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Diagnostic Efficiency of Heat Shocked Protoscoleces Extract Antigens for Human Cystic Echinococcosis by ELISA

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Abstract

Diagnosis of cystic echinococcosis is complex and has to be confirmed by the combination of immunological tests and imaging techniques. In this study heat shock proteins were induced and their immunoreactivity was assessed by ELISA.

Sera were collected from 34 hydatid patients who were admitted to the Rizgary Teaching Hospital through October 2013 to July 2017, in addition to 29 healthy donors and 18 non-hydatid cases. For heat shock response, two batches of 25000 protoscoleces (Ps) were incubated separately at 42°C and 45 °C for 4 hours. Heat treated and normal Ps were disrupted and the extracts were divided into two parts. One part was directly used as source of antigens (PE, PE42 and PE45) and the other one was partially purified on Sephadex G150. The immunoreactivity of these antigens, as well as hydatid fluidwas assessed by ELISA. The cutoff value to differentiate positive from negative sera was established by receiver operating characteristic (ROC) analysis.

Two peaks of PE42 and three peaks of PE45 resulted by Sephadex G150. Extracts of 42ºC treated Ps resulted in two protein peaks and were used as PE42P1 and PE42P2 antigens. For 45°C treated Ps, the chromatography patterns resulted in three protein peaks and were used as PE45P1, PE45P2 and PE45P3 antigens. Highest rates of sensitivity, specificity and diagnostic accuracy were detected with PE42P2 (91.2%) and PE45P2 (91.2%). Sensitivity of ELISA was consistent for liver cysts with all applied antigens.

Hydatid antigens extracted from heat treated Ps markedly raised the sensitivity of ELISA to detect anti-hydatid IgG.

Keywords: Hydatid disease, Heat shocked protoscoleces, Sero-diagnosis, Hydatid antigens, Cystic echinococcosis.

الكفاءة التشخيصية للمستضدات المستخلصة من الرؤيسات الاولية المعاملة بالحرارة في تشخيص داء المشوكات الكيسية في الانسان بتقنية الايلايزا

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الخلاصة

تشخيص داء المشوكات الكيسي معقد ويجب تأكيده من خلال مجموعة من الاختبارات المناعية وتقنيات الاشعة. في هذه الدراسة، تم تكوين بروتينات الصدمة الحرارية و تقييم نشاطها المناعي بواسطة تقنية الامدصاص المناعي المرتبط بالانزيم (ايلايزا). جمعت الأمصال من 34 مصابا بالاكياس المائية، والذين دخلوا مستشفى رزكاري التعليمي من أكتوبر 2013 الى تموز 2017، و 29 متبرعًا سليمًا، و 18 حالات غيرمصابه ب الاكياس المائية. للاستجابة للصدمة الحرارية، حضنت دفعتين من 25000 رؤيسة اولية **(Ps)** بشكل منفصل في 42 درجة مئوية و 45 درجة مئوية لمدة 4 ساعات. ومن ثم تم تكسير الرؤيسات المعالجة بالحرارة والطبيعية ومن ثم تم تقسيم المادة الطافية الناتجة إلى جزأين، أحدهما استخدمت مباشرة كمصدر للمستضدات **PE** ، **PE42** ، و**PE45** ، بينما تم تنقية الجزء الثاني جزئيًا في عمود **Sephadex G150** ، أُستخدمت تقنية ايلايزا في تقييم النشاط المناعي لهذه المستضدات بالإضافة الى السائل العداري ، ب.تم تحديد القيمة الفاصلة للتمييز بين الأمصال الإيجابية والسلبية من خلال اختبار منحنى تشغيل المستقبل **ROC**.

