Original article

Diagnostic value of hydatid cyst antigens using western blotting method

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Abstract

Introduction and objective: Hydatidosis is one of the most important and commonly found parasitic zoonoses in both humans and different animals, which is caused by the cestode helminthes *Echinococcus granulosus*. The diagnosis of the disease is primarily based on imagery techniques. Thus, highly sensitive and reliable serologic methods are required to confirm the diagnosis. AntigenB (AgB) and protoscoleces antigen (PSC Ag) were purified as two specific parasitic antigens and then evaluated against sera from two groups of hydatidosis and non-hydatidosis (control) subjects using the western blotting method in order to identify the most sensitive and specific antigen.

Materials and methods: Sera samples were taken from 22 patients under operation for hydatid cyst. 16 patients were also included as control group. Cyst fluid and protoscoleces were extracted and partially purified in a protein A column. Using SDS-PAGE, subunits of the cyst fluid antigen, AgB, and PSC Ag were identified. Finally, the subunits were transferred from gel to nitrocellulose membrane in a western blot test and reacted with hydatid and control sera in order to assess their sensitivity and specifity.

Results: Three antigens were identified as the subunits of AgB while 10 antigens were identified as PSC Ag. The sensitivity and specifity of AgB subunits in the western blot test were 77% and 100%, respectively. None of the PSC Ag subunits had both high sensitivity and high specifity concurrently.

Conclusion: It has been shown by the western blot test that the AgB 8/12 and 16 KDa subunit components had high diagnostic sensitivity and specifity levels (81% and 100%, respectively) and that they could presumably assist the physician in his pre- and post-operation diagnosis of hydatid cysts.

Keywords: Protoscoleces antigen, Antigen B, Western blotting, Hydatid cyst

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Introduction

Hydatid cyst is produced by the larval cestodes found in canids, called *Echinococcus granulosus*. It is one of the most important parasitic zoonoses universe found in different parts of the world including Europe, Central Asia, China, Australia, Africa, America, the Middle East, and Iran [1]. The adult worm is abundantly found in the small intestine of canids (the definitive host) which contain large numbers of ruptured eggs in the host intestine to set the eggs free. Intermediate hosts (herbivores) and humans may accidentally come into contact with the eggs through vegetables and water whereby the eggs find their way into their host's digestive system. The larval tapeworm then burrows through the intestinal wall and travels to different body organs via the blood. The hydatid cyst develops then in the organ where it resides [2].

Given the importance of specific antigens in confirming the incidence of hydatidosis and also that the disease has a high rate of incidence in Iran [3], it is necessary to identify the specific antigens and to perform accurate serological tests. Of great importance are also post-operation follow-up measures using a standardized sensitive test with specific antigens. Efforts were made in this study to isolate and identify specific antigens including antigen B and protoscoleces antigen and to determine their subunit components. Comparisons were then made to determine the diagnostic values of these two antigens in terms of their sensitivity and specifity in two groups of subjects with hydatidosis (experimental group) and normal subjects (control) [4].

The subunits with the highest sensitivity and specifity levels were then identified, which can be used as a complementary diagnostic test in confirming a case of hydatidosis. This test can also be used as a first step toward identification of active antigens in the hydatid cyst. The aim of present study was to isolate and identify specific antigens including antigen B and protoscoleces antigen and to determine their subunit components.

Materials and methods

The samples collected included cyst fluid and serum samples. Sheep hydatid cysts were collected from slaughterhouse in Isfahan and transferred to the Parasitology laboratory, School of Medicine, Isfahan University of Medical Sciences. The cyst fluid was centrifuged at 1000g for 20min and the supernatant was then collected in sterile containers to be stored at -70°C. Protoscoleces were separated and washed two to three times in a PBS (pH=7.4) and then stored at -70°C.

