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Comparative Evaluation of Different Diagnostic Techniques using Laminated Layer Antigen for Serodiagnosis of Human Hydatidosis

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ABSTRACT

Human hydatidosis is a public health problem in many parts of the world and improvement in diagnosis of the disease is still being pursued. It is diagnosed with radiological methods complemented by serological tests but cross-reactivity with other parasites represents a big limitation. The aim of the present study was to evaluate 3 different serological techniques: enzyme-linked immunoelectrotransfer blot (EITB), indirect enzyme linked immunosorbant assay (ELISA) and Dot-ELISA using Laminated Layer (LL) antigen prepared from hydatid cysts driven from infected sheep, for diagnosis of human hydatidosis in patients living in Menoufia Governorate, Egypt. The indirect ELISA for the detection of specific IgG4 antibody achieved a sensitivity of 92% and specificity of 96%. Dot-ELISA (enzyme linked immunosorbant assay) employed LL antigen demonstrated sensitivity of 96% and specificity of 98%. The pool of positive hydatid sera recognized strong bands at 66, 55 and 27 kDa. The 27 kDa band showed the highest sensitivity (80%) and specificity (96%) when reacted with individual human sera. So, the Dot-ELISA was the most sensitive serological tool which might be of value in serodiagnosis of human hydatidosis.

Key words: *E. granulosus*, LL, EITB, ELISA, DOT-ELISA

INTRODUCTION

Human Cystic Echinococcosis (CE) is a chronic endemic helminthic disease which is caused by the metacestode (larval) stage of the dog tapeworm *Echinococcus granulosus* (Zhang *et al.*, 2006; Eckert *et al.*, 2011). It is an important public health problem worldwide (Schantz, 1991) and one of the most important zoonotic diseases in Egypt (Ibrahim *et al.*, 2007). Herbivores such as sheep, horse, cattle, pig, goat and camel are the intermediate hosts. The highest percentages of fertile cysts are detected in sheep and goat, so they are the most important intermediate hosts for *E. granulosus* (Gottstein and Reichen, 2003).

Human is an accidental intermediate host, who is infected by handling soil, dirt or animal hair contaminated with eggs. Egg hatches in the small intestine, releasing the oncosphere which migrates via the portal system to various organs, mainly the liver developing into the metacestode stage (Eckert and Deplazes, 2004).

Echinococcal cysts locate particularly in the liver, but other organs such as lung, spleen, brain, heart and kidneys can be also affected (Sadjjadi, 2006; Siracusano *et al.*, 2008). The fully developed hydatid cyst is unilocular and full of hydatid cyst fluid (HCF). The hydatid cyst wall (HCW) consists of an inner germinal layer supported extracystally by a noncellular Laminated Layer (LL) which

is surrounded by host-produced fibrous adventitial tissue (Zhang *et al.*, 2003). The LL is the physical barrier against host immune cells and plays a foremost role in *E. granulosus* immune evasion (Margutti *et al.*, 2008).

Human hydatidosis is typically asymptomatic. The clinical symptoms usually become evident 10 years or more after initial infection depending on the organ affected, size of the cyst, number of cysts and complications that result from rupture of the cysts (Sako *et al.*, 2011).

The immunological responses in human CE include both humoral and cellular immune responses against established parasitic infection. In chronic phase, there is frequent elevation of specific antibody levels, particularly IgG, IgM and IgE (Khabiri *et al.*, 2006) with IgG1 and IgG4 subclasses being predominant (Sterla *et al.*, 1999). The IgG4 subtype is considered as an immunological marker during the course of human hydatidosis because it has a higher level in symptomatic CE than asymptomatic patients (Shambesh *et al.*, 1997).

Diagnosis of the CE in humans is established by imaging techniques supplemented with serological methods which are both nowadays widely applied to support the clinical diagnosis (Parija, 1998; Doiz *et al.*, 2001).

The serological tests depend on detection of specific antibodies against various antigens of hydatid (Zhang *et al.*, 2003). Serological tests such as immunoelectrophoresis, double diffusion in agar, indirect haemagglutination (IHA), ELISA, immunoblot (IB) and indirect immunofluorescent antibody test (IFA) are employed for the immunodiagnosis of hydatid disease in human (Virginio *et al.*, 2003).

Accurate immunodiagnosis of the infection requires highly specific and sensitive antigens. The most appropriate antigens have been extracted from the developmental stage of the hydatid worm and the intermediate host species and this is crucial for the improvement of the diagnostic features of the tests (Carmena *et al.*, 2006; Tawfeek *et al.*, 2011). The most common sources of candidate antigens used for the immunodiagnosis of CE are hydatid cyst fluid (HCF), somatic extracts (S-Ag) and excretory-secretory products (ES-Ag) from protoscoleces or adults of *E. granulosus* (Tawfeek *et al.*, 2011).

The LL produced by the germinal layer is rich in carbohydrate (Diaz *et al.*, 2011). It is an attractive source of antigenic molecules which could be tested in immunodiagnosis of hydatid disease because it has acellular nature, it is accessible to the immune system, it may influence the immune response in CE and it supplies a considerable amount of parasitic material (Taherkhani *et al.*, 2007).

There is no standard serodiagnostic test for CE and the recommended sensitive assays are IHA and ELISA for initial screening and follow up. Immunoblotting assay using different antigens were reported to have high sensitivity and specificity in reaction with sera of CE patients (Rashed *et al.*, 2003) and Enzyme Immunosorbent Assay (EITB) method, although it was a time consuming test, it could be considered as an authentic technique due to its high validity (Rokni and Aminian, 2006).

The aim of the current study was to evaluate 3 different serological techniques; EITB, indirect ELISA and Dot-ELISA using LL antigen prepared from hydatid cysts driven from the liver and lung of hydatid infected sheep, for diagnosis of human hydatidosis in patients living in Menoufia Governorate, Egypt.

