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Research Article
Application of Latex Agglutination and Sandwich ELISA in Detection of Human Hydatidosis

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Abstract

Background and Objective: Immunodiagnosis has been found to be useful not only in primary diagnosis of cystic echinococcosis (CE) but also for the follow up of patients after treatment. The present study aimed to prepare and purify paramyosin antigen of *E. granulosus* and evaluate its diagnostic efficacy for detection of echinococcosis by latex agglutination test (LAT) and sandwich Enzyme Linked Immuno-sorbent assay (ELISA) in human serum samples. **Materials and Methods:** The LAT and sandwich ELISA was carried out using purified hydatid cyst paramyosin antigen by gel filtration. The tested sera included sera from patients infected with hydatidosis (n = 41), sera of patients with other parasites; 8 with *Schistosoma mansoni*, 5 with *Schistosoma haematobium*, 10 with fascioliasis, 10 with amoebiasis and 20 tested negative control. **Results:** The results showed that by using LAT; 10 out of 41 *E. granulosus* infected samples showed false negative results and the sensitivity of the assay was 75.6%. All 14 out of 33 of other parasites groups were at the border line of the cut off value giving 73.6% specificity. The 5 false positive cases in the group of patients infected with *Fasciola* and 5 false positive cases in patients with liver amoebiasis. The 3 false positive cases found in patients with *Schistosoma mansoni* and only one false positive case in patients with *Schistosoma haematobium*. By using sandwich ELISA, 37 of *E. granulosus* infected patients had high positive antigen concentration leading to sensitivity of 80.4% while the specificity was 71.7%. **Conclusion:** The LAT is a suitable and applicable diagnostic method especially when followed by Sandwich ELISA as a confirmatory test for diagnosis of cystic echinococcosis.

Key words: Latex agglutination, sandwich ELISA, Immunodiagnosis, *Echinococcus granulosus*, paramyosin antigen, cystic echinococcosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Echinococcosis, also called parasitic disease of tapeworms of the *Echinococcus* type. The two main types of the disease are cystic echinococcosis and alveolar echinococcosis[1]. Hydatid cysts of *E. granulosus* develop in internal organs mainly the liver (in 75% of cases) and lungs (in 5-15% of cases) of humans and intermediate hosts (herbivores such as sheep, horses, cattle, pigs, goats and camels) as unilocular fluid-filled bladders. The disease often starts without symptoms and this may last for years. The symptoms and signs that occur depend on the cyst's site and size. The disease spread when food or water that contains the eggs of the parasite are eaten or by close contact with an infected animal[2]. The eggs are released in the stool of meat-eating animals that are infected by the parasite. Commonly infected animals include dogs, foxes and wolves. For these animals to become infected they must eat the organs of an animal that has the cysts such as sheep or rodents[3]. Prevention of cystic disease is by treating and vaccination of sheep is often difficult. The cystic disease may be drained through the skin, followed by medication. The alveolar type often needs surgery followed by medications. The medication used is albendazole and mebendazole are the only anthelmintic effective against the alveolar disease[4].

Cystic echinococcosis (CE) is re-emerging as a major public health issue, despite efforts to control CE, the disease continues to threaten human health in a number of countries, including Egypt. In the last few years, several cases of human CE have been reported and treated in the hospitals of Cairo and the Delta regions[5]. In upper Egypt, a serological survey showed that 5% of human samples tested positive for *E. granulosus*[6]. It is well-known that the prevalence rates in farm animals are in co-ordinance with the human rate of infection, the spectrum of symptoms depends on the following; involved organs, size of cysts and their sites within the affected organ or organs, interaction between the expanding cysts and adjacent organ structures, particularly bile ducts and the vascular system of the liver, complications caused by rupture of cysts, bacterial infection of cysts and spread of protoscolices and larval material into bile ducts or blood vessels, immunologic reactions such as asthma, anaphylaxis or membranous nephropathy secondary to release of antigenic material. Diagnosis is a basic part of population studies on echinococcosis[7].