مستخلصات **PS** المعالجة بـ 42 درجة مئوية أسفرت عن ذروتين من البروتين واستخدمت كمستضدات **PE42P1**و **PE42P2**.بالنسبة لـ **Ps** المعالجة بـ 45 درجة مئوية ، نتج عن نمط الكروماتوغرافيا ثلاث قمم بروتينية واستخدمت كمستضدات **PE45P1** ، **PE45P2** و **PE45P3** . تم الحصول على أعلى نسبة من الحساسية،النوعية والدقة التشخيصية عند استخدام **PE42P2** و**PE45P2** بقيمة 91.2% لكليهما. كانت حساسية **ELISA** متوافقة مع أكياس الكبد مع جميع المستضدات المستخدمة.المستضدات العدارية المستخرجة من **Ps** المعالجة حرارياً زادت بشكل ملحوظ من حساسية ايلايزا للكشف عن الأجسام المضادة ل للأكياس المائية من نوع **IgG** .

Introduction:

Cystic echinococcosis is a zoonotic; cyst forming disease caused by the metacestode and hydatid cyst of the tapeworm *Echinococcus granulosus*. The disease has spread worldwide especially in sheep-rearing areas [1] and has been reported as an important public health challenge in most the developing countries [1][2]. Hydatid cyst can be developed in almost all body organs and tissues of the intermediate host [3]. However, liver (≈70%) is the most common site of infection followed by the lung (10%-30%) and in about 10% of the cases. The cysts develop in other sites such as spleen, kidney, pancreas, muscle, abdominal wall, eye, brain, bone etc. [4]. All ages are at risk of getting this infection, but in most cases, clinical manifestations do not typically clear until the second or third decade of life [5].

Definitive diagnosis of cystic echinococcosis is complex and has to be confirmed by the combination of immunological investigations and imaging techniques (X-ray, ultrasonography, computed tomography scan and magnetic resonance imaging) in the clinically suspected cases [6][7][8]. For immunological investigations, most studies have depended upon hydatid cyst fluid as a wealthy source of hydatid antigens [9]10][11][12]. Diagnosis of infection by enzyme-linked immunosorbent assay (ELISA) using a variety of standardized hydatid antigenic fractions, was widely adopted by the researchers [7][13][14][15] and was shown to be closely related to ultra-sonographic examination in human and animal carcasses post-mortem examination. For that purpose, all such standardized techniques and their variants have already been investigated. Recently, recombinant antigens technology has also been explored. However, the serological techniques using native hydatid antigens are being more sensitive and specific [16][17][18].

Most of the serological techniques used for diagnosis have their own problems and limitations such as limited availability, variable sensitivity and specificity, laboratory

equipment availability, and source and nature of the used antigens. Therefore, for clinical setting, the results of serological investigations depend upon several factors, such as antigen quality, composition, concentration and stability of the antigen, affected organ, number of cysts (single or multiple) and individual variabilities of immune responses [13][19][20].
Heat shock response is defined by the immediate expression of group of proteins, heat shock proteins (HSPs). Exposure of cells, tissues or organisms to various forms of environmental stresses as well as numerous other forms of cellular stress such as ischemia, oxidants, heavy metals and endotoxins are capable of inducing the same stress response. As it was first described in response to relatively over physiology temperature, it was called the heat shock response. HSPs are typically regarded as intracellular proteins. These proteins primarily function as chaperones guiding proper protein folding and maturation [21][22]. Immune responses to HSPs have been detected in both natural and experimental, bacterial, protozoa, fungal and helminthes infections. Furthermore, HSPs have also been expressed in the infectious pathogens as a protective strategy in response to host defense mechanisms on one hand [23][24] and their role in the maturation and induction of life cycle stages conversion on the other hand [21][22]. These proteins are highly conserved antigenic epitopes among various microbial pathogens [19]. They are known to induce strong humoral and cellular immune responses in various infections [23][24]. In *E.granulosus*, both HSP60 and HSP70 were found to be expressed by heat treated protoscoleces [20][25].

In the present study, heat shock proteins were induced under hyperthermic condition. The immunoreactivity of antigenic fractions extracted from heat treated protoscoleces was investigated by ELISA to be native antigen candidates for the serodiagnosis of human cystic echinococcosis.