MATERIALS AND METHODS

Serum sample collection: The current study was carried out from January to December 2013. A total of 75 subjects participated in this study and they were divided into three groups.

Group (I) included 25 patients having hepatic hydatid cysts who were diagnosed clinically, by ultrasound and proved positive serologically with IHA. Group (II) included 25 patients infected with other parasitic diseases including *Schistosoma mansoni* (n = 10), *Fasciola gigantica* (n = 10) and Hookworms (n = 5), who were proved positive by stool examination. The patients of both groups were attending the Menoufia University Hospitals and Liver Institute. Group (III) Included 25 individuals of the medical staff at Menoufia University who served as parasite-free healthy negative controls. Blood samples (5 mL) were withdrawn from each person participated in this study. Sera were separated from blood samples, aliquoted and kept at -70°C until used.

Preparation of laminated layer antigen: LL antigen was prepared according to Taherkhani (2001) and Farhoodi *et al.* (2010). Viable hydatid cysts were obtained from the livers and lungs of naturally infected sheep slaughtered at the local abattoirs (Menoufia Governorate, Egypt). Hydatid fluid was aspirated from the cyst by a 10 mL syringe following disinfection of host capsule surface with 70% ethanol. The capsule wall was incised and the cyst wall, containing the LL and germinal layers (GL) was removed. The LL was separated from the GL with forceps and scraping with a scalpel using a dissection microscope. The separated tissue were washed several times in phosphate buffer saline (PBS, pH 7.4) and examined by microscopy to confirm the absence of any remnants of the GL and any protoscoleces. Samples were then incubated in 1 M NaCl at 4°C for 30 min. The LL extracts were subjected to freeze/thaw process and then the samples were homogenized in a glass homogenizer with an equal volume of PBS in a total concentration of 1 mM Phenyl Methyl Sulphonyl Fluoride (PMSF). The preparation was then disrupted by sonication in soniprep 150 ultrasonic disintegrator (Sanyo MSE) (10 sec on, 5 sec off) on ice for 15 min. The main part of the sonicated material (milky suspension) was kept at -20°C to be subsequently used.

Analysis of parasite antigens by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): LL was fractionated on SDS according to Laemmli (1970) with slight modifications. LL Protein (10 µg protein per well using mini gel) in 20 µL of sample buffer (0.5% M, Tris-HCl, 0.8 mL glycerol, 1.6 mL 10% SDS, 0.4 mL B-mercaptoethanol and 0.4 mL 0.05% bromophenol blue) was applied to a 12% SDS-PAGE gel using a casting apparatus (Hofer Scientific Instruments, San Francisco, California, USA). The power was applied to the cell and the electrophoresis was run using 120 V constant until the front dye reaches the lower end. The gel was stained with Commasie brilliant blue R-250 stain (Sigma) to visualize protein bands of the separated LL antigen. The standard molecular marker was used for each gel from range 28 upto 250 kDa.

Enzyme Linked Immunoelctro Transfer Blot technique (ELITB): SDS-PAGE electrophoresis was performed as mentioned above then, the electrophoresed LL antigen was blotted according to Tsang *et al.* (1983). The electrophoresed antigen was blotted from the gel onto nitrocellulose sheet (Pharmacia), using transblot cell (Bio-Rad). Nitrocellulose membrane was blocked with 5% non-fat milk in 50 mM PBS/0.05% Tween 20 for 1 h at room temperature. The membrane was washed three times with 50 mM PBS/0.5% T20, then cut into strips of 4 mm wide and placed in a multichannel tray. Nitrocellulose strips were incubated with human serum diluted 1:200 in the washing buffer for 2 h at room temperature. After that, the blots were washed three times and anti-human IgG peroxidase conjugate diluted 1:5000 in washing buffer was added to

the strips and left 1 h at room temperature. The strips were washed with PBS/0.5% T20 for the last time and color development of various bands was detected by adding diaminobenzidine substrate solution (DAB) (Sigma) for 10 min. The reaction was stopped by rinsing the strips with distilled water. Initially, the western blot was performed using pooled sera of infected hydatid patients, group (I) and pooled healthy sera, group (III) and after that, the individual sera from all groups were tested to determine the sensitivity and specificity of the reactive bands.

Detection of anti-hydatid IgG4 antibody by ELISA: ELISA test based on the original method of Engvall and Perlmann (1971) was used with some modifications. Wells of polystyrene microtiter plates (Costar, Corporate Headquarters, Cambridge, MA, USA) were coated with 100 μL of LL antigen at a concentration of 15 $\mu\text{g mL}^{-1}$ diluted in coating buffer (0.06 M carbonate-bicarbonate buffer, pH 9.6) and then incubated overnight at 4°C. Plates were washed 5 times with the washing buffer (PBS/0.05% T20), blocked by dispensing 200 μL /well BSA in PBS and left for 2 h at room temperature. Following washing the wells 5 times, 100 μL /well of human serum at a dilution of 1/200 in washing buffer, were added to the plates and incubated for 1 h at 37°C. Then, the plates were washed 5 times and 100 μL /well of horse radish peroxidase goat anti-human IgG4 at a dilution of 1:1000 in washing buffer, were added to each well and incubated for 1 h at 37°C. After 5 times of washing, a color reaction was developed by incorporating 100 μL /well from freshly prepared O-phenylenediamine dihydrochloride (Sigma) into the reaction till the color appearance. The reaction was stopped by adding 50 μL of 2 M H_2SO_4 (stopping buffer). The optical density (OD) was measured at 492 nm using a microtiter plate ELISA reader (Bio-Rad, Richmond, California, USA. CA 94804). The OD cut-off value was determined as the mean plus 2 standard deviation (SD) of negative controls. The OD readings equal to or less the cut-off value were considered negative, while readings above than this value were considered positive.

