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Purification and Characterization of *Echinococcus granulosus* Cathepsin-B Protein and Evaluation of its Role as a Diagnostic Marker

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Abstract: Cystic echinococcosis (CE) is a chronic, complex and neglected disease caused by the larval stage of *Echinococcus granulosus*. The effects of this neglect have a stronger impact in remote rural areas whose inhabitants have no chances of being diagnosed and treated properly without leaving their jobs and travelling long distances, sometimes taking days to reach the closest referral center. The present study was conducted to evaluate the diagnostic efficacy of purified polyclonal antibody (PAbs) raised against *E. granulosus* Cathepsin B for detection of circulating hydatid antigen using Sandwich ELISA. Cathepsin B from sheep and camel livers was purified by ammonium sulfate fractionation and a series chromatography on DEAE Sepharose CL-6B and Sephadex G-75. The purified protein injected in rabbits to raise specific PAbs against *E. granulosus* Detection of Cathepsin B in human serum by sandwich-ELISA gave a sensitivity of 90.47%, a specificity of 94.64%. On the other hand, detection of Cathepsin B in human serum by Dot- ELISA gave a sensitivity of 90%, a specificity of 93.1%. In conclusion, sandwich ELISA techniques appear to be sufficiently sensitive assays for the detection of human echinococcosis using cathepsin B antigen.

Key words: Echinococcosis-Sepharose CL-6B · Sephadex G-75-Sandwich ELISA technique · Dot-ELISA · Cathepsin B.

INTRODUCTION

Human cystic echinococcosis (CE) is a zoonosis caused by the larval stage of the *Echinococcus granulosus* and the most common sites affected are the liver and lung in approximately 80-90% of cases. The hydatid bone represents the 0.5-2.5% of all cases [1].

Echinococcus granulosus is a worldwide geographic distribution and occurs on all continents [2].

Clinical diagnosis of CE is frequently difficult, hence always supported by imaging and immunological methods. The immunodiagnostic methods detecting the antibodies have the disadvantages of low specificity and sensitivity and the inability to differentiate between recent and past infections [3].

The diagnosis of hydatidosis is based on immunodiagnostic methods along with radiological and ultrasound examinations [4, 5]. Although various imaging techniques such as ultrasonography or radiology easily detect CE in clinical settings, the primary diagnosis needs be confirmed by serological tests since the clinical signs of the disease are non-specific [6, 7]. A great number of immunological assays have been developed for detection of anti-hydatid cyst antibodies and recently, hydatid antigens in the serum [8].

Therefore, immunodiagnosis remains an important tool in the diagnosis of the disease. Chordi and Kagan were the first to use immunoelectrophoresis to identify the antigenic components of sheep hydatid cyst fluid (HCF) and subsequently determined which antigenic components were active in detecting antibodies in the sera of patients with hydatid cysts [9]. A successful immunodiagnostic test depends on the use of highly specific and sensitive antigens, as well as the detection of the appropriate antibody class or subclass [10, 11].

However, ELISA results showed highly variable sensitivities and no cross-reactivity with other parasite species has been frequently reported [12].

The presence of a cyst-like mass in a person with a history of exposure to sheep or dogs in areas where *E. granulosus* is endemic supports the diagnosis of cystic

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echinococcosis. However, echinococcal cysts must be differentiated from benign cysts, cavitary tuberculosis, mycoses, abscesses and benign or malignant neoplasms. A non-invasive confirmation of the diagnosis can usually be accomplished with the combined use of radiologic imaging and immunodiagnostic techniques. Radiography permits detection of echinococcal cysts in the lungs; in other sites, however, calcification is necessary for radiographic visualization. Computed tomography, magnetic resonance imaging and ultrasonography are useful for diagnosis of deep-seated lesions in all organs and also for determination of the extent and condition of the avascular fluid-filled cysts. Abdominal ultrasonography has emerged as the most widely used imaging technique for echinococcosis because of its widespread availability and usefulness for defining number, site, dimensions and vitality of cysts. [11] Portable ultrasonography machines have been applied for field surveys with excellent results [13].

