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Serodiagnosis of human hydatidosis with an ELISA developed based on antigens derived from sheep hydatid cysts and comparison with a commercial human ELISA kit

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ABSTRACT

Objective: To explore the serodiagnosis of hydatid cyst in human using different antigens of sheep (hydatid fluid, Somatic and Excretory/secretory antigens of protoscolex) by ELISA and compares this result with commercial human ELISA kit. **Methods:** One hundred blood samples from patients with history of severe abdominal pain and eosinophilia were obtained. Ten serum samples were obtained from surgically and pathologically confirmed cystic echinococcosis patients from Mashhad university hospital as positive control and 5 serum samples from infant under one year old as negative control. Blood samples were centrifuged at 3 000×g at 20 ℃ for 15 min and sera were stored at −20 ℃. First, these samples were tested for the presence of antibody by commercial human ELISA. Then, ELISA was developed on microplates coated with hydatid fluid, Somatic and Excretory/secretory antigens of protoscolex of sheep. **Results:** The results of this study as analyzed by Kappa test showed that, hydatid fluid antigen could be used as a precise source of detection in indirect ELISA test. **Conclusions:** Hydatid fluid in comparison with Excretory-secretory and somatic antigens showed more compatibility agreement in kappa test which can be used for further studies in development of any ELISA test for diagnosis of human hydatidosis.

1. Introduction

Hydatydosis caused by the larval stage (hydatid cyst) of the dog tapeworm, *Echinococcus granulosus (E. granulosus)*, is a major infection with worldwide distribution and variable geographical incidence[1]. This parasite is one of the most important zoonotic diseases prevalent in different parts of the world including the Middle East[2]. The disease is a global zoonotic infection which is economically important and constitutes a threat to public health in many countries[3].

Human infection is common in countries where sheep and cattle rearing constitute an important industry. As diagnosis of this disease by clinical symptoms and scanning alone is often difficult and confusing, some reliable and sensitive serological tests are required to corroborate the evidence reached. Serological tests using crude antigens for diagnosis of *E. granulosus* are sensitive, however their specificity are not satisfactory[4].

For the time being, specific diagnosis of the disease is based on immunological methods supplemented with radiological and ultrasound examinations. A wide number of immunological tests have been developed for the detection of hydatid antibodies and of late hydatid antigens in the serum[1]. The hydatid anti-based serological tests include indirect haemagglutination (IHA), indirect immunofluorescence (IFA), immunoelectrophoresis, counter-current immune-electrophoresis (CIEP), radio-immunoassay (RIA) and Enzyme linked Immunosorbent Assay (ELISA). The hydatid antigen-based sero-logical tests include mainly the ELISA[1]. Diagnosis of infection by ELISA technique was closely related to diagnosis

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using sonographic examination in human and post mortem examination in animals[5]. Many of these assays with higher sensitivity and specificity require sophisticated equipment and trained technicians. Therefore, there is a need for an immunoassay to be simple and inexpensive[6].

Some researchers showed that ELISA may be adapted successfully for the serological diagnosis of hydatidosis. The sensitivity and accuracy of ELISA depend on the composition, concentration and stability of the antigen used. Early diagnosis of human hydatid disease by detecting the specific antibodies in patients' sera is considered an important step in the treatment of infection. But the diagnostic efficiencies of assays depend greatly on the characteristics of the antigen and the various conditions used[7]. ELISA and immunoblotting are among the tests widely used to follow up cystic echinococcosis (CE) patients conventionally by including crude hydatid cyst fluid, which has so far been the major source of antigen used for this purpose[8,9]. It may be preferable to carry out ELISA using more specific antigens so that sufficient specificity can be achieved to take advantage of the greater sensitivity of ELISA[10]. However, the diagnostic performance of these antigens has been assessed in different laboratories, using different serum collections and different techniques, which made it difficult to draw conclusions[11]. Studies in the field of veterinary medicine have shown that many proteins of *E. granulosus* such as hydatid fluid and protoscoleces are immunogenic, and such antigens have been applied in the serodiagnosis of canine intestinal echinococcosis[8,12], in the detection of CE in sheep[13] and CE patients[6]. Currently, the preferred CE immunodiagnostic techniques are hemagglutination and ELISA[3,14]. The aim of this study was the serodiagnosis of hydatid cyst in human using different antigens of sheep hydatid cysts (hydatid fluid, Somatic and Excretory/secretory antigens of protoscolex) by ELISA and compares this result with commercial human ELISA kit.