Materials and Methods:

Patients

The serum collection used in this study comprised 34 serum samples from patients with surgically confirmed cystic echinococcosis, collected in the surgical theater of Rizgary Teaching Hospital, Erbil, Iraqi Kurdistan Region, with the following cyst locations: 24 liver samples (3 recurrent, 3 multiple liver cysts and 18 single cyst); 3 lung samples; 4 muscle samples (2 anterior abdominal wall muscle, 2 psoas major muscle); 1 spleen sample and 2 multiple organs involved samples. Blood samples were drawn a day before surgical excision of the cysts. In addition, 29 sera from healthy donors and additional 3, 5 and 10 samples were collected from children with dwarf tapeworm infection, female patients with positive anti-phospholipid IgG antibodies (APA IgG) and female patients with positive anti-*Toxoplasma* IgG antibodies respectively. Sera were obtained from the blood samples by centrifugation at 3000xg for 5 min, aliquot and stored at 70°C until tested.

Ethical Consent

This study was approved by Medical Ethics Committee in College of Medicine, Hawler Medical University, Erbil. Informed consent of the patients participating in the study was undertaken. The aim of the study was explained to the participants and that their information would keep confidential and will not be used for any other purpose, apart from the study.

Induction of Heat Shock Proteins Expression

Heat shock response was induced [25]. Briefly, protoscoleces were aseptically collected from affected sheep livers slaughtered at Erbil abattoir. Viability of the protoscoleces was assessed by methylene blue (0.05%) exclusion and flame cell activity. For the induction of heat shock proteins expression, two batches of 25000 protoscoleces per 3 ml of medium 199

(HIMEDIA, India) were incubated separately at 42°C and 45°C for four hours.

Heat treated protoscoleces and normal protoscoleces were disrupted [26]. Briefly, the protoscoleces were washed thrice with cold PBS (0.15 M) and re-suspended in three volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM phenyl methyl sulphonyl fluoride, 0.15 M NaCl, 0.5% (v/v) Triton x-100 and were disrupted by homogenizer. The resultant homogenized protoscoleces were sonicated for two periods of 15 sec with two intervals of 10 sec cooling, using an MSESoniprep fitted with an exponential probe at 4µm amplitude. Disrupted protoscoleces were then centrifuged at 5000xg for 30 min at 4ºC. The resultant supernatant was divided into two parts, one was directly used as a source of antigens in ELISA whereas the second portion was subjected to partial purification by column chromatography.

Partial Purification of Heat Shocked Protoscoleces Antigens

Hydatid antigens were purified from heatshocked protoscoleces by single step purification on Sephadex G 150 chromatography column in accordance with [27]. The purification process was carried out at 4ºC. 10ml of the 42ºC and 45ºC heat shocked protoscoleces extract solution was loaded on to Sephadex G 150 (Pharmacia fine chemicals) column (1.5 x 45 cm) pre-equilibrated with PBS buffer, pH 7.4 (NaCl 7.19 gm, KH2PO41.56 gm, Na2HPO46.74, NaHPO4.2H2O8.45 gm, Na2HPO4.12H2O17.01 gm, 1000 ml distilled water). Hydatid antigens were then eluted from the column with 4-fold PBS buffer. Fractions of 3ml each were collected at a flow rate of 40ml/h. Protein eluting from the column during purification was verified at 280nm of absorbnce.

Hydatid Antigens

Hydatid antigens were obtained by processing the fluid and particulate content of ovine liver cysts, following verification of the absence of bacteria and host cells, only fertile cysts with viable protoscoleces were selected for further proceeding. Hydatid fluid was aspirated aseptically, dialyzed against two changes of phosphate buffered saline pH 7.4 and centrifuged at 3000xg. The resultant supernatant was used as a source of hydatid fluid (HF) antigens [28]. In addition to HF antigens, eight further protoscoleces extract antigens were assessed by ELISA, namely: normal protoscoleces extract (PE), 42ºC exposed protoscoleces extract (PE42), 45ºC exposed protoscoleces extract (PE45) and five antigenic preparations eluted from the chromatography column. The protein content in the extract solutions was estimated by the method of Lowry [29]. Anti-hydatid IgG antibodies were tested by ELISA as described previously [13].