Specific diagnosis of the disease is based on immunological methods include indirect haemagglutination (IHA), indirect immunofluorescence (IFA), immunoelectrophoresis, counter-current immune-electrophoresis (CIEP), radioimmunoassay (RIA) and Enzyme linked Immunosorbent. Assay [14]. 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. [15].

Antibody assays are useful to confirm presumptive radiologic diagnoses, although some patients with cystic echinococcosis do not demonstrate a detectable immune response [16]. Hepatic cysts are more likely to elicit an immune response than pulmonary cysts. Regardless of location, the sensitivity of serologic tests is inversely related to the degree of sequestration of the echinococcal antigens inside cysts; for example, healthy, intact cysts can elicit a minimally detect-able response, whereas previously ruptured or leaking cysts are associated with strong responses. The indirect hemagglutination test is sensitive but has now been replaced by (ELISA) for initial screening of sera. Specific confirmation of reactivity can be obtained by demonstration of specific echinococcal antigens by immunoblot assays. Eosinophilia is present in <25% of infected persons [13].

To overcome this several immunological tests, depending on antigen detection, have been developed as an alternative for echinococcosis diagnosis [16]. Detection of parasite antigen also helps to demonstrate the effect of treatment and has a high specificity [18].

The present study was conducted to evaluate the diagnostic efficacy of purified polyclonal antibody (PAbs) raised against *E. granulosus* Cathepsin B for detection of circulating hydatid antigen using Sandwich ELIZA.

MATERIALS AND METHODS

Animal: Two New Zealand white male rabbits, weighting approximately 1.5 Kg and about 2 months age, were purchased from rabbit research unit (RRU), Agriculture Faculty, Cairo University. They were examined before the start of the experiments and found free from parasitic infection and were used in the production of the antibodies [19]. They were housed in the animal house in Theodore Bilharz Research Institute (TBRI), Giza, Egypt. They were kept for 4 weeks (experiment duration) under standard laboratory care at 21°C, 16% moisture, filtered drinking water with additional salt (1cm/5 liter) and vitamin (1cm/10 liter). Diet that contains 15% protein, 3% fat and 22% fiber was purchased from RRU. Animal experiments were carried out according to the Internationally Valid Guidelines.

Parasite: Hydatid cysts were removed from liver and lungs of both sheep and camels from an abattoir in Cairo Governorate and were transferred to our laboratory in TBRI in Hanks' buffer **(**Hanks' Balanced Salt Solution) (HBSS) to stimulate normal ion concentration under physiological tissue conditions [20].

Sera Samples

Collection of Samples from Human Subjects: Forty two *E. granulosus* infected human patients from highly endemic areas in Alexandria Governorate who were admitted to tropical and surgical departments of TBRI and El-Azhar University Hospitals were diagnosed by sonography, CT and MR imaging to have CE in their livers. Besides, 24 patients infected with other parasites (*Schistosoma mansoni*, *Fasciola gigantica* and Hookworms) were included. In addition, 20 individuals of the medical staff at TBRI served as parasite free-healthy negative controls. Blood samples were collected from all cases and sera were separated, aliquoted and kept at -20 °C until used.

Collection of Samples from Naturally Infected Animals: A total of 207 animals (sheep and camels) samples serum were collected during several visits to local abattoir (127) animals were infected by *E. granulosus*, 50 animals were positive for other parasites (*Schistosoma mansoni*, *Fasciola gigantica* and *Hookworms*) than *E. granulosus* and 30 animals were healthy control). Sera were collected during slaughtering. The livers and gall bladders of animals undertaken in the study were checked for the adult flukes. Sera were stored at -20°C until used.

Preparation of Parasite Antigen: *Echinococcus granulosus* hydatid fluid was collected from ovine fertile cysts for subsequent use as a specific parasite antigen and clarified by centrifugation at 10,000 *g* at 4°C for 60 min., dialyzed against phosphate buffer saline (PBS) pH 7.2. Protoscoleces were prepared following the method of Rafiei and Craig [20]. In brief, protoscolex were collected and the viability was determined by the vital coloration approach with 0.2% eosin staining. The protoscoleces were subjected to three cycles of freezing and thawing and suspension in 10 times their volume of 0.15 M PBS, pH 7.2. Subsequently, the protoscolex were suspended in 4 times their volume of PBS containing 0.1 mg aprotinin/ mL, then sonicated on ice in a 150 W ultrasonic disintegrator, until no intact protoscolex were visible microscopically and the supernatant solution was split into aliquots and stored at -20°C until further processing.