2. Materials and methods

2.1. Serum samples

One hundred blood samples from patients from Mashhad University of Medical Science with history of severe abdominal pain and eosinophilia were obtained. Ten serum samples were obtained from surgically and pathologically confirmed cystic echinococcosis (CE) patients as positive control and 5 serum samples from infant under one year old as negative control. Blood samples were centrifuged at 3 000×*g* at 20℃ for 15 min and sera were stored at −20℃.

2.2. Preparation of antigen

2.2.1. Hydatid fluid

Hydatid-infected livers and lungs of the sheep slaughtered in Mashhad Abattoir (Iran) were collected and transported to the Parasitology Department, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad (Iran). Then, the surface of the cysts was disinfected by iodine alcohol and the cyst fluid was aspirated under sterile condition[6]. To remove the protoscoleces and large particles, hydatid cyst fluid was centrifuged at 1 000 *g* for 30 min. and the supernatant was collected as hydatid fluid and stored at −20 ℃ until used.

2.2.2. Somatic antigen

Somatic antigens were obtained from protoscoleces removed by aseptic cyst puncture as described by Smyth and Davies[15]. The protoscoleces are washed three times with phosphate-buffered saline (PBS; pH 7.2). The washed protoscoleces are then subjected to three cycles of freezing and thawing and resuspended in 10 volumes of PBS 7.2 containing 0.5M PMSF (at a ratio of 1 in 100). Samples were sonicated in vicinity of ice (1 min, 0.5 amplitude) (Tommy Seiko model UP-200P, Tokyo) until no intact protoscoleces are visible microscopically. Sonicate is left at 4℃ overnight and then centrifuged at 10 000 rpm for 30 min. The supernatant is collected, dispensed in small aliquots and stored as protoscolex antigen at −20℃.

2.2.3. Excretory/secretory antigens

Excretory-secretory antigens (ES-Ag) were obtained as described by Carmena *et al*[12]. Breifly, protoscoleces with viability higher than 90% were selected. Viability was assessed by morphological appearance, flame cell motility and general contractile movements. Protoscoleces were cultured in PBS complemented with 10% glucose, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in 5% CO_2, which promoted parasite survival for several days. Every 8 h the medium was removed and replaced with fresh medium. Protein recovery from the media was achieved by using Ultrafree 15 filters with a 5 kDa pore diameter membrane (Millipore, Bedford, US). EDTA (5 mM) and PMSF (2 mM) were added, and the ES products were aliquotted and stored at −20℃. The concentration of each antigen was measured using Bradford method, and then the samples were stored at −20℃ until used.

2.3. ELISA

Indirect ELISA, using different antigens, was used for the detection of antibodies in sera of CE patients[4]. First, 100 samples were tested for the presence of antibody by commercial human ELISA kit (EUROIMMUN). Ten positive

