Evaluation of Enzyme Linked Immunosorbent Assay, Utilizing Native Antigen B for Serodiagnosis of Human Hydatidosis

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ABSTRACT

Background: Hydatidosis is one of the cosmopolitan parasitic zoonoses caused by the larval stage of *Echinococcus granulosus*. Diagnosis of hydatidosis is still an unresolved problem. Serological tests using crude antigens for diagnosis of *E. granulosus* are sensitive, however their specificity are not satisfactory. Therefore, WHO recommended specific serological methods using specific antigens, specially native AgB for proper diagnosis. **Objectives:** This study was designed to evaluate the ELISA and counter current immunoelectrophresis (CCIEP) method using native antigen B (Ag B) for serodiagnosis of human hydatidosis in Fars Province, Iran, an endemic area for this parasitic disease. **Methods:** Native AgB was purified from sheep hydatid fluid. Serum samples obtained from 40 pathologically confirmed cases of hydatidosis along with samples from patients with fascioliasis, toxocariasis, taeniasis and cancer patients and sera from healthy individuals were tested by ELISA using native antigen B or tested by countercurrent immunoelectrophresis (CCIEP) using crude sheep hydatid cyst fluid. **Results:** Sensitivity of the ELISA system was determined to be 92.5% and the specificity was found to be 97.3%. Positive and negative predictive values of the system were 92.5% and 97.3%, respectively. For countercurrent immunoelectrophresis the sensitivity of the assay was 97.5% and its specificity was 58.18%. This ELISA system is much more specific in detecting anti hydatid cyst antibody than CCIEP, while CCIEP is more sensitive in detecting anti hydatid cyst antibody. **Conclusion:** The new ELISA system using native antigen B is a suitable method and preferable to CCIEP for immunodiagnosis of human hydatidosis.

Keywords: Hydatidosis, Native Antigen, CCIEP, ELISA, Serodiagnosis, Antigen B

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INTRODUCTION

Cystic echinococcosis (CE), which is caused by the larval stage of *Echinoccocus granulosus*, is one of the most important parasitic diseases in the world and Eastern Mediterranean region (1). Human Infections occur during the natural transmission of the parasite between the canid, as the definitive host, and the domestic livestock, as the intermediate host. Diagnosis of CE is mainly based on a positive serological test along with an imaging finding as recommended by WHO (2). Among different serological techniques, ELISA has been reported to be a relatively reliable test for diagnosis of human hydatidosis. However using crude hydatid cyst fluid (CHF) as an antigen in ELISA reduces the specificity of this test since CHF contains various metabolites of the host and the parasite (3). Antigen B (AgB), one of the main antigens of HCF which is a 160 KDa lipoprotein antigen, has been the focus of many studies for serodiagnosis of hydatidosis (4-8).However counter current immunoelectrophoresis (CCIEP) using crude hydatid cyst fluid, has been used for many years in different centers for serodiagnosis of hydatid cyst (9-10). This study was designed to assess the ELISA method using purified AgB from sheep hydatid cyst fluid for immunodiagnosis of human hydatid cyst. Furthermore the study aimed to compare the validity of the ELISA and CCIEP for the diagnosis of human hydatidosis.

MATERIALS AND METHODS

Preparation of Hydatid Cyst Fluid. Hydatid cyst fluid (HCF) of *Echinococcus granulosus* was collected from cysts developed in the liver and the lung of sheep. To remove the protoscoleces and large particles, HCF was centrifuged at 1000 g for 30 min. Protein content of the sample was determined by Bradford protein assay (12).

Preparation of Antigen B. Antigen B was prepared from HCF as originally described by Oriol(11). Briefly, 100 ml of HCF were dialyzed overnight against 5 mM acetate buffer (pH 5) at 4ºC. The samples were centrifuged at 50,000 g for 30 min to remove the albumin. The supernatant was removed and the pellet was dissolved in 0.2M phosphate buffer (pH 8). Saturated ammonium sulfate was used to remove the globulin from the sample. Finally the sample was boiled in a water bath for 15 min and centrifuged at 50000g for 60 min to isolate AgB, which is heat stable, from other antigens.