Data Analysis

Cutoff value to differentiate positive from negative sera was established by a receiver operating characteristic (ROC) analysis [16], using SPSS software (ver. 20.0, Chicago, IL, USA). ROC curves were generated by plotting sensitivity versus 1 - specificity and the area under the curve were used to determine the diagnostic performance of each antigens batch. Accordingly, sensitivity, specificity and Youden's index as well as confidence interval of 95% at *P*-value≤ 0.05 were estimated for each antigenic preparation, then were used to compare the immunoreactivity of different antigens tested in this study [13][30].

Results

Partial Purification of Heat Shocked Protoscoleces Extracts Antigens

Extracts of 42ºC treated protoscoleces resulted in two protein peaks, 1 and 2 (Figure 1). Eluted fractions in peak 1 (fractions 6-19) and peak 2 (fractions 20-25) were collected and

used as PE42P1 and PE42P2 antigens, respectively. For 45°C treated protoscoleces extract, the chromatography pattern (Figure 2) resulted in three protein peaks: 1 (fractions 6-18), 2 (fractions 19-28) and 3 (fractions 29-32). The eluted fractions were collected and used as PE45P1, PE45P2 and PE45P3 antigens respectively in ELISA.

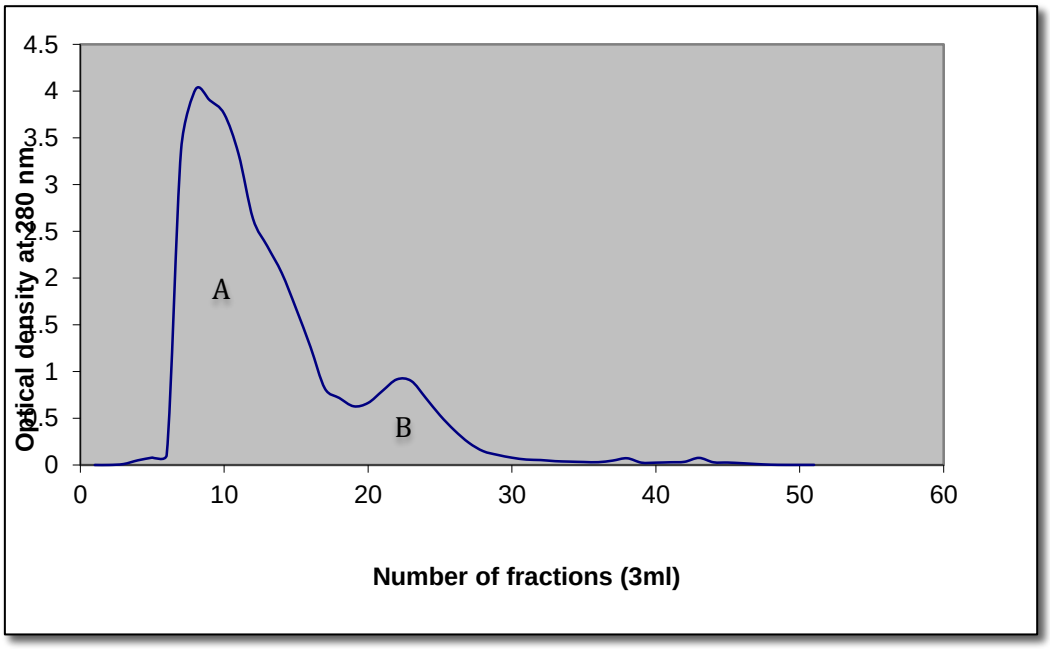

Figure 1: Elution pattern of extracts of 42 ºC treated protoscoleces, on Sephadex G-150 chromatography column (1.5 x 45 cm). Two protein peaks (A, B) at 280 nm

Cut off Value, Sensitivity and Specificity

The ROC curve for all used antigen batches was obtained by plotting the optical density of each tested serum by ELISA. This curve and corresponding data were used to estimate cutoff values, sensitivity specificity and diagnostic accuracy.