and 5 negative samples were used. Then, ELISA was developed in flat-bottom 96-well microplates (Nunc, Nalge, Nunc International, Roskilde, Denmark) coated with different amount of hydatid fluid, Somatic and Excretory/secretory antigens of protoscolex of sheep. After optimizing the ELISA kit, one hundred blood samples were tested by indirect ELISA. All the solutions were used at 100 μL per well, except the blocking solution which was used at 300 μL. After optimizing the ELISA kit, one hundred blood samples were tested by indirect ELISA. The plates were coated with 5 μg/well of three antigens of sheep in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated at room temperature (RT) overnight. The following day, after three times washing the wells, they were blocked with 300 μL of 2% (w/v) bovine serum albumin (BSA) in PBS and then incubated for 1 h at RT to block any remaining unblocked attachment sites on the wells. After washing the plates, 1 μL of serum samples diluted in 100 μL of sample buffer were added and then the plates incubated 30 min at RT. Each serum sample was tested in duplicate. Negative and positive controls were used. The wells were washed as above and then 100/well of the secondary antibody (anti-human IgG) in blocking solution which was conjugated to horseradish peroxidase was added to plates and incubated for 30 min at RT. Then 100 μL of substrate solution containing TMB/H_2O_2 was added to each well and the plate was incubated for 15 min in darkness at room temperature. The reaction was stopped by adding 100 μL/well of a solution containing 0.01% w/v sodium azide in 0.1 M citric acid. The absorbance at 490 nm was monitored with a microplate reader (Bio-Rad).

2.4. Statistical analysis

Kappa statistic test was used for agreement of tests with human ELISA kit. According to EUROIMMUN kit, samples with absorbance less than 0.8 were suggested as negative, samples between 0.8-1.1 were suggested as borderline and samples more than 1.1 were suggested as positive. The cut off values for all the ELISAs performed for each antigen were established as the mean of all the tested normal human sera plus three standard deviations.

3. Results

Using commercial human ELISA kit, from 100 blood samples, 3, 45 and 52 samples were positive, negative and borderline, respectively according to kit manual (Table 1). All 10 positive (100%) and 5 negative (100%) control samples showed positive and negative reaction by EUROIMMUN kit. In another experiment, plates coated with hydatid fluid of sheep and from 100 samples, 7, 15 and 78 samples were positive, negative and borderline, respectively. All 10 positive (100%) and 5 negative (100%) control samples showed positive and negative reaction with hydatid fluid. In plates coated with somatic antigen of protoscolex, 13, 13 and 74 samples were positive, negative and borderline, respectively. Eight positive (80%) and 5 negative (100%) control samples showed positive and negative reaction with somatic antigen of protoscolex. Also in plates coated with excretory/secretory antigens of protoscolex, 52 and 48 samples were positive and borderline, respectively with no negative sample (Table 1). Six positive (60%) and 5 negative (100%) control samples showed positive and negative reaction with excretory/secretory antigens of protoscolex. According to the results of 10 and 5 positive and negative samples, amount of the compatibility of hydatid fluid, excretory/secretory antigens of protoscolex and somatic antigen of protoscolex with human ELISA kit were 1, 0.727 and 0.5, respectively by kappa test. Also according to the results of 100 samples, amount of the compatibility of hydatid fluid, excretory/secretory antigens of protoscolex and somatic antigen of protoscolex with human ELISA kit were 0.506, 0.231 and 0, respectively by kappa test.

Table 1
The results of serological test for detection of human hydatidosis with different antigens of sheep along with commercial human ELISA kit.

Sera-borderline	Sera tested negative	Sera tested positive	Antigen
52	45	3	Commercial Ag
	5/5	10/10	Control sera
78	15	7	Hydatid fliud
	5/5	10/10	Control sera
74	13	13	Protoscolex- Somatic Ag
	5/5	8/10	Control sera
48	0	52	Protoscolex-ES Ag
	5/5	6/10	Control sera

Table 2
Comparison of sensitivity (se) and specificity (sp) between three sheep hydatid cyst derived antigens and commercial human ELISA kit.

