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Original Article

Isolation and purification of *Echinococcus granulosus* antigen B from hydatid cyst fluid using three different methods

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ABSTRACT

Hydatid cyst, the larval stage of cestodes *Echinococcus* spp., is recognized as a zoonotic infection in the world. The World Health Organization (WHO) has recently classified echinococcosis in a group of neglected tropical diseases. The prevalence of *Echinococcus granulosus* infection is high in Iran due to the presence of various intermediate hosts in this country. Considering the rising trend of this zoonotic parasitic disease based on national epidemiological studies, diagnosis is of great significance. WHO has suggested the use of specific antigens, especially antigen B (AgB) for serological diagnostic tests. In general, AgB is a polymeric lipoprotein, which disintegrates into 8.12, 16, and 20.24 kDa subunits. In the present study, we applied three different methods for AgB isolation from hydatid cyst fluid (HCF) and compared their efficacy in AgB isolation. Finally, the protein concentration of this antigen was measured by Bradford assay and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that the application of polyethylene glycol (PEG 4000) as a thickener agent beside purification of HCF in dialysis bag and filtering and also dialysis against acetate buffer leading to the best quantity in purified antigen B.

Keywords: Antigen B, *Echinococcus granulosus*, Hydatid cyst, Isolation

INTRODUCTION

Echinococcus granulosus is a cestode with two types of hosts, i.e., carnivores as definitive hosts and herbivores and omnivores as intermediate hosts. This parasite has a worldwide distribution, with various cases reported from different countries (Barnes et al., 2012). In the larval stage, *E. granulosus* causes cysts in most organs of intermediate hosts, especially the liver, lung, and brain (Silva, 2011). The external layer of hydatid cyst, fibrotic tissues, formed due to the reaction of the host's immune system and the large volume of cyst fluid, prevent drug effectiveness (Rigano et al., 2001). Over the past decade, introduction of specific and sensitive diagnostic methods has been helpful for the treatment and management of cystic echinococcosis (CE). CE, as a zoonotic disease, is known as a health problem in human communities and is considered as a recently discovered, recurrent disease in some countries (Moro and Schantz, 2009). Various research studies

and statistics reported by the surgical units of Iranian hospitals, Ministry of Health, and disease management centers have revealed the rising trend and relatively high prevalence of cystic echinococcosis in different regions (Rokni, 2009). Considering the importance of CE in human health due to the involvement of vital organs, rapid and precise diagnostic methods are highly required. Serological methods are among the common methods for the diagnosis of hydatidosis in humans (Barnes et al., 2012). In fact, use of specific and protective antigens (Ags) in diagnostic kits is necessary for determining the serum antibody titer. Overall, Ag5 and AgB are two important and familiar Ags in CE (Rigano et al., 2001). Despite the prevalent use of Ag5 in the past, today it has lost its diagnostic value due to its low specificity and sensitivity in enzyme-linked immunosorbent assay (ELISA) and its cross-reaction with other antibodies against cestodes, nematodes, and trematodes (Gillespie and Hawkey, 1995). The World Health Organization (WHO) has recommended the application of specific serological methods with specific Ags, particularly AgB (Sadjjadi et al., 2007). AgB allocates more than 10% of hydatid cysts fluid (Rigano et al., 2001)and is synthesized and released by the germinal layer of these cysts. In addition, protoscolex, a protein with significant immunological properties, is thought to play an important role in the interaction between parasites and hosts through inhibiting the chemotaxis of polymorphonuclear leukocytes and shifting the immune response to a non-protective Th_2 response (Gonzalez-Sapienza and Cachau, 2003; Zhang et al., 2010). AgB is a lipoprotein (Mamuti et al., 2006) with several subunits of 8, 16, 24, 32 kDa; also, in the literature, 12 and 20 kDa subunits have been identified, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Carmena et al., 2006; Rahimi et al., 2011). In this study, we aimed to examine and compare three different methods for AgB isolation. We also measured the amount of AgB protein by Bradford assay and confirmed the findings by SDS-PAGE in order to propose an optimal method.

MATERIALS AND METHODS

Preparation of hydatid cyst fluid (HCF). For the extraction of AgB from HCF, first, cyststic liver of sheep were obtained from a slaughterhouse, and the surface of cysts was disinfected with alcohol and heat; the hydatid fluid of cysts was aspirated with a syringe (Figure 1).

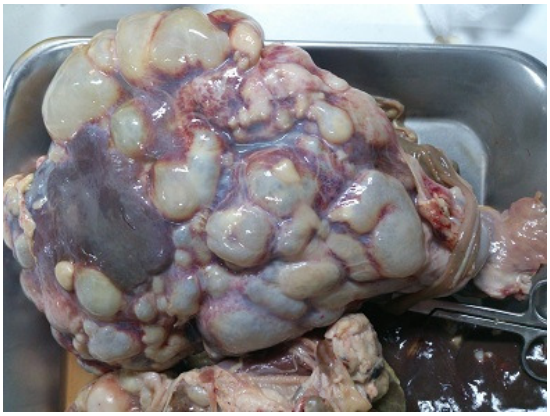

Figure 1. Infected liver by hydatid cyst

By using an optical microscope, the fluid was examined to determine the presence of protoscoleces (Figure 2) and assess the turbidity of the fluid (since bacteria turn the fluid turbid). The controlled fluid was stored in sterile dishes at -70°C until isolation (Sarkari et al., 2007; Rafiei et al., 2008).