Serum Samples. Forty serum samples were obtained from surgically and pathologically confirmed CE patients from Shiraz university hospitals as well as Dena hospital in Shiraz. Also at total of 40 samples were collected from fasciolosis (9 samples), taeniasis (2 samples), toxocariasis (11 samples) and malignant patients (20 samples) together with 70 samples from healthy subjects as negative controls.

Enzyme linked Immunosorbent Assay (ELISA). ELISA was carried out in flat-bottom 96-well microplates (Nunc, Nalge, Nunc International, Roskilde, Denmark). The plates were coated with 5 µg/ml of purified AgB (100 µl/well) in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated at 4ºC overnight. Excess antigen was removed by washing the plate five times in phosphate buffered saline-Tween 20 (PBST, pH 7.4 containing 0.05% Tween 20). Blocking was done with 3% skimmed milk in PBST for 2 hours. The wells were washed and 100 µl of serum samples (1/50 dilution in PBST) from surgically confirmed cystic echinococcosis patients along with samples from healthy subjects as negative controls and sera from fasciolosis, taeniasis

and malignant patients were applied to the plates and incubated for 1.5 hour. The plates were washed as before and 100 µl aliquots of horseradish peroxidase conjugated polyclonal antibody against human immunglobulin (Dako) at a 1/1000 dilution in PBST were added to the plates and incubated for 1 hour at room temperature. After washing as before, the plates were incubated with chromogen/substrate (100 µl/well of OPD, 0.025% H_2O_2 in 0.1 M citrate buffer, pH 5) and the reaction terminated with 1mM sulphuric acid after 30 min. The absorbance at 490 nm was monitored with a microplate reader. The cut off point was set as 2SD above the mean of control samples.

Countercurrent Immunoelectrophoresis (CCIEP). Countercurrent immunoelectrophoresis (CCIE) was carried out by a modified Kelkar and Kotwal method, using HCF (8). Briefly, Agarose-gel slides were prepared using 1% agarose and wells of 4 mm diameter were punched. Anodal wells were filled with patients' sera (15 µl of undiluted serum) and cathodal wells with 15 µl of CHF having 50 µg/ml proteins. Electrophoresis was run for 60 min at 40 mA .A line of precipitation between antigen and antibody well was considered as a positive result. To remove any unrelated precipitation lines, the slide was incubated in saline overnight.

RESULTS

In CHFAg-CCIEP method, 39 (97.5%) of hydatidosis patients were found to be positive and 46 (41.8%) cases of controls were false positive. Therefore, the sensitivity and specificity of this method for diagnosis of human hydatidosis were 97.5% (95% CI=85.2-99.8%) and 58.18% (95% CI=48.3-67.3%). Positive predictive value and negative predictive value of the test were 45.8% (95% CI=35-56%) and 98.5% (95%CI=90.6-99.9%), respectively (Table 1).

Table 1. Performance of CCIEP in diagnosis of human hydatidosis in hydatidosis patients, other diseases and normal groups

Type of serums	No.	No. of Positive cases in CHFAg-CCIEP	
		Number	Percentage
Hydatidosis	40	39	97.5
Fascioliasis	9	4	3.64
Toxocariasis	9	2	1.82
Taeniasis	2	2	1.82
Malignancies	20	3	11.82
Normal	70	25	22.73
Total	110	46	41.8

In AgB-ELISA method 37 (92.5%) of hydatidosis patients were found to be positive while only 3 cases (2.73%) of controls (2 healthy control and one fasciolosis) were positive by this system. Accordingly the sensitivity and specificity of the test were 92.5% (95% CI=78.5-98-1%) and 97.3% (95% CI=91.6-99.3%), respectively. Positive and negative predictive value of the assay were 92.5% (95% CI=78.5-98.1%) and 97.3% (95% CI= 91.6-99.2%), respectively (Table 2).

Statistical analysis of the data showed a moderate agreement (kappa=0.481) between ELISA and CCIEP. Table 3 summaries the results obtained by ELISA and CCIEP.