Purification and Characterization of Cathepsin *B* Antigen: Ammonium Sulfate, DEAE- Sepharose CL-6B and gel filtration chromatography on Sephadex G-75column, these techniques for purification of antigen by separating proteins on the basis of charge and molecular size according to Smith *et al*. [21]. Then protein content was estimated by a Bio-Rad protein assay as shown by Bradford [23]. Finally characterization of protoscolex antigen by SDS-PAGE according to Laemmli [24].

Characterization of Cathepsin B Antigen: The purified **cathepsin B antigen** protein was assayed by sodium dodecyl sulfate. polyacrylamide gel electrophoresis (SDSPAGE) under reducing conditions [24].

Assessment of Reactivity of Cathepsin B by Indirect ELISA: This method was performed, with some modifications from the original method of Engvall and Perlman [24].

Production and Purification of Polyclonal Antibodies: Before immunization, rabbits were assayed by ELISA for hydatid Abs and cross reactivity with other parasites. Rabbits were injected intramuscularly (i.m.) at four sites according to Fagbemi *et al.*[25] with 100ìg of purified cathepsin *B* antigen mixed with equal volume of complete Freund's adjuvant (CFA, Pierce, Rockford, IL, USA). Then, 3 booster doses (0.5 mg of purified cathepsin *B* antigen with equal vol. of incomplete Freund's adjuvant (IFA, Pierce) were given at one week intervals. One week after the last booster dose, the rabbit's sera were obtained and PAb fraction was purified by 50% ammonium sulfate precipitation method [26]. More purification of PAb was performed by caprilic acid method [27]. The reactivity of anti-cathepsin B IgG PAb against Echinococcus antigens was assessed using indirect ELISA.

Detection of Circulating cathepsin B in Human Sera by Sandwich ELISA: The microtitration plates were coated with 100 μl/well of 1/25anti-purified **cathepsin B** IgG pAb, incubated overnight at room temperature and washed 3 times with 0.1 M PBS/T, pH 7.4. Wells were blocked with 100 μl/well of 2.5% FCS/PBS/T, incubated for2 h at 37°C and washed 3 times with PBS/T. 100 μl of human serum samples was pipette into the wells in duplicate, incubated for 2 h at 37°C and washed 3 times. 100 μl/well of peroxidase-conjugated PAb of 1/100 for IgG was then added, plates were incubated for 1 h at room temperature. The plates were washed 5 times with washing buffer, 100ìl of substrate solution were added to each well and the plates were incubated in the dark at room temperature for 30 min., 50 ìl/well of 8 N H_2SO_4 were added to stop the enzyme substrate solution. The absorbance was measured at 492 nm using ELISA reader.

Statistical Analysis: The data are presented as mean±standard deviation of mean (X±SD). The mean values of each group were calculated from the mean values of individual patients. The mean groups were compared by analysis of variance [26]. This may be accomplished by changing the selection of the reference value (i.e. cut-off) for the particular test [28].

- Sensitivity= (no. of true +ve cases/ no. of true +ve cases + no. of false -ve cases).
- Specificity= (no. of true -ve cases/ no. of true -ve cases + no. of false +ve cases).
- Positive predictive value (PPV) = (No. of true +ve cases/ no. of true +ve cases + no. of false +ve cases).
- Negative predictive value (NPV) = (No. of true -ve cases/ no. of true -ve cases + no. of false -ve cases).

