-ELISA and Eg-IHA (sensitivity 100% and 86%, respectively) and showed the usefulness of their combined application for the diagnosis and post-operative surveillance of human alveolar and cystic echinococcosis. IgG-ELISA and IHA have been also demonstrated as the most adequate test for post-surgical follow-up in clinical outcoming hydatidosis patients, by Force et al 1992 (6). Our results using AgB in IHA at the standardized conditions show a high diagnostic value with noticeable sensitivity

(93.75%) and specificity (100%) with no cross-reaction when it apply by sera from the patients with other diseases. Then it can be used as a valid serodiagnostic method for laboratory diagnosis and screening studies in human cystic echinococcosis. According to our results, IHA test would be useful for serological laboratories, sero-epidemiological studies and educational purposes.

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