Diagnosis of hydatidosis is still difficult, which is based mainly on serodiagnostic techniques. So, in recent years, the validity of serological techniques for diagnosis of hydatidosis has been improved. A variety of techniques are applied for such diagnosis in different animals and human as enzyme-linked immunosorbent assay (ELISA)[8] and Enzyme-linked immune-electro-transfer blot (EITB)[9]. The serological techniques are still lacking the diagnostic specificity, especially in endemic areas therefore, evaluation and purification of antigens are needed to increase the sensitivity of these serological techniques for the detection and confirmation of the disease in its early stages[10]. Immunoblotting has been reported to yield very sensitive and specific results in diagnosis of Hydatidosis[11]. Both camel and sheep hydatid fluid and protoscoleces antigens, beside the immune-reactive study may be helpful for diagnosis of hydatidosis in human. However, immunological procedures for the detection of antibodies to hydatid infection need epidemiological studies, further examination and treatment so effective measurements might become implemented for a better control of this zoonotic cystic echinococcosis[12].

The use of multiple parallel testing or additional confirmatory tests in a diagnostic strategy can overcome the lack of a perfect gold standard. This will yield valuable information at both population and individual levels[13]. Antibody detection assays have many drawbacks, for example, they cannot distinguish between past and present infections and cannot be used for assessment of the efficacy of treatments. Antigen detection assay may circumvent this problem[14]. It has been shown that hydatid cyst antigen can be detected in the serum or urine of hydatidosis patients. Circulating hydatid antigens are present in the serum only during active infection and the levels of these antigens continue to decrease after surgical removal of the hydatid cyst or successful chemotherapy[15]. The detection of circulating antigens rather than antibodies may be very useful in the Immunodiagnosis of hydatid disease[16]. So, the present study aimed to prepare and purify paramyosin antigen of *E. granulosus* and evaluate its diagnostic efficacy for detection of Echinococcosis by latex agglutination and sandwich ELISA in human serum samples.

MATERIALS AND METHODS

Animal: Two New Zealand white male rabbits, weighting approximately 1.5 Kg and about 2 months age, purchased from rabbit research unit (RRU), Agriculture Faculty, Cairo University. They were examined before the start of the experiments and were used in the production of the antibodies[17].

Parasite: Hydatid cysts were removed from sheep and camel liver and lungs from an abattoir in Cairo Governorate and were

transferred to the laboratory in TBRI in Hanks' buffer (Hanks' Balanced Salt Solution) (HBSS) to stimulate normal ion concentration under physiological tissue conditions[18].

Serum samples: For assessing diagnostic sensitivity of paramyosin antigen in diagnosis of human echinococcosis, 41 blood samples from patients with echinococcosis were used (28 patients suffered from cysts in the liver and 13 patients had pulmonary cysts). All of these were clinically asymptomatic. Other proven parasitic diseases: 8 infected with *Schistosoma mansoni* and 5 with *Schistosoma haematobium*, fascioliasis (10 patients) and liver amoebiasis (10 patients). About 20 blood samples used as negative control were from healthy individuals. Serum samples were separated, aliquoted and kept at -20°C until used.

Preparation of parasite antigen: The hydatid cyst fluid (HCF) was aseptically obtained from the hydatid cysts in sheep collected from an abattoirs in Cairo Governorate. To remove the protoscolices and large materials, HCF was centrifuged (1,000 g for 30 min). Protein content of the sample was determined[19].

Protein extraction in high salt concentration: Hydatid cyst fluid were homogenized in PBS buffer, pH 7.0 followed by 30 sec pauses, using an Ultrasonic Homogenizer 4710 (Cole-Palmer Instrument, Ill.). The sonicated hydatid cyst fluid was centrifuged at 48,000 g for 30 min at 4°C and the pellet obtained was suspended in 1 vol of 20 mM Tris-HCl with pH 8. Aliquots of supernatants, were analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of comparable composition were pooled and concentrated four fold by ultra-filtration on a PM10 membrane (Amicon, Mass.). The concentrated material was then subjected to gel filtration chromatography as described[20].