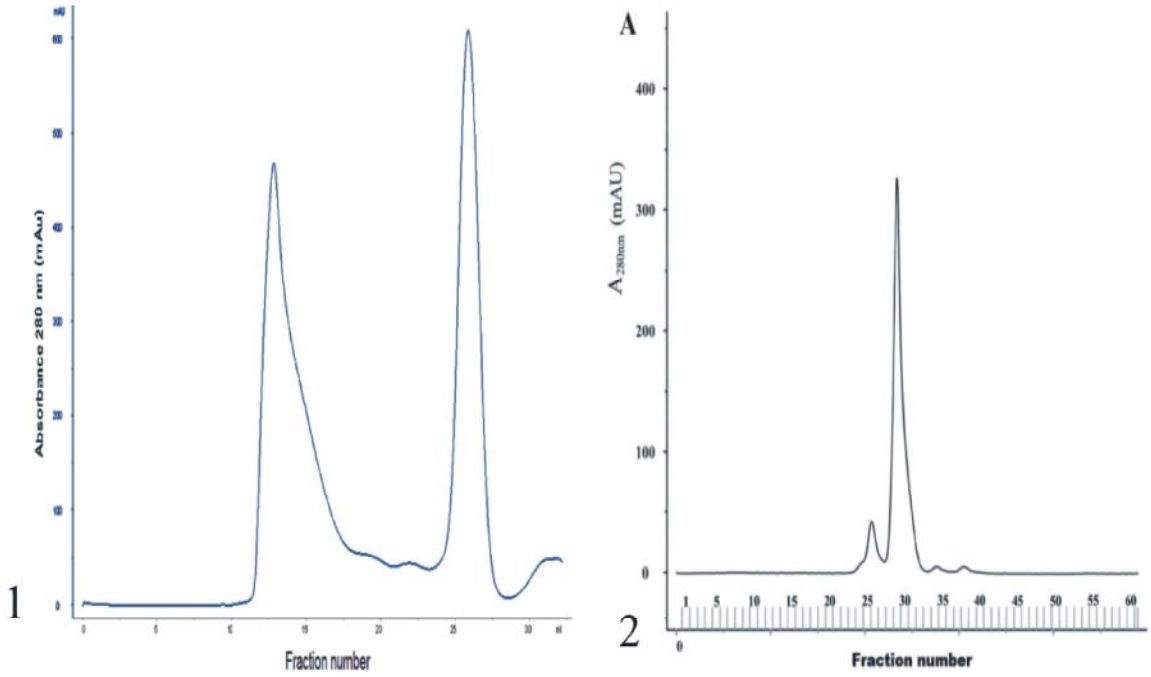

Fig. 1: Elute profile for chromatography of Cathepsin B antigen on Sepharose CL-6B (1) and DEAE SephadexG-75 chromatography (2).

RESULTS

Calculation of Total Protein Content of cathepsin B: Cathepsin B obtained from hydatid cyst fluid contains 7.7 mg/ml of total protein as measured by Bio-Rad protein assay while it was 4.5 mg/ml after precipitation.

Purification and Characterization of Cathepsin B from Hydatid Cyst Fluid of *Echinococcus granulosus*

1. Purification by DEAE-Sepharose CL-6B and gel filtration chromatography on DEAE SephadexG-75: The fractions collected from Sepharose CL-6B chromatography were further purified by DEAE SephadexG-75 gel filtration column chromatography and one peak (a) was obtained represents the column elution volume fractions which contain Cathepsin B with OD value 1.30 at fraction number 29 (Fig. 1).

Characterization of Cathepsin B antigen by SDS-Gel Electrophoresis: The eluted protein fractions resulted from the different purifications methods was analyzed by 12.5% SDS-PAGE under reducing condition and stained with Coomassie Blue. Protein band were appeared at 3 different bands at 13, 26 and 29 kDa which representing purified cathepsin B antigen (Fig. 2).