In order to identify and evaluate the purified and unpurified antigens (and their subunits) by using the immunoblotting test, a total number of 38 sera samples were collected and tested. The samples were collected in the following distinct groups: 22 sera samples from patients with hydatidosis diagnosed post-operationally as confirmed by parasitological and histological tests. The samples were collected from patients in hospitals in Isfahan, Hamedan, and Shahrekord, four sera samples from patients with acute toxoplasmosis, four samples from patients infected with cestodes other than *E. granulosus*, and four samples from patients infected with leishmaniasis or Kala-azar. These samples were used for the purposes of cross and nonspecific reactions; and four sera samples were from normal healthy subjects as control.

Antigen B was prepared by using Oriol *et al.* [5] method. 100ml of fresh collected cyst fluid was centrifuged at 1500g for 30min. The supernatant was dialyzed against PBS at 4°C overnight. The dialyzed solution was centrifuged at 50000g in 4°C and the sediment re-suspended in 10ml of PBS (0.2mol pH=8). The solution was boiled in water bath for 15min and re-centrifuged at 50000g. Supernatant containing Ag B stored at -70°C for subsequent treatment [5-7]. Purification of Ag B and removal of host immunoglobulins were performed on protein A column (Pharmacia-Biotech) [8] equilibrated with two column volume of binding buffer (sodium phosphate 20Mm, pH=7). The sample (supernatant containing Ag B) applied to the column then washed with five column volume of elution buffer (citric acid 0.1M pH=3-6).

A desirable volume (200µl) of the protoscoleces was homogenized at 4°C. The solution was then sonicated four times in an ice bath using a sonicator set at 5cps and at maximum tone for 30sec. The sonicated and homogenized product was then centrifuged (1000g for 30min) to remove the supernatant from the sediments (the sediments consisted of protoscoleces membranes and hooks while the solution contained protoscoleces cell protein content). The solution obtained from the centrifuge was then dialyzed against PBS overnight at 4°C [9].

Bradford method was used in the assessment of proteins [10]. The solution from dialysis was poured into eppendorf tube and stored at -70°C for subsequent treatments. Low molecular weight sigma markers (including eight proteins with known molecular weights in the range of 6500/66000 Daltons) were subjected to immunoelectrophoresis along with the samples under identical conditions using the SDS-PAGE immunoelectrophoresis and, subsequently, stained using silver nitrate. The western blotting method was then used to determine the diagnostic value of the antigens obtained [11,12]. The number of the samples in this study was calculated with confidence limits 95% (Z=1.64), to be about 38 sera samples and to compare sensitivity of AgB and PSC Ag, Chi-squared test was used.

Results

Cyst fluid ingredients including albumin, globulins, and other parasite antigens such as antigen 5 were each removed from the fluid in different stages of the purification process. The high albumin content in the cyst fluid caused albumin to be observed in rather considerable quantities in the immunoelectrophoresis process (66KDa band) although great amounts had been removed in the purification process. AgB was broken down into three bands with molecular weights of 8-12, 16, and 20-24 KDa. The PSC Ag was also broken down into 10 bands with molecular weights of 90-110, 77, 65, 56, 45, 35, 32, 24-29, 14/20, and 10 KDa (Fig. 1).

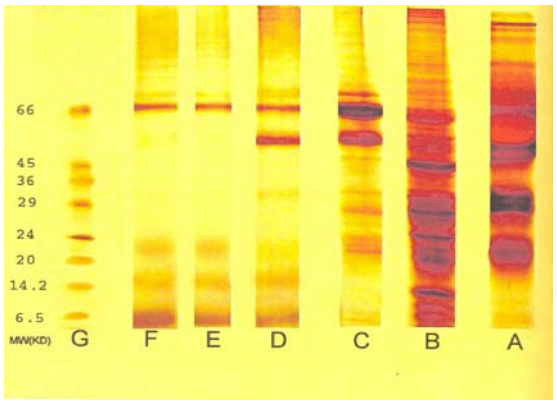

Fig. 1: Monitoring of the AgB and PSC Ag purification trend by SDS-PAGE

A: Raw hydatid fluid antigen (CHF Ag), **B**: PSC Ag, **C**: Supernatant containing albumin, **D**: Sediments containing parasite antigens and globulins, **E, F**: Supernatant containing AgB, **G**: Marker size