Gel filtration chromatography: Gel filtration of the crude paramyosin extract was performed on a Pharmacia Bio-Pilot system using a Superdex 200 HR16/50 column (Pharmacia Biotech, Uppsala, Sweden). Approximately 10 mg of protein in 2 mL were applied and eluted in the same buffer at a flow rate of 2.5 mL min^{-1}. Protein elution was monitored at 280 nm and 1 mL-fractions were collected. Protein fractions were analyzed by 12.5% SDS-PAGE[21].

SDS-PAGE and protein staining: Electrophoresis was performed, where proteins run under non-reducing and reducing conditions (10 mM DTT) were stained using Coomassie brilliant blue R250 (CBB-R250) protocol[22].

Production and purification of polyclonal antibodies: Rabbits were used for production of antibodies, by injected intramuscularly (i.m.) at four sites according to Fagbemi *et al.*[23]. Gel-filtration was performed by caprylic acid method caprylic acid method of McKinney and Parkinson[24]. The reactivity of anti-paramyosin antigen IgGpAb against *Echinococcus* antigens was assessed using indirect ELISA.

Labeling antibodies with horseradish peroxidase: Conjugation of antibodies was performed as mainly described by Wilson and Nakane[25]. The solution was dialyzed against PBS overnight and the conjugated antibody was stored at -20 in small aliquots until use.

Indirect ELISA for detection of IgG4 antibody: The indirect ELISA was carried out according to method described by Hassan *et al.*[26] and the optimal antigen, serum and conjugate concentrations were determined after preliminary checkerboard titration according to Shaapan *et al.*[27]. In this study, the optimum conditions were 10 μg mL^{-1} coating buffer for paramyosin antigen, 1:100 serum dilution, 1:3000 peroxidase labeled rabbit anti-human, (Sigma) as conjugate and 1 mg p-nitrophenyl-phosphate (p-NPP) dissolved in 1 mL substrate buffer as substrate.

Detection of human echinococcosis in serum by latex agglutination test (LAT): A polystyrene latex suspension (0.81 μm; Sigma, St. Louis, MO) was used in this test. About 1% standardized polystyrene latex suspension was prepared. An equal volume of sensitized latex reagent was added to the tested serum on one half. The same volume of control latex suspension was added to the serum on the other half as a negative control. The intensity of agglutination accumulated around the edge of the according to reaction zone, the positivity was classified into high (+++), moderate (++), low (+). When no agglutination was seen, the result was considered negative (-)[28].

Detection of human echinococcosis in sera by sandwich ELISA: The micro titration plates were coated with 100 μL $well^{-1}$ of 1/25 anti-purified paramyosin IgGp Ab. About 100 μL of human serum samples was pipette into the wells. The plates were washed 5 times with washing buffer. About 100 mL of substrate solution were added to each well and the plates were incubated in the dark at room temperature for 30 min. The absorbance was measured at 492 nm using ELISA reader[29].

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. One-way of ANOVA test conducted and the level of significance was p>0.05.

- **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

Ethical approval: The study was approved by National Research Centre Ethics Committee (approved at date of 1/7/2015). Written consents were taken from all the participants in the study before collecting the data and all had the right to withdraw from the study if they refuse to participate.

RESULTS

Purification of paramyosin by column chromatography: Superdex 200 HR16/50 column chromatography further purified the partially purified antigen by high salt concentration method. The eluted antigen was represented by multiple peaks (from a to e) with maximum OD value equal to 0.93 at fraction (e). The eluted fractions contained the paramyosin antigen (Fig. 1).

SDS-PAGE of purified paramyosin hydatid cyst antigen: The migration pattern of hydatid cyst fluid antigens in SDS-PAGE gel was analyzed. Hyd-crude antigen revealed several protein bands with molecular weight ranged from 14-98 kDa. Hyd-partially purified antigen after homogenization revealed many protein bands with molecular weight of 14-97 kDa. Hyd-purified antigen after high salt concentration revealed major bands at 14, 42, 45 and 96 and many minor bands. Hyd- paramyosin antigen after gel filtration chromatography revealed a major single band at 95 KDa (Fig. 2).