Table 2. Performance of Ag B ELISA in diagnosis of human hydatidosis in hydatidosis patients, other diseases and normal groups.

Type of serums	No.	No. of positive cases in Ag B-ELISA	
		Number	Percentage
Hydatidosis	40	37	92.5
Fascioliasis	9	1	0.91
Toxocariasis	9	0	0
Taeniasis	2	0	0
Malignancies	20	0	0
Normal	70	2	1.82
Total	110	3	2.73

Table 3. Importance of Ag B ELISA compared to CCIEP test for the diagnosis of human hydatidosis

Cases	ELISA		CCIEP		
	Positive	Negative	Positive	Negative	Total
Hydatidosis	37	3	39	1	40
Controls	3	107	46	64	110
True Positive	37		39		
True Negative	107		64		
False Positive	3		46		
False Negative	3		1		
Sensitivity (%)	92.5		97.5		
Specificity (%)	97.3		58.2		
Validity (%)	94.9		77.8		
Positive predictive value (%)	92.5		45.9		
Negative predictive value (%)	97.3		98.5		

DISCUSSION

The diagnosis of CE mainly depends on radiological and immunological procedures. Imaging methods are sometimes limited by the small size of the lesion and the atypical images which are not easy to be distinguished from abscesses or neoplasms. Routine laboratory diagnosis of CE is dependent on detection of specific antibody response. Serum is generally used for detection of specific antibody although some studies showed that detection of antibody in urine might also be a good alternative (13).

The present study reports the use of native antigen B for detection of anti-hydatid cyst antibody and compares it with results obtained by CCIEP using HCF.

The most common antigenic sources used for the immunodiagnosis of CE are HCF, somatic and excretory products from protoscoleces or adult worm (7).

Hydatid cyst fluid (HCF) is a complex mixture of glyco- and lipoproteins, carbohydrates and salts. Some of its components derive from the host (mainly albumin and immunoglobulins), while the remaining are the products of the metacestode. HCF is considered the main antigenic source for the immunodiagnosis of human CE. For clinical practice, crude HCF has a high sensitivity, ranging typically from 75% to 95% (2). However, its specificity is often unsatisfactory and cross-reactivity with sera from patients infected with other cestode, nematode, and trematode species is commonly reported (14).

A sensitivity of 95.5% was reported for CCIEP in the study of Hira et al. using crude hydatid antigen (10). Ardehali et al. reported a sensitivity of 100% for CCIEP using human hydatid cyst fluid and 61% suing sheep hydatid cyst fluid (15). Sadjjadi et al.

evaluated the result of imaging and CCIEP in diagnosis of 1227 surgically confirmed hydatid patients and found CCIEP to be positive in only 62% of the cases (16).
In our study, high false positivity with control serum samples was observed in CCIEP. One reason for this high range of false positivity might be the use of crude antigen (HCF). It has now become more common to purify components of HCF such as the lipoprotein antigens B and 5, the most appropriate components of HCF for immunodiagnosis of CE. Antigen B is a polymeric lipoprotein of 160 KDa composed of several subunits with molecular weight of 8, 16, 24, and 32 KDa (8). Antigen B has been used for serodiagnosis of CE with sensitivities ranging from 50 to 92%.(6) The use of antigen B for serodiagnosis of human CE was evaluated by Williams et al. Antibodies to antigen B was found in 62 percent of CE patients while sera from non-hydatid diseases and controls including a variety of other parasitic infections were non- reactive to the antigen (17).
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 obtain different results using AgB prepared from different host species.
In our study, native antigen B was prepared from sheep HCF and evaluated for detection of anti-hydatid cyst antibody in an ELISA system. Results showed satisfactory sensitivity and specificity for the system. Based on our findings, the specificity of the ELISA system was quite high while the specificity of CCIEP was no more than 60%. Our findings showed that ELISA, using native antigen B, is a reliable method for the diagnosis of CE. In conclusion, CCIEP using HCF must be replaced with ELISA in clinical laboratory settings for the diagnosis of CE.

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