The immunoblotting (western blotting) test was performed in order to determine the diagnostic value of each of the Ag B subunits in the cyst fluid and PSC Ag of hydatid cyst in diagnosis of hydatidosis and also to determine the diagnostic value of each individual subunit. In the case of AgB, of the 22 samples from patients with hydatidosis, 18 samples (82%) reacted positively with 16KDa subunits and 18 samples (82%) reacted positively with 20-24 KDa subunits. Observations also showed that 19 samples (86%) had a positive reaction with at least one band (8-12 or 16 KDa subunits) and 20 samples (91%) with at least one band (8-12, 16, or 20-24 KDa subunits) (Fig. 2).

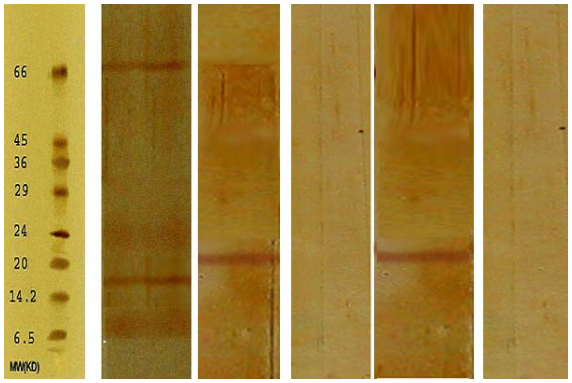

Fig. 2: Reactions of AgB subunit components with various serum samples in hydatidosis diagnosis using the immunoblotting test

A: Marker size, **B**: Hydatidosis, **C**: cestodes other than *Echinococcus granulosus,* **D**: leishmaniasis, **E**: Toxoplasmosis, **F**: Healthy subject serum

The 8-12 and 16 KDa bands had no false positive reactions with none of the control sera samples. In one case (25%), the sera sample infected with *Hymenolepis nana* had a false positive reaction with the 20-24 KDa subunit and in one sample infected with *Taenia saginata* (25%), a false positive reaction was recorded with the 20-24 KDa subunit. Also, positive reactions were recorded with the 20/24 KDa subunit for two samples infected with acute toxoplasmosis. No false positive reactions were observed for the samples infected with *Leishmania*. Neither did any of the samples from healthy subjects exhibited false positive reactions with AgB subunits (Table 1).

With regard to protoscoleces antigen, among the 22 hydatidosis serum samples examined, the following positive reactions were recorded: 14 samples (64%) with the 90-110 KDa band, 16 samples (73%) with the 77 KDa band, 15 samples (68%) with the 65 KDa band, 14 samples (64%) with the 56 KDa band, 13 samples (59%) with the 4560 KDa band, 19 samples (86%) with the 35 KDa band, 15 samples (68%) with the 32 KDa band, 10 samples (45%) with the 24-29 KDa band, 15 samples (68%) with the 14-20 KDa band, and finally, 10 samples (45%) with 10 KDa band (Fig. 3).

Table 1: Results from reactions of serum samples with AgB subunits of hydatid fluid using the immunoblotting test

Sample	Number	No. (%) of cases of positive reactions with various bands (KDa)				
		8/12	16	20/24	8-/2 or 16	8/2 or 16 or 20/4
Hydatidosis	22	17 (77)	18 (82)	18 (82)	19 (86)	20 (91)
Toxoplasmosis	4	0(0)	0(0)	2 (50)	0(0)	2 (50)
Other cestodes	4	0(0)	0(0)	2 (50)	0(0)	2 (50)
Leishmaiasis	4	0(0)	0(0)	0(0)	0(0)	0(0)
Healthy subjects	4	0(0)	0(0)	0(0)	0(0)	0(0)
Total (%)	16 (100)	0(0)	0(0)	4 (25)	0(0)	4 (25)