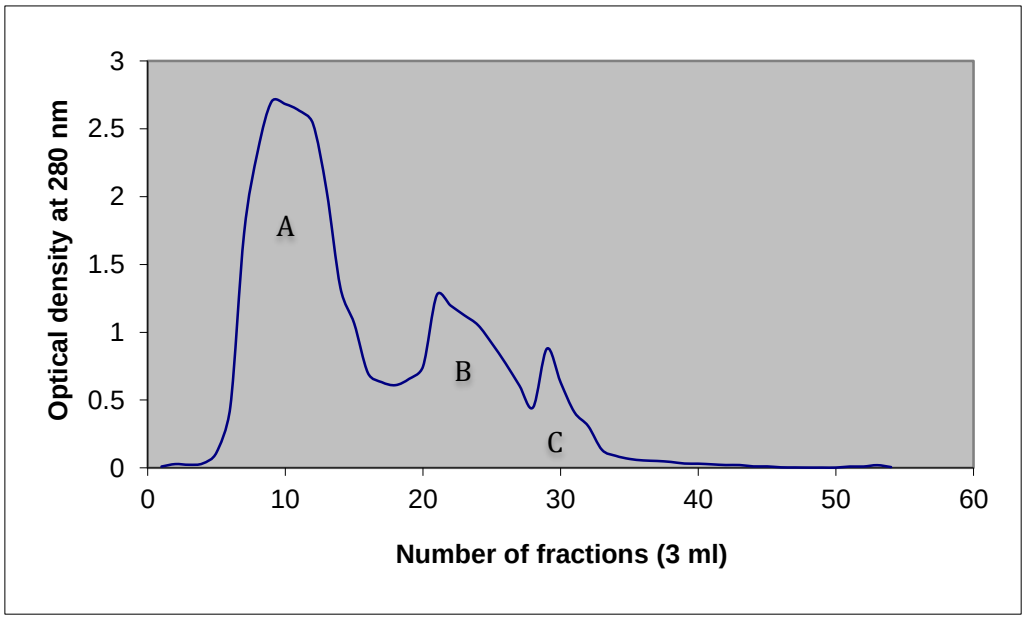

Figure 2: Elution pattern of extracts of 45 ºC treated protoscoleces on Sephadex G-150 chromatography column (1.5 x 45 cm). Three protein peaks (A, B, C) at 280 nm

All antigenic preparations used in this study revealed a significant rate of sensitivity, specificity and diagnostic accuracy. However, the sensitivity of ELISA to detect anti-hydatid IgG antibodies using PE42P2 (91.2%) and PE45P2 (91.2%) markedly raised than that observed with all other antigenic preparations including HF, PE45, PE42P1 and PE45P3 which detected anti-hydatid IgG antibodies each with sensitivity, specificity and diagnostic accuracy of 85.3%, 100% and 85.3% respectively (Table 1 and Figure 3). The diagnostic sensitivity of ELISA to detect anti-hydatid IgG antibodies in the sera of surgically confirmed hydatid patients was consistent for liver and lung cysts with all antigenic preparations used in this study. However, less consistent results were obtained with the other involved organs, except PE42P2 and PE45P2 that provided equivalent diagnostic accuracy (91.2 %) which detected two of the cases, one with psoas major muscle involvement and the other with multiple cysts that missed by HF, PE, PE42, PE 45, PE42P1, PE45P1 and PE 45P3 (Table 2). The specificity of all antigenic preparations applied in this study was detected as 100% when cross-reacted with sera of 18 non-cystic echinococcosis patients and 29 healthy controls (Table 1 and Figure 3).