S antigen	E.S antigen	HF antigen	Human kit	Sesological results
60%	80%	100%	100%	Sensitivity
100%	100%	100%	100%	Specificity

4. Discussion

Immune response in hydatidosis, the basis of laboratory diagnosis, is quantitatively small and frequently insufficiently intense to be detected serologically. This has caused a constant search for increasingly sensitive techniques to detect very low antibody levels. To achieve this, several immunological methods have been evaluated in recent years[16,17]. Many studies have been carried out concerning immunoserologic tests of hydatidosis. These

include studies reporting that the sensitivity of IgG ELISA is excellent[18,19]. El-Shazly *et al*[20] by using ELISA and IHA tests found that the sensitivity and specificity of ELISA was more than IHA test.

Most of the serological tests such as ELISA performed on patients' sera for detection of specific antibodies gave rise to variable results of sensitivity and specificity. False negative results in human hydatidosis composes a serious enigma in attaining a conclusive result, as its rate may be 3%-5% of hydatid patients and even up to 35%-40% in hyper-endemic areas[21]. The antibody is not raised in some of the hydatidosis patients or the titer is low especially in old persons and infants. Also in cerebral, ocular, and calcified cysts, the antibody titer is low and cannot be easily detected[22]. On the other hand, the long persistence of anti-*E. granulosus* antibodies after surgical removal of the cysts results in unreliable diagnosis of relapse in patients[23]. Findings of this study indicated that antibody detection assay in serum samples of CE patients is a sensitive approach for diagnosis of hydatid cyst. In the present study, the results of antibody detection by indirect ELISA, using different antigens, showed that the hydatid fluid was the most effective antigen of those assessed for detection of infection with hydatidosis in human. The reason of this different response might be due to using different antigens or due to different response of people to these antigens. For this reason, these antigens could influence the sensitivity and specificity of the test. When different antigens including hydatid fluid, excretory/secretory and somatic antigens of protoscolex were used, the false positive of samples was 4%, 10% and 41%, respectively. Also, the false negative of hydatid fluid, excretory/secretory and somatic antigens of protoscolex was 30%, 32% and 45%, respectively. In our previous study, the results of antibody detection by indirect ELISA showed that the hydatid fluid was the most effective antigen for detection of hydatidosis in sheep when compared with excretory/secretory and somatic antigens of protoscolex. Previous studies were done to compare between the values of crude or purified hydatid cyst fluid antigens in diagnosis of hydatid disease. Some authors advocate the use of purified or partially purified antigens in serologic systems[24,25]. However, El-On *et al*[26] reported that IgG ELISA based on a purified antigen or crude HCF antigens has comparable sensitivity and specificity, so that either can be used for sero-epidemiological surveys. In contrast to our results, three ELISAs were developed and validated, using as antigen purified 8 kDa antigen B (AgB) hydatid cyst fluid protein (8 kDa ELISA), recombinant EG95 oncosphere protein (OncELISA) or a crude protoscolex preparation (ProtELISA). This study demonstrated that the ProtELISA was the most effective immunological method of those assessed for detection of infection with *E. granulosus* in sheep. Because of its limited diagnostic sensitivity of about 50%-60%, the assay would be useful for the detection of the presence of infected sheep on a flock basis but not for reliable identification of individual animals infected with E. granulosus[13]. Two previous studies by other investigators had used protoscoleces in the primary serodiagnosis of CE. Both showed high diagnostic sensitivities of ELISA of 90.5%[27] and 90%[28].

In conclusion, we have established that serologic tests are valuable tools in hydatid cyst cases and that it would be appropriate to use these tests together with radiology for diagnosis and postoperative follow-up of the disease. We believe that if at least 2 serologic tests are applied, sensitivity in the diagnosis will reach higher values. The nature and quality of the antigens are variable among the host species. This may be one of the reasons why different laboratories obtain different results for the detection of anti-CE antibodies with antigens prepared from different host species[29]. Taken together, findings of this study indicated that antibody detection assay is a sensitive approach for diagnosis of hydatid cyst in human and the sensitivity and specificity of the test using hydatid fluid as the antigen of choice in developing ELISA is accurate more than excretory/secretory and somatic antigens of protoscolex.

Conflict of interest statement

We declare that we have no conflict of interest.

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