Dot enzyme-linked immunosorbant assay test (Dot-ELISA): Dot-ELISA was performed by the method described by Zheng *et al.* (1986) with slight modifications. Briefly, 5 μg of the prepared LL antigen was dotted on each nitrocellulose membrane discs and allowed to dry at air thoroughly. The discs were placed into flat bottom micrometer plate well. The excess unbound antigen is removed by the washing buffer (PBS/ 0.5% T20). Non-specific binding sites were blocked by adding 100 μL of PBS/ 0.5% T20/1% bovine serum albumin (BSA) to each well and left for 1 h at 37°C. The discs were washed by shaking the plate. Then, 100 μL of the serum samples diluted 1:200 in washing buffer were added to the discs which were incubated at room temperature for 1 h. The discs were washed again and 100 μL of the 1:1000 rabbit anti-human IgG peroxidase conjugate (Sigma) was added to each well and the discs were incubated for 2 h at 37°C in the dark. After washing, 100 μL of substrate solution (3 mg DAB (Diamino Benzidine Tetrahydrochloride, Sigma) in 5 mL PBS/0.5% T20 and 5 μL H_2O_2) were added into each disc and left for 30 min at 37°C in the dark. Sera that gave visible brown spots on discs were considered positive.

Statistical analysis: Sensitivity, specificity, diagnostic accuracy, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were determined:

$$\text{Sensitivity} = \frac{A}{A + C} \times 100$$

$$\text{Specificity} = \frac{D}{B+D} \times 100$$

$$\text{PPV} = \frac{A}{A+B} \times 100$$

$$\text{NPV} = \frac{D}{C+D} \times 100$$

$$\text{Diagnostic accuracy} = \frac{A+D}{A+B+C+D} \times 100$$

where, A is true positive, B is false positive, C is false negative and D is true negative.

Ethical consideration: An informed written consent was taken from all subjects for participating in this study.

RESULTS

SDS-PAGE analysis of sheep LL antigen: Fractionation of the crude extract of sheep LL in SDS-PAGE was done under reducing conditions. It revealed 9 discrete bands approximately at 97, 72, 66, 55, 50, 42, 34, 29 and 27 kDa while purified LL antigen gave less number of bands at 66, 55, 34 and 27 kDa (Fig. 1).

EITB assay: Immunoblotting of purified LL antigen with a pool of human hydatid positive and negative sera and probed with anti-human IgG is shown in Fig. 2. Pooled human sera infected with

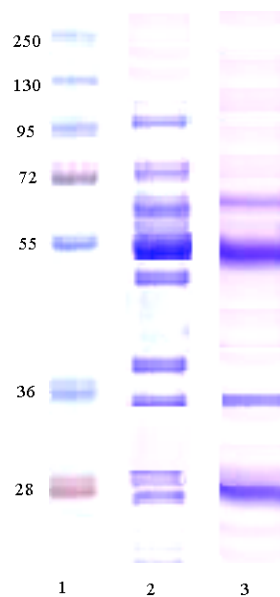


Fig. 1: SDS-PAGE of laminated layer (LL) antigen from hydatid cysts, Lane 1: Molecular weight standard, Lane 2: Crude LL antigen and Lane 3: Purified LL antigen

hydatid recognized bands of purified LL at 66, 55 and 27 kDa, whereas pool of hydatid negative human sera did not react with any band.

When individual human serum from CE patients (Group I) were tested against LL, total IgG responses showed strong reactivity at bands of 66, 55 and 27 kDa. The 66 kDa band was recognized by the sera of 13 CE patients with sensitivity of 52% and cross-reacted with the sera of 4 cases (16%) of parasitic infected control group (Group II) with specificity of 92%. No cross-reaction was recognized with sera from healthy controls (Group III). The PPV, NPP and diagnostic accuracy were 76.5%, 79.3 and 78.7%, respectively (Table 1 and 3). The 55 kDa band was recognized by the sera of 7 CE patients with sensitivity of 28% and cross-reacted with the 9 sera of parasitic infected control (Group II) with specificity of 82%. No cross-reaction was shown with sera from healthy controls (Group III). The PPV, NPP and diagnostic accuracy were 43.8, 69.5 and 64%, respectively

Table 1: Reactivity of different Molecular Weight (MW) bands of sheep LL antigen among different groups by EITB

MW band (kDa)	Group I (Hydatid infected, No. 25)		Group II (Parasitic infected, No. 25)		Group III (Healthy controls, No. 25)	
	No.	(%)	No.	(%)	No.	(%)
66	13	52	4	16	0	0
55	7	28	9	36	0	0
27	20	80	2	8	0	0

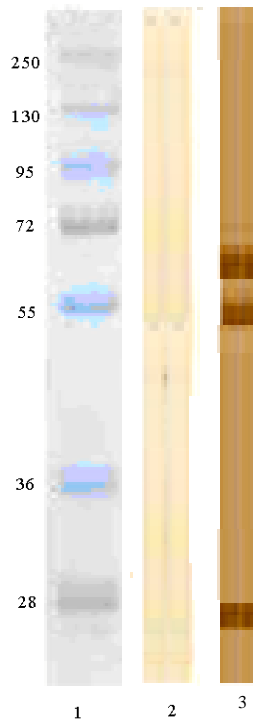


Fig. 2: EITB of purified LL antigen from sheep hydatid cysts with pooled positive hydatid sera and pooled negative sera, Lane 1: Molecular weight standard, 2: Negative human sera and Lane 3: Positive human sera

Table 2: Detection of anti-hydatid IgG4 antibodies in serum of CE, other parasitic infection and control groups by Dot-ELISA and indirect ELISA