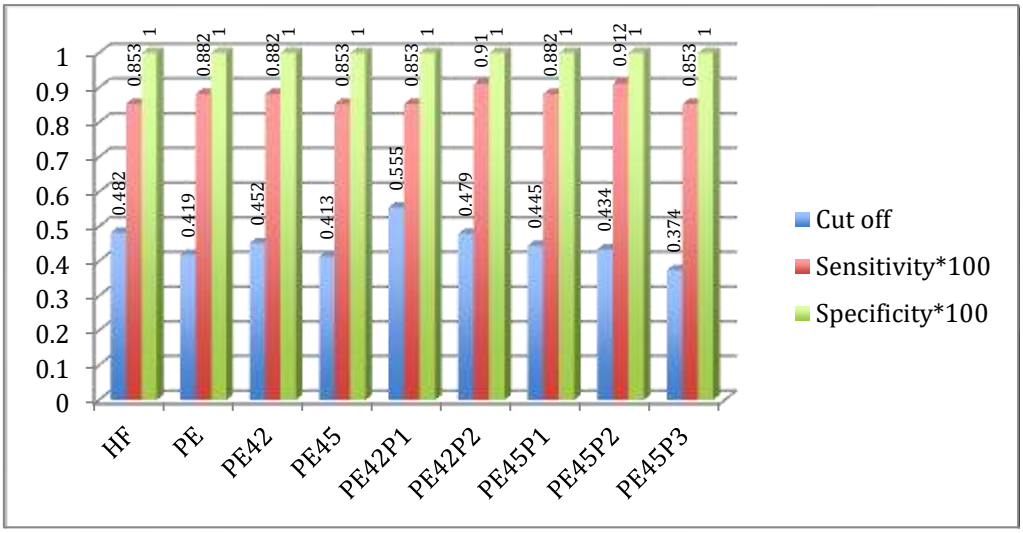

Figure 3: Cutoff value, sensitivity and specificity of ELISA utilizing different antigenic preparations, obtained by ROC curve.

Table 1: Cutoff value, sensitivity, specificity and diagnostic accuracy of ELISA to detect anti hydatid IgG antibodies in the sera of surgically confirmed hydatid patients, utilizing different hydatid antigen preparations.

Antigen	Cut off value	Sensitivity (%)	Specificity (%)	Youden's index (%)	*P* value	95% Confidence Interval		
						Lower Bound	Upper Bound	AUC
HF	0.482	85.3	100	85.3	<0.001	0.868	0.993	0.931
PE	0.419	88.2	100	88.2	<0.001	0.867	0.998	0.932
PE42	0.452	88.2	100	88.2	<0.001	0.000	1.000	0.954
PE45	0.413	85.3	100	85.3	<0.001	0.832	0.990	0.911
PE42P1	0.555	85.3	100	85.3	<0.001	0.864	1.000	0.934
PE42P2	0.479	91.2	100	91.2	<0.001	0.000	1.000	0.964
PE45P1	0.455	88.2	100	88.2	<0.001	0.924	1.000	0.966
PE45P2	0.434	91.2	100	91.2	<0.001	0.902	1.000	0.960
PE45P3	0.489	85.3	100	85.3	<0.001	0.830	0.980	0.905

AUC: area under the curve
Youden's index =(Sensitivity + Specificity) – 1

Table 2: Sensitivity of ELISA to detect anti-hydatid IgG antibodies in the sera of surgically confirmed hydatid patients utilizing different antigenic preparations, with details of involved organs.

	Antigen	HF (Positive)	PE (Positive)	PE42 (Positive)	PE45 (Positive)	PE42P1 (Positive)	PE42P2 (Positive)	PE45P1 (Positive)	PE45P2 (Positive)	PE45P3 (Positive)
Affected Organs	No.									
Liver	24	22	22	22	22	22	22	22	22	22
Lung	3	2	2	2	2	2	2	2	2	2
Muscle	4	3	3	3	3	3	4	3	4	3
Spleen	1	1	1	1	1	1	1	1	1	1
Multiple Organs	2	1	2	2	1	2	2	2	2	1
Total (%)	34	29 (85.3)	30 (88.2)	30 (88.2)	29 (85.3)	30 (88.2)	31 (91.2)	30 (88.2)	31 (91.2)	29 (85.3)
Healthy control	29	0	0	0	0	0	0	0	0	0
Toxoplasmosis	10	0	0	0	0	0	0	0	0	0
Dwarftape worm infection	3	0	0	0	0	0	0	0	0	0
APA IgG positive	5	0	0	0	0	0	0	0	0	0