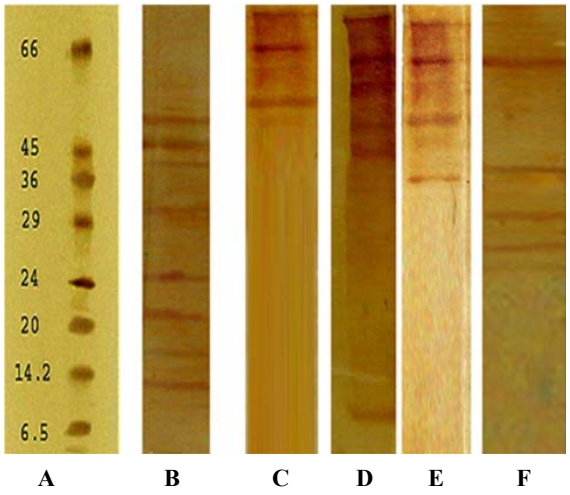

Fig. 3: Reactions of SPC Ag subunit components with various serum samples in hydatidosis diagnosis using the immunoblotting test

A: Marker size, **B**: Hydatidosis, **C**: Leishmaiasis, **D**: Normal subject serum, **E**: Toxoplasmosis, **F**: Cestodes other than *Echinococcus granulosus*

The 10 KDa and 32 KDa bands had no false positive reactions with the control sera samples. One sample (25%) of the sera taken from subjects with *H. nana* had false positive reactions with the subunits 77, 65, 56, 45, and 35. Also, one sample (25%) had false positive reactions with the bands 77, 65, and 56 and one sample (25%) with 65, 24-29, 14-20 bands. One sample from the subjects with toxoplasmosis exhibited false positive reactions with the 77, 65, and 45 KDa bands and one sample (25%) with the subunits 90, 77, 65, and 35 KDa. One sample (25%) from the patients with leishmaiasis had false positive reactions with the subunits 77, 65, and 56 KDa. The control sera samples also reacted with the 56-110, 65, 77, and 90 KDa bands and only one had false positive reactions with the 45 and 24-29 KDa bands (Table 2).

Table 2: Results from reactions of serum samples with SPC Ag subunits of hydatid fluid using the immunoblotting test

Sample	No.	No. (%) of cases of positive reactions with various bands (KDa)									
		90/110	77	65	56	45	35	32	24/29	14/20	10
Hydatidosis	22	14(64)	16(73)	15(68)	14(64)	1359)	19(86)	15(68)	10(45)	15(68)	22
Toxoplasmosis	4	1 (25)	2 (50)	2 (50)	0(0)	1(25)	1(25)	1(25)	0(0)	0(0)	0(0)
Other cestodes	4	0(0)	2 (50)	3 (75)	2 (50)	1(25)	1(25)	1(25)	1 (25)	1 (25)	0(0)
Leishmaiasis	4	1 (25)	1 (25)	1 (25)	1 (25)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Healthy subjects	4	2 (50)	2 (50)	2 (50)	2 (50)	1(25)	0(0)	0(0)	1 (25)	0(0)	0(0)
Total (%)	16	4 (25)	7 (44)	8 (50)	5 (31)	3(19)	2(12)	0(0)	2 (12)	1 (6)	0(0)

Sensitivity and specifity levels of each of the subunits of the different antigens were determined and compared using the western blotting test. The results showed that the sensitivity of Ag B for the 8-12, 16, and 20-24 KDa bands were 77%, 82%, and 82%, respectively. The highest sensitivity level obtained in the interactive evaluations of all three bands, i.e. 8-12, 16, or 20-24, was 91%. In the case of protoscoleces, the highest sensitivity level (86%) belonged to the 35 KDa band while the lowest sensitivity level was 45% related to the 10 and 24-29 KDa bands. The specifity levels of the 8/12, 16, and 20/24 KDa bands were 100%, 100%, and 75% and in the interactive evaluations of all three bands the specifity level was found to be 75% (Table 3).