Subject groups	Indirect ELISA				Dot-ELISA			
	No.		%		No.		%	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Group I (Hydatid infected, No. 25)	23	2	92	8	24	1	96	4
Group II (Parasitic infected, No. 25)	2	23	8	92	1	24	4	96
Group III (Healthy controls, No. 25)	0	25	0	100	0	25	0	100

Table 3: Sensitivity, specificity, PPV, NPP and accuracy of different immunological assays using sheep LL antigen for detection of IgG among total examined individuals

Criteria	EITB (kDa)			Indirect ELISA	Dot-ELISA
	66	55	27		
Sensitivity (%)	52.0	28.0	80.0	92.0	96.0
Specificity (%)	92.0	82.0	96.0	96.0	98.0
PPV (%)	76.5	43.8	90.9	92.0	96.0
NPV (%)	79.3	69.5	90.6	96.0	98.0
Diagnostic accuracy	78.7	64.0	90.6	94.7	97.3

(Table 1 and 3). The 27 kDa band was recognized by the sera of 20 CE patients with sensitivity of 80% and cross-reacted with the sera of 2 cases (8%) of parasitic control group (Group II) with specificity of 96%. No cross-reaction was demonstrated using sera from healthy controls (Group III). The PPV, NPP and diagnostic accuracy are 90.9, 90.6 and 90.6%, respectively (Table 1 and 3). The results are shown in Fig. 3.

Indirect ELISA using anti-LL IgG4: The LL of hydatid was used as antigen in indirect ELISA for the detection of IgG4 antibody in the sera of patients with proven CE. The Mean±SD and ranges of ODs at 492 nm of the three groups of sera are shown in Fig. 4. The ELISA cut-off value was calculated as the mean OD of control non-infected sera plus 2 SD, which was 0.41 so, a serum with an OD≥0.41 was considered to be positive for anti-hydatid antibody IgG4. The mean OD±SD of positive cases from CE (Group I) was 1.11±0.22, while the mean OD±SD of subjects of parasitic infected group (Group II) was 0.32±0.1. Specific IgG4 antibodies were detectable in 23 of 25 (92%) sera of CE group, in 2 of 25 (8%) sera of parasitic group and were not detected among healthy controls sera (0%). Accordingly, a sensitivity of 92%, specificity of 96%, PPV of 92%, NPV of 96% and accuracy of 94.7% were determined (Table 2 and 3).

Dot-ELISA: Using sheep hydatid LL antigen for detection of hydatid antibodies (total IgG) in sera of CE, other parasitic infected patients and healthy controls are summarized in Table 2 and 3. An optimum concentration of 5 µg antigen per dot detected specific anti-hydatid IgG antibodies at 1: 200 serum dilutions of CE cases. It demonstrated diagnostic levels of antibodies in 24 out of 25 (96%) sera of cases with confirmed CE (Group I). The test cross-reacted with only in one sera out of 25 sera parasitic infected control group 4%, (Group II) and no reaction was detected with sera

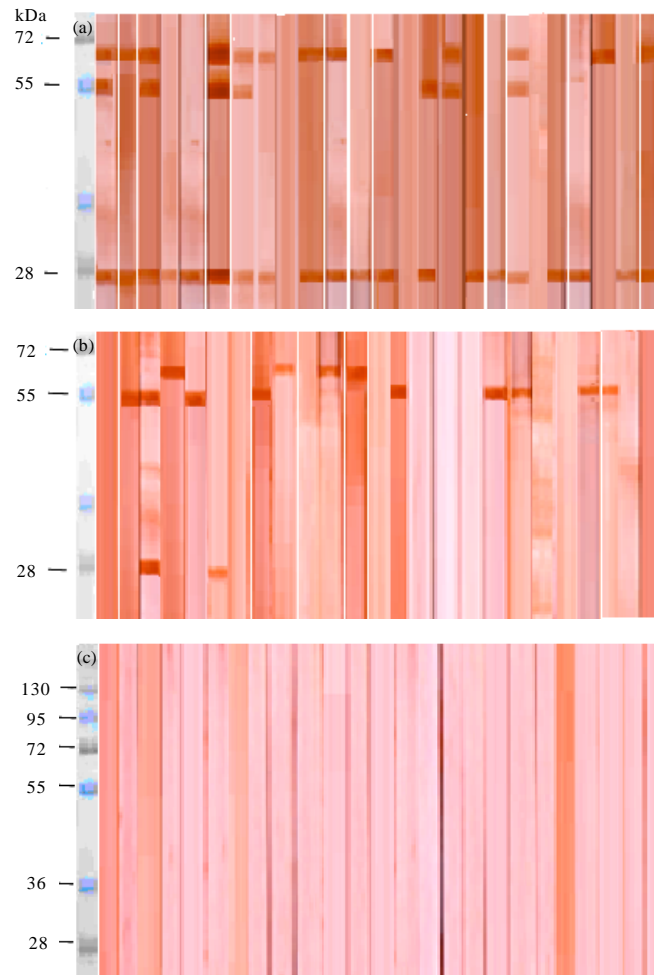


Fig. 3(a-c): EITB of purified LL antigen with individual human sera from (a) CE patients (Group I), (b) Parasitic infection (Group II) and (c) Healthy controls (Group III)