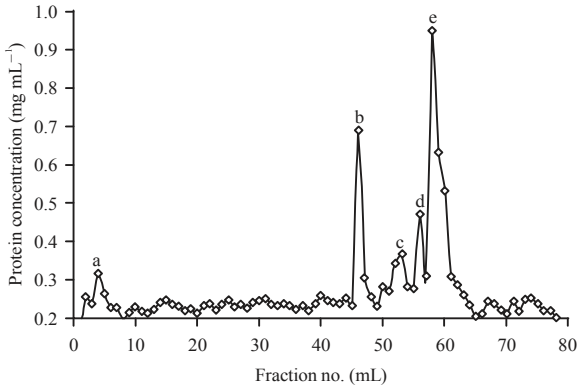

Fig. 1: Profile of superdex 200 HR16/50 column chromatography elution fractions

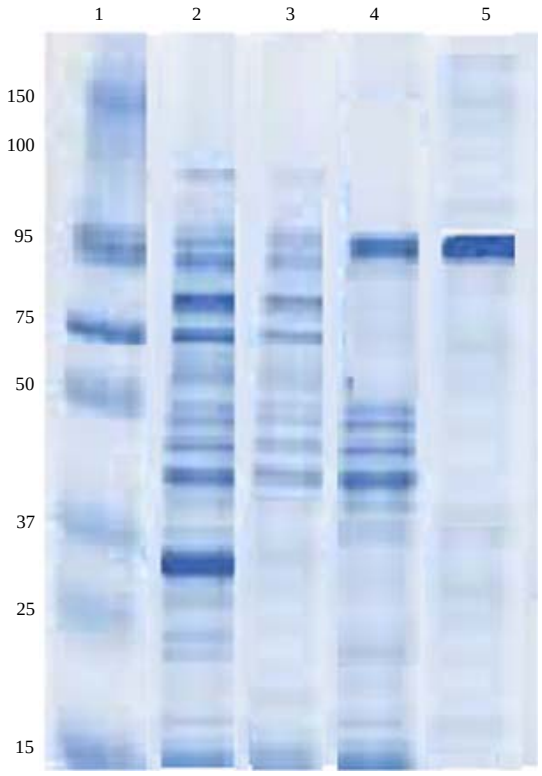

Fig. 2: SDS-PAGE of purified paramyosin antigen eluted from affinity chromatography columns
Lane 1: Low MW standard, Lane 2: Crude antigen, Lane 3: Partially purified antigen after homogenization, Lane 4: Purified antigen after high salt concentration and Lane 5: Paramyosin HC-antigen

Production of pAbs against *E. granulosus* tegument-associated antigen: Blood samples were withdrawn from New Zealand white rabbits before the injection of each immunizing dose. They were tested for the presence of

specific anti-paramyosin antibodies by indirect ELISA. An increasing antibody level started one week after the first booster dose. A high titer of specific anti-paramyosin antibodies was observed at 4th week from the start of immunization dose with OD of 1.88 at 1/100 dilution (Fig. 3).

Detection of anti-hydatid IgG4 antibody by Indirect ELISA: All cases of negative controls had negative results while 15 out of 33 other parasites group showed positive ELISA results for anti-hydatid IgG4 antibody 4 Schistosoma mansoni, 2 Schistosoma haematobium, 5 fascioliasis, 4 liver amoebiasis. The sensitivity and specificity of the indirect ELISA was calculated as 80.5 and 71.7%, respectively. A highly significance in the level of anti-hydatid antibodies was observed in the hydatid group than the other studied groups represented by the area under the curve reached to 0.686 (Fig. 4).

Detection of human echinococcosis in serum by latex agglutination test (LAT): The levels of circulating hydatid antigens in serum samples of different studied groups were measured by the purified IgG anti-paramyosin pAb-based LAT. The false negativity *E. granulosis* infected samples was 24.4% and the sensitivity of the assay was 75.6%, while 14 out of 33 of other parasites groups were at the border line of the cut off value giving 73.6% specificity. Five false positive cases were belonged to the group of patient infected with *Fasciola* and other five false positive cases where belonged to the group of patient infected liver amoebiasis. Three false positive cases were belonged to the group of patient infected with *Schistosoma mansoni* and only one false positive case were belonged to the group of patient infected *Schistosoma haematobium* (Table 1).