The highest diagnostic specifity obtained for protoscoleces antigen (PSC Ag) was 100% belonging to the 10-KDa band while the lowest one was 50% belonging to the 65-KDa band (Table 3).

Table 3: Diagnostic values of AgB subunits and PSC Ag for hydatidosis using the immunoblotting test

	AgB & PSC Ag subunits (KDa)	True positive	True negative	False positive	False negative	Sensitivity (%)	Specificity (%)	Validity (%)	Predictive value	
									Positive (%)	Negative (%)
AgB	8/12	17	16	0	5	77	100	88.5	100	76
	16	18	16	0	4	82	100	91	100	80
	20/24	18	12	4	4	82	75	78.5	82	75
	8/12 or 16	18	16	0	4	82	100	90.5	100	80
	8/12 or 16 or 20/24	20	12	4	2	91	75	83	83	86
PSC Ag	90/110	14	12	4	8	64	75	69.5	78	60
	77	16	9	7	6	73	56	64.5	70	60
	65	15	8	8	7	68	50	59	65	53
	56	14	11	5	8	34	69	66.5	74	58
	45	13	13	3	9	59	81	70	81	59
	35	19	14	2	3	86	88	87	90	82
	32	15	14	2	7	68	88	79	88	70
	24/29	10	14	2	12	45	81	63	83	54
	14/20	15	15	1	7	68	94	81	94	68
	10	10	16	0	12	45	100	72.5	100	57

Due to the variety of bands in the protoscoleces antigen and also since there was not a simultaneous diagnostic specifity and sensitivity in the bands obtained from immunoelectrophoresis of the protoscoleces antigen, interactive evaluation of bands does not have any diagnostic value for this antigen (Table 3). The highest negative predictive value was 100% and belonged to the 8-12, 16 and 8-12 or 16 KDa bands of the Ag B. The same value in the case of protoscoleces antigen was 100% and belonged to the 10-KDa band.

The highest negative predictive value was 86% and belonged to the 8-12, 16, or 20-24 KDa bands of Ag B while it was 82% for the 35 KDa band of the protoscoleces antigen (Table 3). The highest validity levels for the Ag B and PSC Ag subunits obtained and comparison of them showed a significant difference ($P<0.05$). The higher the number of the samples, the difference was the greater. It can, therefore, be concluded that the difference Ag B has a higher validity compared to PSC Ag. The highest validity was obtained with either 8-12 or 16 KDa bands (Table 3).

Discussion

Ag B was broken down into three bands. Other components present in the hydatid fluid such as host albumin with molecular weights of 66KDa were removed in a great extent. Glubolins including light and heavy chains were removed by being passed through protein A chromatographic column. The Ag 5 subunits were removed during one stage of the purification process. The protoscoleces Ag was subdivided into 10 bands after removing the membranes and rostellar hooks through SDS-PAGE. Poretti *et al*. [13] in their study on differential diagnosis of hydatidosis used western blotting to separate the raw antigen in the hydatid fluid based on SDS-PAGE and immunoblotting. They reported the 29, 8, and 34 KDa bands as the most immunogenic bands. It seems, however, that the 29 KDa band should belong to Ag5.

Leggart *et al*. [14] reported the 12KDa subunit components as the smallest Ag B subunit. But Rogan *et al*. [15] identified the 8-KDa subunit component as the smallest AgB subunit. In the present study, three bands of 8-12, 16, and 20-24 KDa were identified as the subunit composition of Ag B. The slight differences reported on the size of the subunit components may be ascribed to the different conditions and the specific implementations of SDS-PAGE such as the composition and percentages of the poly-acrylamide gel used. Moreover, such antigenic differences expected among different strains of the parasite as those reported in Gottenstein [16] for different isolates of *Echinococcus* multi-locularis should not be overlooked.