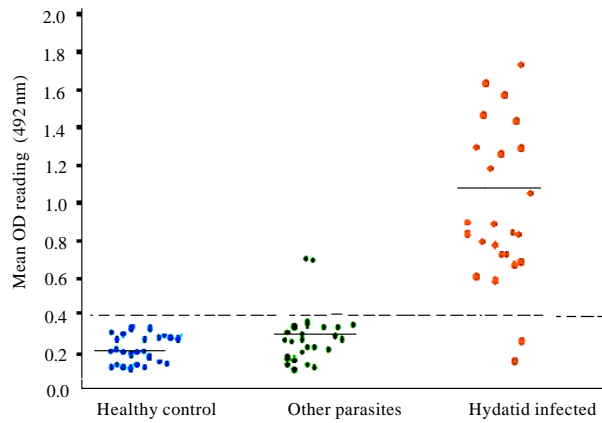


Fig. 4: Indirect ELISA using anti LL IgG4

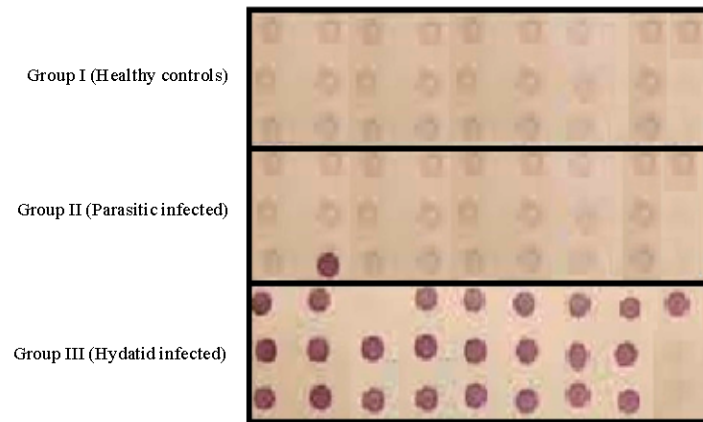


Fig. 5: Dot-ELISA reactions in different groups, p, n = 25 in each group. Brown dot indicate positive reaction where for each group, n = 25

of healthy controls (Group III). The sensitivity, specificity, PPV, NPV and diagnostic accuracy of Dot-ELISA were 96, 98, 96, 98 and 97.3%, respectively (Table 3). The positive reactions were shown in Fig. 5.

DISCUSSION

Human CE is recognized globally as a major zoonotic disease with increasing prevalence all over the world (Eckert *et al.*, 2001; Da Silva, 2010). In Arab countries, it still remains as a significant health problem (Ahmadi, 2004).

Diagnosis of CE in humans is carried on by clinical examination, imaging techniques and confirmed with serological methods (Doiz *et al.*, 2001). Serodiagnosis of hydatidosis is still problematic (Sako *et al.*, 2011). So, highly sensitive and specific *Echinococcus* antigens must be selected to increase the sensitivity and specificity of currently available immunological assays (Carmena *et al.*, 2006). Different antigens obtained from hydatid cysts derived from various intermediate hosts were used for diagnosis of human hydatidosis (Sabry, 2007). The serodiagnosis has an important role in diagnosis because of its low cost and easily performance (Ortona *et al.*, 2003; Swarna and Parija, 2008).

The LL extract of *E. granulosus* has proven to be of an antigenic nature (Taherkhani and Rogan, 2000) and it is attractive diagnostic candidate antigen.

In this study, the efficacy of purified LL antigen prepared from the hydatid cyst of infected sheep for serodiagnosis of hydatidosis in patients infected with *E. granulosus* living in Menoufia Governorate, Egypt was evaluated with three different serological tests which were EITB, indirect ELISA and Dot-ELISA.

In the present study, fractionation of the crude extract of LL antigen in SDS-PAGE gave bands at 97, 72, 66, 55, 50, 42, 34, 29 and 27 kDa while the purified LL antigen gave less number of bands at 66, 55, 34 and 27 kDa which is quite similar to that reported by Taherkhani and Rogan (2000) and Taherkhani *et al.* (2007). Kordafshari *et al.* (2010) demonstrated that LL of hydatid cyst gave 5 observed bands, the most prominent was 35-38, 50 and 60 kDa. The band of 38 kDa was reported by Hoseini (2006).

In the current work, when testing the reactivity of hydatid infected human sera against the purified LL antigen using EITB technique, the pooled positive human hydatid sera demonstrated an intense antibody reaction (IgG) against protein bands at 66, 55 and 27 kDa. There was no reactivity against protein band of 34 kDa. This is in agreement with Taherkhani and Rogan (2000) who indicated that the bands of 66, 55 and 27 kDa were of significance for serodiagnosis of CE.

When individual human sera of CE patients (Group I), parasitic infected control (Group II) and healthy control (Group III) were blotted against purified LL, total IgG responses showed sensitivity of 52% with 66 kDa, 28% with 55 kDa and 80% with 27 kDa. The highest sensitivity with purified LL antigen was reported with 27 kDa band and this was better than sensitivity (67%) that reported with 25 kDa, the nearest in molecular weight (Taherkhani *et al.*, 2007). The higher sensitivity detected in the present study could be attributed to the purified nature of sheep extract of LL. The specificity of the detected bands for diagnosis of CE in humans were 92% with 66 kDa, 82% with 55 kDa and 96% with 27 kDa and accuracy was 78.7, 64 and 90.6%, respectively. The lower specificity of 55 kDa band and higher specificity of 27 kDa band could be related to the parasitic nature of 27 kDa band and host nature of 55 kDa band, host specific (Taherkhani and Rogan, 2000). Taherkhani and Rogan (2001), reported that 27 kDa band was obvious in semi-purified LL.

In our study, indirect ELISA detected specific anti-LL IgG4 in 23 out of 25 positive hydatid sera achieving sensitivity of 92%. Cross-reaction was detected among 2 out of 25 sera from parasitic infected control group (8%) and no cross-reaction was shown among healthy controls sera revealing a specificity of 96% when using both groups as the negatives for hydatid infection. Metwally and Al-Olayan (2013) reported that indirect ELISA detected anti-hydatid antibodies (total IgG) in 65% of hydatid patients with sensitivity of 72.2% and specificity of 75% (using commercial ELISA kits). Another study was performed by Sadjjadi *et al.* (2009) and they found that 94.2% of patients with CE have antibodies in their sera by indirect ELISA when using antigen B. In addition, Younis *et al.* (2008) reported 90% sensitivity and 75% specificity by conventional ELISA when using crude hydatid fluid (CHF) antigen.