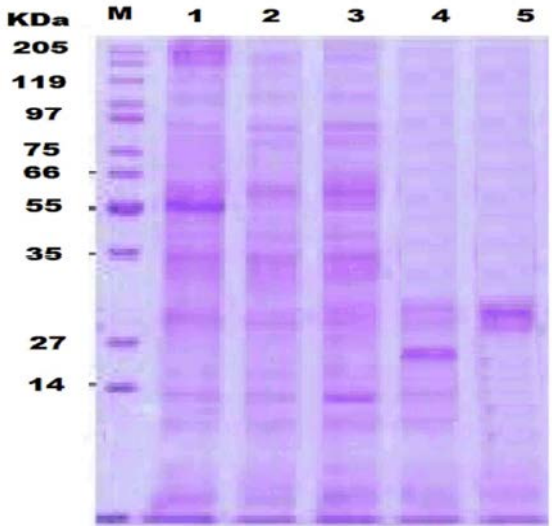

Fig. 2: 12.5% SDS-PAGE of cathepsin B antigen before and after purification (stained with Coomassie Blue).
Lane 1: Crude extracts of *E. granulosus* adult worms.
Lane 2: The homogenized cyst.
Lane 3: Purified Cathepsin B after ammonium sulphate.
Lane 4: Purified CathepsinB after CL-6B column.
Lane 5: Purified Cathepsin B after G-75 column

Table 1: Reactivity of purified cathepsin B antigen by indirect ELISA.

Serum samples	MOD readings (human) at 492 nm±SD
Echinocucuss granulosus	1.33±0.205
Shistosoma mansoni	0.226±0.191
Fasciola gigantica	0.196±0.103
Hook worms	0.213±0.191

Table 2: Reactivity of rabbit anti- *E. granulosus* IgG PAb against many parasitic antigens by indirect ELISA

Parasitic antigen	MOD readings (human) at 492 nm±SD
E. granulosus	2.11±0.152
F.gigantica	0.263±0.224
S. mansoni	0.352±0.201
Hookworms	0.217±0.112

Reactivity of Cathepsin B antigen by Indirect ELISA: Serum samples from human infected with *E. granulosus* gave a strong reaction against Cathepsin B antigen with mean OD reading equal to 1.33 and no cross reactions were recorded with sera of animals or patients infected with other parasites e.g., *S. mansoni*, *F. gigantica* and hookworms (Table 1).

Production and Purification of Polyclonal Antibodies: Test blood samples were withdrawn from rabbit before the injection of each immunizing dose. They were tested for the presence of specific anti-*E. granulosus* antibodies (Abs) by indirect ELISA. Three days after the 2nd booster dose immune sera gave a high titre against cathepsin B antigen with OD of 2.66 (Fig. 3). The total protein content of crude rabbit serum containing anti- *E. granulosus* antibody was 9.6 mg/ml. The yield of purified anti-*E.granulosus* IgG PAb following each purification step was determined by the assessment of protein content. Purification using the 50% ammonium sulfate precipitation method [29], the protein content was 5.1 mg/ml, while following 7% caprylic acid [30] precipitation method the content dropped to 3.8 mg/ml.

Characterization and Reactivity of anti-*E. granulosus* IgG PAb: The purity of IgG after each steps of purification was assayed by 12.5% SDS-PAGE under reducing condition. The purified Pab IgG was represented by H- and L-chain bands at 50 and 31 kDa respectively (Fig. 4). The PAb appears free from other proteins. Reactivity of anti-*E. granulosus* antibodies against protoscolex antigen and other parasitic antigens (*S. mansoni*, hookworms, *F. gigantica*) was determined by indirect ELISA. The produced anti- *E. granulosus* antibodies diluted in PBS buffer gave strong reactivity to protoscolex antigen. The OD readings at 492 nm for *E. granulosus* were 2.11 compared to 0.352, 0.217 and 0.263 for *S. mansoni*, hookworms and *F. gigantica*, respectively (Table 2).