APA IgG: anti-phospholipid immunoglobulin G antibodies

Discussion:

Due to asymptomatic features during the early stages of hydatid infection and for a long period after the cysts being established, the early detection of hydatid disease in human is therefore difficult. Although the definitive diagnosis for most human cases of hydatidosis is based mainly upon imaging techniques, such as X-ray, ultrasonography, CT scanning and magnetic resonance imaging [7][10] . However, these procedures are often not readily available in most communities. These usually provide effective diagnosis of the late stages of clinical infection. Hence, in addition to imaging techniques, these reliable serological techniques provide opportunities for early treatment and to improve the prognosis of patients in terms of whether, surgical or medical treatment should be adopted [31][32].

In the current study, the antigenic preparations applied in ELISA technique interacted differently for detection of anti-hydatid antibodies in the sera of surgically confirmed hydatid patients. Hydatid fluid antigens widely applied for serodiagnosis [33][34], revealed lowest sensitivity when compared with antigens extracted from heat shocked protoscoleces. The sensitivity and the diagnostic accuracy of the ELISA markedly increased with partially purified hydatid antigens derived from 42ºC and 45ºC heat shocked protoscoleces. Exposure of protoscoleces to relatively high temperature led to the over-expression of various stress-induced proteins which increased the polyclonal antigenic targets detected by specific serum antibodies [18][35]. These proteins could also be expressed *in vivo* on exposure of the parasite to hostile environment within the host [21][24]. Detection of specific antibodies produced against those stress-induced proteins could be missed when normal protoscoleces antigens and even crude hydatid fluid antigens are used in the immunodiagnostic procedures.

In this study, the highest sensitivity and specificity of ELISA was detected when PE42P2 and PE45P2 antigens were applied, indicating that purification of crude hydatid antigens will decrease non-specific reactions and chemical inhibitors [28].

Using different sources of hydatid antigens applied in various techniques, the sensitivity and specificity were variable. Of the 65 patients with confirmed hydatidosis, sera from 62 (95%) yielded positive results with ELISA using partially purified hydatid antigens. Whereas this rate was sharply reduced by counter-current immunoelectrophoresis (55%) [28].
Hydatid antigens enriched with antigen B by ELISA showed controversial results with variable sensitivity and specificity observed [13]. In the present study the sensitivity of ELISA was also affected by the localization of the cysts which was in consistent with the results previously obtained [8][31]. The sensitivity of ELISA to detect liver cysts ranged from 72% to 100% while the sensitivity of this test is around 50%-60% for lung cysts. Liver cysts release more antigenic materials into surrounding host tissues than lung cysts which are usually enveloped by an intact hyaline cyst wall [36]. The specificity of IgG ELISA was found to be 100% for all antigenic preparations applied in this study. In regions where cystic echinococcosis, alveolar echinococcosis and cysticercosis are endemic, potential cross-reactivity could be detected [37]. In Iran, almost all detected cases were due to *Echinococcus Granulosus.* As the consumption of pork, due to religious aspects, is prohibited, human cysticercosis is very rare. However, testing the cross-reactivity of both infections, in respect of heat shocked protoscoleces extracts antigens is highly recommended in areas with both species endemicity [38].

Conclusion:

Hydatid antigens extracted from heat treated *Echinococcus Granulosus* protoscoleces markedly increased the sensitivity of ELISA to detect anti-hydatid IgG antibodies in the sera of cystic echinococcosis patients. However, testing the specificity of those antigens for alveolar echinococcosisis is highly recommended in areas where both cystic and alveolar echinococcosis are endemic.

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Conflict of Interest:

The author declares that there is no conflict of interest.

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