On the other side, there were 2 hydatid positive sera which did not react with LL antigen when applying indirect ELISA assay and this may be attributed to the presence of small cysts early after parasitic infection, the cysts might be large but intact with no leakage of immunogenic cyst materials, existence of heavily calcified cysts and/or cysts in extrahepatic location.

Kaur *et al.* (1999) compared the sensitivity and specificity of standard and rapid ELISA using CHF as antigen in detection of antibodies in hydatid disease, the specificity was 90.27% and 100%, respectively while sensitivity of standard ELISA was 88.23% and that of rapid was 82.3%. The ELISA assay using the CHF antigen revealed sensitivity and specificity of 92.22 and 98.75%, respectively (Siavashi *et al.*, 2005). In a study conducted in Egypt, Romia *et al.* (1992) reported the sensitivity and specificity of ELISA assay using CHF as antigen were 88 and 96.9% in detection of anti-hydatid antibodies, respectively.

Dot-ELISA has been considered as one of the valuable methods in diagnosis of different parasitic diseases (Pappas, 1988; Biswas *et al.*, 2004; Roldan *et al.*, 2009) including hydatidosis (Hadighi *et al.*, 2003). The results of the current work showed that Dot-ELISA detected specific anti-hydatid IgG antibodies in 24 out of 25 positive hydatid sera achieving sensitivity of 96%, while only 1 serum from parasitic infected group (4%) cross-reacted with Dot-ELISA showing specificity of 98%. The high sensitivity of Dot-ELISA could be attributed to using of nitrocellulose paper which can detect trace amounts of antibody. A lot of reports have evaluated the efficacy of Dot-ELISA in

diagnosis of human CE using various hydatid cyst antigens. Rogan *et al.* (1991) reported a sensitivity of 94% and specificity of 90.5% of Dot-ELISA using Ag B whereas, crude sheep HCF gave lower specificity (52%) and higher sensitivity (97%) in the Turkana region, Iran. The lower specificity observed by this study was due to cross-reaction with sera of patients infected with other parasitic diseases such as cysticercosis, filariasis and toxocariasis.

Zhang and McManus (1996) employed CHF antigen in Dot-ELISA assay using titer of 1:400 and reported a sensitivity of 92.3% and specificity of 89.9%. Also, Siavashi *et al.* (2005) compared Dot-ELISA and Sandwich ELISA using CHF antigen and found that Dot-ELISA showed 100% sensitivity and 98.75% specificity. A recent study employed Dot-ELISA test using different antigens; cyst wall, protoscolex and cyst fluid antigens showed the sensitivity of Dot-ELISA was 96.66, 86.66 and 93.33%, respectively and the specificity of the assay was 70% for Dot-ELISA using cyst fluid, protoscolex and cyst wall antigens (Swarna and Parija, 2008).

The Dot-ELISA in our hands showed the highest sensitivity, specificity and other diagnostic criteria of a serological test in comparing with ELISA or EITB. It has many advantages over the other two tested techniques in our study such as it is rapid, the membrane conserving the antigen requires a trace amounts of the antigen reaching few nanograms, it is easy and it needs few microliters of serum to proceed the assay (Swarna and Parija, 2008). Until now, there is no recommended standard serological test for human CE having high sensitivity and specificity (Moghadam *et al.*, 2013).

CONCLUSION

It is concluded from this study that the purified LL antigen is easily prepared from the animal hydatid cyst and could be obtained with large volume, also, Dot-ELISA technique has advantages which are of low cost, quick and does not require highly skilled personals. In addition to all of that, results indicated that detection of specific antibodies using purified LL antigen employing Dot-ELISA is a sensitive serological tool that might be valuable in serodiagnosis of human hydatid to complement and confirm the imaging diagnosis. The LL antigen-Dot-ELISA assay is recommended to be tested in larger samples and in patients with cysts affecting other organs addition to liver. Lastly, further study of LL antigen is recommended to purify its component of high antigen nature which may give better sensitivity and specificity.

REFERENCES

- Ahmadi, N.A., 2004. Using morphometry of the larval rostellar hooks to distinguish Iranian strains of *Echinococcus granulosus*. *Ann. Trop. Med. Parasitol.*, 98: 211-220.
- Biswas, R., S.C. Parija and S.K. Narayan, 2004. Dot-ELISA for the diagnosis of neurocysticercosis. *Rev. Inst. Med. Trop. Sao Paulo*, 46: 249-252.
- Carmena, D., A. Benito and E. Eraso, 2006. Antigens for the immunodiagnosis of *Echinococcus granulosus* infection: An update. *Acta Trop.*, 98: 74-86.
- Da Silva, A.M., 2010. Human echinococcosis: A neglected disease. *Gastroenterol. Res. Pract.*, Vol. 2010. 10.1155/2010/583297
- Diaz, A., C. Casaravilla, F. Irigoien, G. Lin, J.O. Previato and F. Ferreira, 2011. Understanding the laminated layer of larval *Echinococcus* I: Structure. *Trends Parasitol.*, 27: 204-213.
- Doiz, O., R. Benito, Y. Sbihi, A. Osuna, A. Clavel and R. Gomez-Lus, 2001. Western blot applied to the diagnosis and post-treatment monitoring of human hydatidosis. *Diagn. Microbiol. Infect. Dis.*, 41: 139-142.