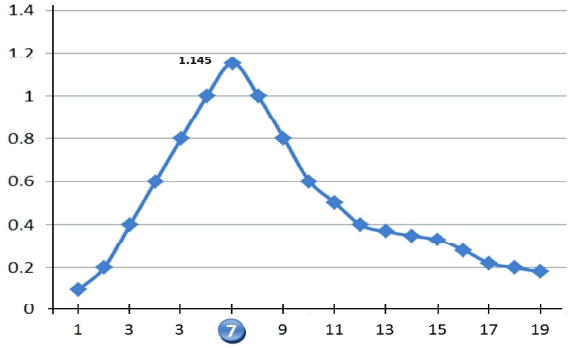

Fig. 3: Elute profile for chromatography of polyclonal antibody on DEAE sephadex A-50 ion exchange chromatograph

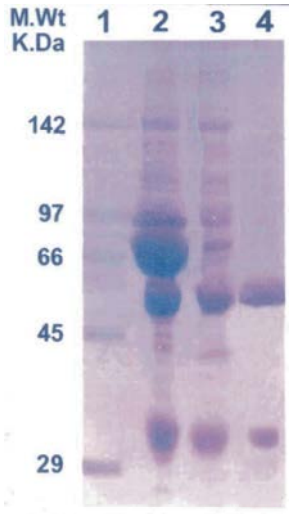

Fig. 4: 12.5% SDS-PAGE of anti- *E. granulosus* IgG polyclonal antibodies before and after polyclonal antibody (stained with coomassie blue).
Lane 1: Molecular weight of standard protein.
Lane 2: Crude anti- *E. granulosus* IgG polyclonal antibody.
Lane 3: Precpitated proteins after 50% ammonium sulfate treatment.
Lane 4: Purified IgG antibody after 7% caprylic acid treatment.

Detection of Cathepsin B antigen in human serum

1. Detection of circulating Cathepsin B antigen in human and animal serum using sandwich ELISA:

Tables 3 show the results of OD value of the human serum samples group. The cut off values for positivity was 0.26 and 0.27 respectively. The OD values of *E. granulosus* infected group (2.23±0.358) were significantly higher than both the negative control group (0.279±0.119) and the other parasites groups (0.519±0.193) (Table 3).

Table 3: Detection of circulating CathepsinB antigen in sera of human subjects infected with *E. granulosus* or other parasite in comparison to healthy control

	Positive Samples		Negative Samples	
Group (no. of human)	*No.*	*M±SD*	*No.*	*M±SD*
Healthy control (n= 20)			20	0.209±0.121
E. granulosus (n= 42)	38	2.23±0.358	4	0.279±0.119
S. mansoni (n= 8)	2	0.519±0.193	6	0.231±0.108
F. gigantica (n= 8)	1	0.622±0.233	7	0.257±0.180
Hookworm (n= 9)	1	0.659±0.139	8	0.311±0.152

Table 4: etection of circulating Cathepsin B in serum of naturally infected animals

	Positive cases		Negative cases	
Group (no. of animals)	No.	X±SD	No.	X±SD
Healthy control (n= 30)			30	0.273±0.198
Echinococcus (n= 127)	112	1.98±0.326	15	0.311±0.117
Schistosoma (n= 16)	5	0.619±0.222	11	0.219±0.149*
Hookworm (n= 12)	--	--	12	0.275±0.091*
Fasciola(n= 9)	1	0.433±0.251	8	0.301±0.109*
Trichostrongyloid (n= 13)	1	0.508±0.171	12	0.196±0.111*

Table 5: Summarizes of the sensitivity, specificity, PPV and NPV of sandwich ELISA that used for detection of cathepsin B antigen in human serum

Echinococcus antigen detected in:		Sensitivity	Specificity	PPV	NPV
Animal	Serum	90.38%	91.95%	95.27%	82.95%
Human	Serum	90.47%	94.64%	92.68%	92.45%

Table (4) shows the results of Cathepsin B detection among different studied groups in animal serum samples. The cut off values for positivity was calculated as mean + 2 SD and recorded 0.37±0.20.

The OD values of *Echinococcus* infected animals group (1.98±0.326) was significantly higher (P< 0.001) than both the negative control group (0.273±0.198) and other parasite group (0.29±0.32).

112 cases were detected as positive samples of Echinococcus infected animals from 127 cases. Those 15 samples were among the light infection subgroup and the sensitivity of the assay was 90.38%. All the 30 negative controls were below the cut off value, while 7 out of 50 (other parasites group) were above the cut off value recording a 91.95% specificity. (Table. 5)

DISCUSSIONS

Human cystic echinococcosis (CE) is regarded as a significant public health problem with high morbidity and mortality rates in endemic areas worldwide [31].