Detection of human *Echinococcus* antigen in serum by sandwich ELISA: The cut-off values were 0.393. The high positive *E. granulosus* infected patients was 37 had leading to sensitivity (80.4%) while the specificity (71.7%) which was

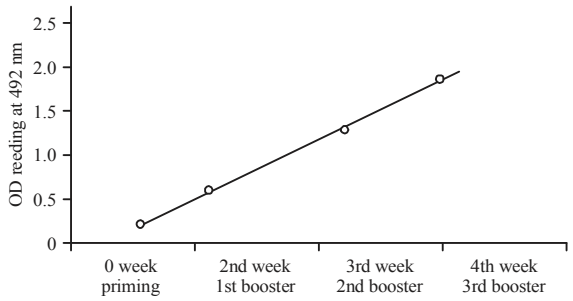

Fig. 3: Reactivity of immunized rabbit anti-hydatid IgG (pAb) against paramyosin by indirect ELISA

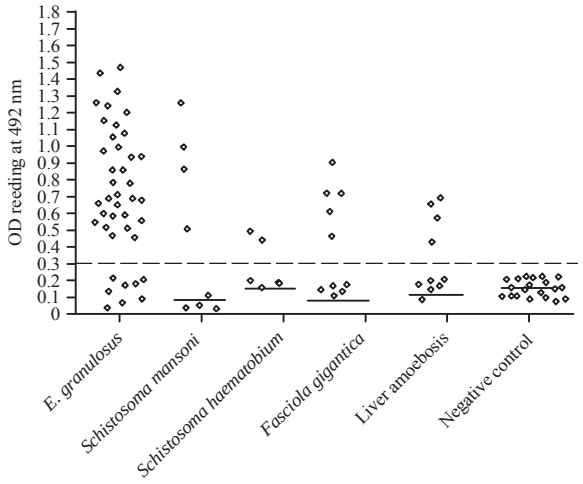

Fig. 4: Anti-hydatid IgG4 antibodies using purified paramyosin antigens in different studied groups

Table 1: Detection of human echinococcosis in serum by Latex agglutination test

	Positive cases			Negative cases		
Groups	Score of the color range	No.	Percentage of positivity (sensitivity)	Score of the color range	No.	Percentage of negativity (specificity)
Echinococcosis (n = 41)	++	11	26.8	-	-	-
	+++	18	43.9	-	-	-
	++++	2	4.9	-	-	-
Total		31	75.6	-	10	24.4
Healthy control (n = 20)	-	0	0.0	-	20	100.0
Other parasites (n = 33)						
Fascioliasis (n = 10)	++	5	50.0	-	5	50.0
Schistosoma mansoni (n = 8)	++	3	37.5	-	5	62.5
Schistosoma haematobium (n = 5)	+++	1	20.0	-	4	80.0
Liver amoebiasis (n = 10)	++	5	50.0	-	5	50.0
Total (n = 53)		14	26.4		39	73.6

Table 2: Detection of human echinococcosis in serum by sandwich ELISA

	Positive cases		Negative cases	
Group (no. of animals)	No.	X±SD	No.	X±SD
Healthy control (n = 20)			20	0.303±0.108
Echinococcus (n = 41)	37	0.940±0.322	4	0.311±0.117
Schistosoma mansoni (n = 8)	1	0.619±0.222	7	0.219±0.149
Schistosoma haematobium (n = 5)	1	--	4	0.215±0.083
Fasciola (n = 10)	2	0.433±0.251	8	0.317±0.133
Liver amoebiasis (n = 10)	1	0.508±0.171	9	0.168±0.141
Overall sensitivity (%)		80.4		
Overall specificity (%)		71.7		

determined as the sum of results of negative control group (n = 20) and the total other parasites group number (n = 33); as *S. mansoni* (n = 8), *S. haematobium* (n = 5), *Fasciola* (n = 10) and liver amoebiasis (n = 10) (Table 2).