- Eckert, J., M.A. Gemmel, F.X. Meslin and Z.S. Pawlowski, 2001. WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern. World Organisation for Animal Health, Paris, France, ISBN: 9789290445227, Pages: 265.
- Eckert, J. and P. Deplazes, 2004. Biological, epidemiological and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin. Microbiol. Rev., 17: 107-135.
- Eckert, J., P. Deplazes and P. Kern, 2011. Alveolar Echinococcosis (*Echinococcus Multilocularis*) and other Forms of Echinococcosis (*Echinococcus vogeli* and *Echinococcus Oligarthrus*). In: Zoonoses, Brown, D., S.R. Palmer, P.R. Torgerson and E.J.L. Soulsby (Eds.). Oxford University Press, Oxford, pp: 671-701.
- Engvall, E. and P. Perlmann, 1971. Enzyme-Linked Immunosorbent Assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry, 8: 871-874.
- Farhoodi, R., S.H. Hosseini, M. Abolhasani and P. Shayan, 2010. Identification and purification of a specific and immunogenic antigen of the laminated layer of the hydatid cyst and production of an antigen-specific monoclonal antibody. Iran. J. Vet. Med., 4: 237-243.
- Gottstein, B. and J. Reichen, 2003. Echinococcosis/Hydatidosis. In: Manson's Tropical Diseases, Manson, S.P., G.G.C. Cook and A.I. Zumla (Eds.). 21st Edn., W.B. Saunders, London, ISBN: 9780702026409, pp: 1561-1582.
- Hadighi, R., F. Mirhadi and M.B. Rokni, 2003. Evaluation of a dot-ELISA for the serodiagnosis of human hydatid disease. Pak. J. Med. Sci., 19: 268-271.
- Hoseini, R., 2006. Determination of electrophoretic pattern of different antigens of *Echinococcus granulosus* and immunization of them. Ph.D. Thesis, Tehran University, Tehran.
- Ibrahim, B.B., F.M. Haridy, M.M. Hegazi and T.A. Morsy, 2007. Human hydatidosis granulosus in greater Cairo, Egypt: With general review. J. Egypt. Soc. Parasitol., 37: 681-688.
- Kaur, M., R.C. Mahajan and N. Malla, 1999. Diagnostic accuracy of rapid enzyme linked immunosorbent assay for the diagnosis of human hydatidosis. Indian J. Med. Res., 110: 18-21.
- Khabiri, A.R., F. Bagheri, M. Assmar and M.R. Siavashi, 2006. Analysis of specific IgE and IgG subclass antibodies for diagnosis of *Echinococcus granulosus*. Parasite Immunol., 28: 357-362.
- Kordafshari, S., S.H. Hosseine, B. Meshgi and M.R. Youssefi, 2010. Comparison of electrophoretic patterns of larval stages of taeniidae and determination of specific antigens of hydatid cyst by Western blotting technique. Global Veterinaria, 4: 601-606.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
- Margutti, P., E. Ortona, F. Delunardo, A. Tagliani and E. Profumo *et al.*, 2008. Thioredoxin peroxidase from *Echinococcus granulosus*: A candidate to extend the antigenic panel for the immunodiagnosis of human cystic echinococcosis. Diagn. Microbiol. Infect. Dis., 60: 279-285.
- Metwally, D.M. and E.M. Al-Olayan, 2013. Serum antibody detection in echinococcosis: Specificity of Hydatidosis Enzyme-Linked Immunosorbent Assay (ELISA) IgG. Life Sci. J., 10: 2107-2110.
- Moghadam, Z.K., F. Ghaffarifar, A. Khalilpour, F.A. Aziz, G. Saadatnia and R. Noordin, 2013. IgG4 detection of *Echinococcus granulosus* paramyosin is a useful diagnostic test for human hydatidosis. Clin. Vaccine Immunol., 20: 501-505.
- Ortona, E., R. Rigano, B. Buttari, F. Delunardo and S. Ioppolo *et al.*, 2003. An update on immunodiagnosis of cystic echinococcosis. Acta Trop., 85: 165-171.
- Pappas, M.G., 1988. Recent applications of the Dot-ELISA in immunoparasitology. Vet. Parasitol., 29: 105-129.