Cystic hydatid disease (CHD) is detectable clinically through various imaging techniques such as ultrasonography or radiology. The primary diagnosis must be confirmed by more specific testing, such as serological tests based on the discovery of antibodies against the organism antigens in the patient's serum [32]. Ordinary serological tests such as immunoelectrophoresis, double diffusion in agar, or indirect hemagglutination are being replaced by more sensitive assay methods such as (ELISA), immunoblot (IB) and indirect immunofluorescent antibody test (IFA) [32]. ELISA is a high-sensitivity test that is strongly recommended for the detection of specific antibodies in cystic human disease (CHD) cases [4, 25]. Antigen detection assay in serum is generally performed by sandwich ELISA [35].

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. 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 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. Purified cathepsins *B* was a glycoprotein consisting of mixtures of 1-chain and 2- chain forms [36]. The purified cathepsin B from bovine brain demonstrated an apparent molecular weight of 27 kDa on gel filtration, but was resolved into 3 bands of 30, 25 and 5 kDa by SDS-PAGE [37, 38].

The molecular mass of the cathepsin *B* from hepatopancreas of carp was 29- 30 kDa by GPC and the enzyme migrated as 2 protein bands on SDS-PAGE with molecular weights of 30 and 26 The homogeneity of the final preparation of cathepsin *B* was confirmed on Native-PAGE, which gave a single protein band However, it migrated as 2 protein bands on SDS-PAGE with estimated molecular weight of 23 and 26 kDa [38].

Cathepsin B antigen used for immunization of rabbit for preparation of rabbit anti-E.granulosus IgG PAb.1mg of cathepsin B antigen were given to each rabbit in entire course of immunization in the first dose [1mg cathepsin B antigen mixed 1:1 in Freund's complete adjuvant (Sigma)]and 0.5 mg emulsified in incomplete Freund's adjuvant in the second and third booster doses injection.

The first boosting was two wk. after priming dose. The following boosting doses were given at weekly intervals according to *Tendler et al.* [39]

The purification procedures followed in this study were satisfactory, for IgG PAb two purification methods undertaken; ammonium sulfate precipitation which showed that, most of albumin was removed from rabbit anti-E. granulosus IgG PAb, 7% caprylic acid according to Goding [39]. The purity of IgG PAb was assayed by 12.5% SDS-PAGE. The purified IgG PAb was represented by H- and L- chain bands at 50 and 31 kDa respectively, indicating that, the purified PAb appears free from other proteins. The yield of PAb as protein content by these methods was 6.4 mg/ml IgG from starting protein content of 16.3 mg/ml.

In the present study, reactivity of purified PAb demonstrated the reactivity of PAb as determined by indirect ELISA, gave a strong reactivity to cathepsin B antigen. The purified PAb was further used as a primary capture to coat ELISA plates. The secondary capture of PAb was by conjugation with Horse-Raddish Peroxidase enzyme (HRP), sandwich ELISA was adopted using a pair of PAbs against cathepsin B antigen, anti- E. granulosus IgG PAb and peroxidase-conjugated IgG polyclonal antibodies.

The cut-off value for positivity in sandwich ELISA for cathepsin B antigen was equal to 0.26 in serum. All values equal to or above these cut-off values were considered positive. On detection of E. granulosus

circulating antigen by sandwich ELISA in human serum samples, 38 out of 42 echinococcosis cases gave positive results with 90.48% sensitivity and 91.3% specifity. In healthy control serum samples, no positive results were obtained giving specificity 100%, but PPV and NPV were 95%, 91.3% respectively, as for sandwich ELISA with paramagnetic nanoparticles the sensitivity, specificity, PPV and NPV were 95.2%, 95.5%, 97.6% and 95.5% respectively.

The standard double antibody sandwich ELISA is a common method for measuring the presence and/or concentration of circulating parasite antigens [41].

In conclusion, sandwich ELISA techniques appear to be sufficiently sensitive assays for the detection of human echinococcosis using cathepsin B antigen.

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