DISCUSSION

Diagnosis of CE can provide substantial improvements in the quality of the management and treatment of disease. In livestock, infection with hydatid cyst is asymptomatic and diagnosis is made usually at necropsy. Immunodiagnosis has been found to be useful not only in primary diagnosis but also for follow up of patients after surgical or pharmacological treatment in man. However assays using crude hydatid antigens have been non-specific due to cross reaction with C*ysticercus* coenurus and other helminthic infections[30]. In order to overcome these difficulties various novel tests using purified antigens are essential for confirmative diagnosis of hydatidosis in man and animals[29,31].

Immunodiagnostic tests using crude hydatid cyst antigen are far from satisfactory. Thus, purification of hydatid antigens is mandatory to remove host components and other cross reactive proteins. Various types of chromatography have been used for purification of hydatid antigens. In the present study, Superdex 200 HR16/50 column gel filtration chromatography had been commonly used to purify the antigens of from hydatid fluid of liver and lung of infected sheep and camel. Elution profiles showed multiple represented by multiple peaks (from A-E) contained the paramyosin antigen. *Echinococcus granulosus* hydatid cyst fluid was purified 24 and 30 fold by Superdex 200 HR16/50 column[32]. The purification was followed by SDS-PAGE (12.5%) analysis. The use of this method proved previously to yield a highly antigen fraction. Many bands were appeared in the SDS-PAGE analysis of paramyosin antigen, the most prominent of which were 32, 55 and 65 kDa[33]. The detected SDS–PAGE analysis of hydatid cyst fluid indicated that 6 specific protein bands were detected at molecular weights 29, 45, 58, 68, 98 and 116 kDa, the 68 and 116 kDa bands had been detected by some researchers[34,35].

The ELISA is the test of choice for the diagnosis of CE in humans and particularly in the follow up of surgical cases using hydatid antigens[36]. However, there are several conflicting reports on the suitability of the ELISA for the diagnosis of hydatidosis[37,38], some researchers showed that, the ELISA may be successfully adapted for the serological diagnosis of hydatidosis[39,40]. The present results are in agreement with those of Kanwar and Vinayak[41], who reported that, the sensitivity, specificity and diagnostic efficacy of detection of circulating antigens in acid treated serum samples was 100% by ELISA. Sensitivity and specificity of EITB assay were determined as 88 and 84% whereas corresponding rates for ELISA were 60 and 94% respectively[9] but among purified antigens, ELISA for IgG was the most sensitive (96.5%) and ELISA for IgE was least specific (24.1%)[39]. In human, the sensitivity of ELISA in detecting anti- PA IgG and circulating protoscoleces antigen (CPA) was 62.5 and 52.5%, respectively while the specificity of the assay was 66.7 and 75%, respectively[29]. Other researchers agree with the present results which indicate that, the sensitivity and specificity of ELISA with hydatid fluid antigen were 91.5 and 96%, respectively. The antigen cross-reacted with the serum of some cysticercosis or clonorchiasis patients. ELISA system using hydatid cyst fluid antigen from Uzbekistan sheep is sensitive and specific for diagnosis of echinococcosis cases[42].

CONCLUSION

The present work is unique study that use Latex Agglutination and Sandwich ELISA in detection of human hydatidosis and revealed that the high positive purified paramyosin antigen of *E. granulosus* antigen concentration used leading to high sensitivity and specificity of diagnostic efficacy.

SIGNIFICANCE STATEMENT

The present study revealed good diagnostic efficacy for detection of echinococcosis by latex agglutination test (LAT) and sandwich Enzyme Linked Immuno-Sorbent assay (ELISA)

in human serum samples using prepared and purified paramyosin antigen of *E. granulosus*. So, the authors suggest that LAT is a suitable and applicable diagnostic method especially when followed by Sandwich ELISA as a confirmatory test for diagnosis of cystic echinococcosis in human and intermediate host animals.

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