- Parija, S.C., 1998. A review of some simple immunoassays in the serodiagnosis of cystic hydatid disease. *Acta Trop.*, 70: 17-24.
- Rashed, S.M., I.A. El-Hyawan, I.M. Nagaty, A.H. Hussein and A. Saad, 2003. Demonstration of species-specific and cross-reactive components of hydatid cyst fluid antigens. *J. Egypt. Soc. Parasitol.*, 33: 315-328.
- Rogan, M.T., P.S. Craig, E. Zeyhle, T. Romig, G.M. Lubano and L. Deshan, 1991. Evaluation of a rapid dot-ELISA as a field test for the diagnosis of cystic hydatid disease. *Trans. R. Soc. Trop. Med. Hygiene*, 85: 773-777.
- Rokni, M.B. and B. Aminian, 2006. Evaluation of the Enzyme-linked Immuno-electro Transfer Blot (EITB) technique using hydatid cyst antigens B/5 and total IgG antibodies in laboratory diagnosis of human hydatidosis. *Pak. J. Med. Sci.*, 22: 127-131.
- Roldan, W.H., Y.A. Espinoza, P.E. Huapaya, A.F. Huiza, C.R. Sevilla and S. Jimenez, 2009. Frequency of human toxocariasis in a rural population from Cajamarca, Peru determined by DOT-ELISA test. *Revista Instituto Medicina Tropical Sao Paulo*, 51: 67-71.
- Romia, S.A., M.E. Youssef, A.E. Handoussa, H.M. Rizk and S.M. Sallam, 1992. Dot-ELISA as a diagnostic test in hydatid disease. *J. Egypt. Soc. Parasitol.*, 22: 603-610.
- Sabry, M.A., 2007. Advanced concepts in diagnosis of hydatidosis in human and living animals. *J. Biol. Sci.*, 7: 720-728.
- Sadjjadi, S.M., 2006. Present situation of echinococcosis in the Middle East and Arabic North Africa. *Parasitol. Int.*, 55: S197-S202.
- Sadjjadi, S.M., F. Sedaghat, S.V. Hosseini and B. Sarkari, 2009. Serum antigen and antibody detection in echinococcosis: application in serodiagnosis of human hydatidosis. *Korean J. Parasitol.*, 47: 153-157.
- Sako, Y., D. Tappe, K. Fukuda, Y. Kobayashi and S. Itoh *et al.*, 2011. Immunochromatographic test with recombinant Em18 antigen for the follow-up study of alveolar echinococcosis. *Clin. Vaccine Immunol.*, 18: 1302-1305.
- Schantz, P.M., 1991. Parasitic zoonoses in perspective. *Int. J. Parasitol.*, 21: 161-170.
- Shambesh, M.K., P.S. Craig, H. Wen, M.T. Rogan and E. Paolillo, 1997. IgG1 and IgG4 serum antibody responses in asymptomatic and clinically expressed cystic echinococcosis patients. *Acta Tropica*, 64: 53-63.
- Siavashi, M.R., H. Taherkhani, K.R. Rezaei, M.R.R. Deligani and M. Assmar, 2005. Comparison of Dot-ELISA and sandwich ELISA diagnostic tests in detection of human hydatidosis. *Iran. Biomed. J.*, 9: 91-94.
- Siracusano, A., R. Rigano, E. Ortona, E. Profumo and P. Margutti *et al.*, 2008. Immunomodulatory mechanisms during *Echinococcus granulosus* infection. *Exp. Parasitol.*, 119: 483-489.
- Sterla, S., H. Sato and A. Nieto, 1999. *Echinococcus granulosus* human infection stimulates low avidity anticarbohydrate IgG2 and high avidity antipeptide IgG4 antibodies. *Parasite Immunol.*, 21: 27-34.
- Swarna, S.R. and S.C. Parija, 2008. Dot-ELISA for evaluation of hydatid cyst wall, protoscoleces and hydatid cyst fluid antigens in the serodiagnosis of cystic echinococcosis. *Revista Instituto Medicina Tropical Sao Paulo*, 50: 233-236.
- Taherkhani, H. and M.T. Rogan, 2000. General characterization of laminated layer of *Echinococcus granulosus*. *Iran. J. Med. Sci.*, 25: 95-104.
- Taherkhani, H., 2001. Analysis of the *Echinococcus granulosus* laminated layer carbohydrates by Lectin blotting. *Iran. Biomed. J.*, 5: 47-51.

- Taherkhani, H. and M.T. Rogan, 2001. Preparation of semi-purified laminated layer of hydatid cyst for diagnosis of infection. *Arch. Razi Inst.*, 52: 61-73.
- Taherkhani, H., E. Zeyhle and M.T. Rogan, 2007. Antibody responses in human cystic hydatid disease to the laminated layer of *Echinococcus granulosus*. *Parasitol. Res.*, 101: 647-652.
- Tawfeek, G.M., H.S. Elwakil, L. El-Hoseiny, H.S. Thabet, R.M. Sarhan, N.S. Awad and W.A. Anwar, 2011. Comparative analysis of the diagnostic performance of crude sheep hydatid cyst fluid, purified antigen B and its subunit (12 Kda), assessed by ELISA, in the diagnosis of human cystic echinococcosis. *Parasitol. Res.*, 108: 371-376.
- Tsang, V., J. Peralta and A. Simons, 1983. Enzyme-Linked Immuno-electro-Transfer Blot Techniques (EITB) for Studying the Specificities of Antigens and Antibodies Separated by Gel Electrophoresis. In: *Methods in Enzymology, Volume 92: Immunochemical Techniques, Part E: Monoclonal Antibodies and General Immunoassay Methods*, Langone, J.J. and H.V. Vunakie (Eds.). Academic Press, New York, USA., ISBN-13: 9780121819927, pp: 337-391.
- Virginio, V.G., A. Hernandez, M.B. Rott, K.M. Monteiro and A.F. Zandonai *et al.*, 2003. A set of recombinant antigens from *Echinococcus granulosus* with potential for use in the immunodiagnosis of human cystic hydatid disease. *Clin. Exp. Immunol.*, 132: 309-315.
- Younis, T., I. Fahmy, S. El-Wakil, M.A. Nihad and A. Ibrahim, 2008. Attempt to improve the diagnostic efficacy of enzyme-linked immunosorbent technique for the serodiagnosis of cystic echinococcosis. *Parasitol. United J.*, 1: 129-136.
- Zhang, L.H. and D.P. McManus, 1996. Purification and N-terminal amino acid sequencing of *Echinococcus granulosus* antigen 5. *Parasite Immunol.*, 18: 597-606.
- Zhang, W., J. Li and D.P. McManus, 2003. Concepts in Immunology and diagnosis of hydatid disease. *Clin. Microbiol. Rev.*, 16: 18-36.
- Zhang, W., Z. Zhang, B. Shi, J. Li and H. You *et al.*, 2006. Vaccination of dogs against *Echinococcus granulosus* the cause of cystic hydatid disease in humans. *J. Infect. Dis.*, 194: 966-974.
- Zheng, G.Y., R.L. Zhao and X.H. Feng, 1986. Dot-immunobinding assay in the serodiagnosis of human hydatid disease. *Am. J. Trop. Med. Hygiene*, 35: 812-814.