According to Sbihi *et al*. [9], PSC Ag can also be subdivided into 12 bands in the SDS-PAGE process, three of which (37, 42, and 110 KDa) are referred to in Sbihi *et al*. [9] and match the three bands identified in the present study. Sbihi maintains that the 110KDa band is probably the same 116 KDa band referred to in Knawar *et al*. [17]. In our study, the PSC Ag was homogenized, sonicated, and finally dialyzed. The membranes and hooks were removed and the larval part was subjected to immunoelectrophoresis to determine the number of bands.

Differences in experimental conditions and implementation of SDS-PAGE can explain the slight differences in the sizes of PSC Ag subunit components obtained here. Also, it must be borne in mind that antigenic differences exist among various strains of the parasite as there are also

differences in the hosts nurturing the cysts. Thus, the 10, 14-20, and 24-29 KDa bands can be conveniently assigned to Ag B [18]. The 32, 35, 45, 56, and 65 KDa bands should probably belong to Ag 5 [9-14] while the 90-110 and 77 KDa bands may be polymers of the lighter subunit components of Ag B [19].

In the light of the above considerations and due to our urgent need for a more specific serological diagnosis of hydatidosis, evaluation of Ag B and PSC Ag subunit compositions was performed using the western blot test based on serum samples prepared from (experimental) hydatidosis patients and (control) healthy subjects. Diagnostic specifity of 8-12 and 16 KDa bands were determined to be 100% without cross reactions with control serum samples. The diagnostic specifity of the 22 KDa band was estimated at 75% due to two serum samples of patients infected with cestodes other than *E. granulosus* and two serum samples of patients with toxoplasmosis infections.

The overall diagnostic specifity levels of the 8-12, 16, or 20-24 KDa bands were also estimated at 75% for the same reason. This results in the observation because mathematical summation of the bands from 8 to 22 KDa leads to increasing sensitivity from 77% to 82% while in the reverse direction, it leads to an decreasing specifity from 100% to 75%. It is concluded that the 8-12 and 16 KDa subunit components of Ag B are highly specific in diagnosing hydatidosis and show no cross reactions with other diseases investigated.

Reports in the literature have identified the smaller subunit of Ag B (8KDa, or according to some other studies the 12 KDa) as peculiar to the *Echinococcus* genus which only cross reacts with the varieties in this genus [13,20,21] and probably with cysticercosis (*Taenia solium*) [18]. Ito *et al.* [21] reported 79% and Poretti *et al*. [13] reported that 53% of the serum samples from patients infected with alveolar cysts showed positive reactions with subunit components of 8 KDa. Fortunately, as the alveolar cyst is limited to a special region in Iran and due to lack of reports on cysticercosis cases in Iran, differential and specific diagnosis of hydatidosis can be solely based on the 8-12 and 16KDa subunit components.

Ortona *et al.* [22] propose a diagnostic sensitivity level of 80% for the smaller Ag B subunits through immunoblotting test while Leggart *et al*. [14] propose a 90.2% sensitivity level for the 12KDa Ag B subunit using the same diagnostic method. Sbihi *et al.* [9] use the same method and propose a sensitivity level of 95% and a specifity level of 100% for the smaller Ag B subunit components.

The false positive reactions of the 22KDa subunit with serum samples from patients infected with other cestodes such as *H. nana* possibly indicate common antigens found in the two groups of patients (infected with hydatidosis and other cestodes). Probably the lack of antigenic similarities or inaccessibility of the antigens by the immunity system to produce antibodies in patients with leishmaiasis may be the cause for the absence of false positive reactions with Ag B subunit components observed in this case (especially with regard to the fact that in patients with cutaneous leishmaiasis, the host systemic antibodies are too low to allow for any false positive reactions with hydatid antigens).

False positive reactions with the 20-/24-KDa subunit are also observed in the case of toxoplasmosis which can be probably explained by the antigenic similarity

between this Ag B subunit component and toxoplasmosis antigens, as also reported in Ortona *et al*. [22]. It needs to be mentioned that the diagnostic value of Ag B increases as the likelihood of simultaneous incidence of hydatidosis and acute toxoplasmosis or cysticercosis decrease.

The diagnostic specifity of the 10KDa band was determined at 100% without cross reactions with control serum samples. This band can probably belong to the antigens secreted by the protoscoleces parenchyma tissue which is similar to Ag B. Other bands, however, show increasingly more cross reactions with serum samples from other patients or even from healthy subjects as the molecular weight increases which, in turn, decreases the sensitivity of these bands. In the PSC Ag, the highest sensitivity level was observed in the 35KDa subunit component.

The false positive reactions observed in this experiment might be due to antigenic similarity with other infections such as *Toxoplasma*, *Leishmania* and cestodes except *E. granulosus*. Moreover, the antibody studied here is IgG which is likely to have cross reactions with other infections [22]. If other specific antibodies such as IgE with a shorter half-life are traced, the percentage of false positive reactions is expected to be considerably lower.

As reported by Sbihi *et al*. [9], PSC Ag also showed cross reactions with patients infected with cysticercosis, leishmaiasis, and toxoplasmosis, thereby decreasing the specifity of the subunits of this antigen. Rafiei and Craig [18] identified the 10KDa subunit of the PSC Ag using 60% of the serum samples taken from patients with hydatidosis in their immunoblotting test. This subunit also positively reacted with samples from patients with alveolar cysts or with trypanosomiasis while no positive reactions were reported with samples from other patients. These researchers concluded that this subunit probably belonged to Ag B [18].

The 38KDa band of the PSC Ag, in their study, exhibited satisfactory sensitivity levels with serum samples from other patients infected with cysticercosis, trypanosomiasis, etc. These researchers claimed identical antigenic compounds to exist in both trypanosomiasis and *E. granulosus* but that the false positive reactions in patients with trypanosomiasis were due to the presence of large amounts of non-specific antibodies, or hypergammaglobulinemia, in such patients [18].

Although trypanosomiasis is non-existent in Iran, the results of this study on patients with Kala-azar in which hypergammaglobulinemia is also observed match the results on patients with trypanosomiasis to the effect that the observation of false positive reactions in serum samples from patients with Kala-azar are due to the presence of hypergammaglobulinemia. While PSC Ag was formerly used only for preparing the antigen in IFAT and with respect to the fact that little, if any, attention has been paid to the characteristics of PSC Ag in the immunological diagnosis of *E. granulosus*. The present study has succeeded in evaluating this antigen and its subunit components while also determining its relatively high sensitivity but low specificity.

In this study, we evaluated the subunit components of PSC Ag using the western blotting test to find different sensitivity and specificity levels. The best subunit in terms of sensitivity was the 35KDa band and the best in terms of specifity was found to be the 10KDa band. Based on our findings, the PSC Ag is of lower diagnostic value compared to Ag B for a variety of reasons,

as reported by Rafiei and Craig [18] and elsewhere indicated that the 10KDa band probably belongs to Ag B and the 35KDa to Ag 5, their rather high sensitivity and specificity in the PSC Ag, the difficulty of preparing PSC Ag as opposed to the easiness of obtaining Ag 5 and Ag B via hydatid fluid, and finally, the reportedly low overall reliability (validity) of the subunit components of this antigen [9,23-25].

In our study and based on previous studies [9,13,22,25,26], the immunoblotting assays, especially in the case of the smaller subunits of 8/12 and 16 KDa of Ag B, enjoy a high diagnostic (100%) specifity in diagnosing hydatidosis in that the subunits do not react with any of the control serum samples under study or the toxoplasmosis, cestodes except *Echinococcus*, or leishmaiasis, which are all endemic parasitic diseases in Iran.

Conclusion

Three subunit components of 8/12, 16, and 20-24KDa bands show a high diagnostic sensitivity and specifity. The results also indicated that the utilization of PSC Ag and Ag B subunits increases diagnostic sensitivity and specificity in the diagnosis of hydatidosis, with higher levels of sensitivity and specifity belonging to Ag B compared to PSC Ag. Finally, it was found that the three subunit components of Ag B can be used as a complementary diagnostic test for special cases of hydatidosis.

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