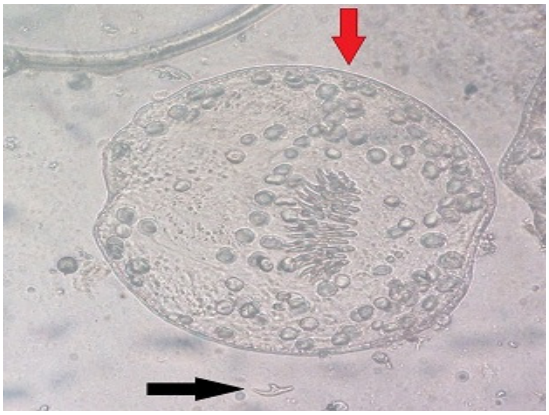

Figure 2. Content of hydatid cyst fluid (HCF). The red arrow shows protoscoleces and the black one indicates the hooks.

Preparation of AgB. We used three different isolation methods for purifying AgB from HCF, as described below:

First method: To eliminate particles, 100 ml of HCF was centrifuged at 1500 g for 30 min. The supernatant was dialyzed against 0.005 M acetate buffer (pH=5) overnight at 4 °C; this step was repeated once. The content of dialysis bags was centrifuged, using an ultracentrifuge at 30,000 g at 4 °C for 30 min; this procedure allowes insoluble proteins such as AgB and Ag5 to settle. The precipitates were then dissolved in 10 ml of 0.2 M phosphate buffer (pH=8) in order to eliminate globulins. The preparation was saturated with ammonium sulfate 40% by gradually adding 2.31 g of ammonium sulfate powder and shaking the mixture. After a short interval, the preparation was centrifuged at 3000 g for 30 min. The supernatant was incubated in a water bath for 15 min. At this stage, Ag5 became denatured and insoluble owing to its heat sensitivity. The mixture was centrifuged by an ultracentrifuge at 30,000 g for 1 h. Finally, the supernatant containing AgB was collected and filtered (using a 0.2 µm filter). Then, sodium azide (NaN_3) was added and the mixture was stored at -70 °C until further use.

Second method: In this method, 100 ml of HFC was centrifuged at 1500 g for 30 min. The preparation was dialyzed against 0.005 M acetate buffer (pH=5) overnight at 4°C. The content of dialysis bags was centrifuged with an ultracentrifuge at 30,000 g at 4°C for 30 min. The precipitates were then dissolved in 10 ml of 0.2 M phosphate buffer (pH=8). The preparation was saturated with ammonium sulfate 40% and centrifuged after a short interval at 3000 g for 30 min.

The supernatant obtained from the salting-out process was incubated in a water bath for 15 min in order to denature Ag5. The mixture was centrifuged by an ultracentrifuge at 30,000 g for 1 h. The supernatant contains of the soluble AgB. After filtration with a 0.2 µm filter and adding NaN_3, the mixture was stored at -70°C until further use (Mohammadzadeh et al., 2012; Asghari et al., 2013).

Third method: In this method, 100 ml of HCF was poured in a dialysis bag and placed in a dish containing polyethylene glycol (PEG 4000) for 1 h in order to concentrate of HCF proteins. The preparation was filtered by a 0.2 microfilter and centrifuged at 1500 g for 30 min. Afterwards, the preparation was dialyzed against 0.005 M acetate buffer (pH=8) overnight at 4 °C. The content of dialysis bags was centrifuged at 30,000 g at 4 °C by an ultracentrifuge for 30 min. The precipitates were then dissolved in 10 ml of 0.2 M phosphate buffer (pH=8). The preparation was saturated with ammonium sulfate 40% and centrifuged at 3000 g for 30 min after a short time interval. The supernatant obtained from the salting-out process was incubated in a water bath for 15 min. the mixture was centrifuged by an ultracentrifuge at 30,000 g for 1 h; finally, the soluble AgB was collected in the supernatant. After filtration (using a 0.2 µm filter), NaN_3 was added and stored at -70°C until further use.

Finally, the protein concentration of the three solutions was determined by Bradford assay, and AgB purity was analyzed by SDS-PAGE.

Bradford protein assay. In this assay, we prepared six dilutions (5-100 µg) of a standard protein (bovine serum albumin) to draw the standard curve and three preparation solutions (M1, M2, and M3) in properly labeled test tubes; then, 100 ml of buffer solution was added to each tube. Moreover, 5 ml of Bradford reagent (Coomassie Brilliant Blue G-250, ethanol 95%, and phosphoric acid) was added and completely mixed. For preparing the blank tube, 100 ml of phosphate-buffered saline (PBS) and 5 ml of Bradford regent were mixed and incubated at room temperature for 20 min. Absorbance was measured at 595 nm with an ELISA reader (BioRad) for plotting the standard curve with standard samples (concentration plotted on the X-axis and absorbance plotted on the Y-axis) and determining the concentration of isolated proteins (Bradford, 1976).

SDS-PADE analysis of AgB. The purity of AgB preparation was assessed by SDS-PAGE (2.5% gel), as reported in a study by Laemmli in 1970. Afterwards, 10 µl of AgB (M1, M2, and M3) was dissolved in the same sample buffer (0.0625 M Tris HCl, pH=6.8, 10% glycerol, 2% SDS, 0.06% bromophenol blue, and 5% ß-mercaptoethanol) and boiled for 10 min; the product was then loaded on the electrophoresis gel.

Electrophoresis was performed at a constant current of 110 V for 1 h. After this period, the separated bands were stained by Coomassie brilliant blue staining solution (0.1 cc Coomassie blue in 70, 15, and 15 of methanol, water, and acetic acid, respectively) and decolorized in decolorizer buffer (70 ml of methanol, 15 ml of water, and 15 ml of acetic acid).

RESULTS

In total, 300 ml of *E. granulosus* HCF was collected from the developed cysts in sheep livers. AgB was successfully isolated by using the mentioned methods, and its concentration was measured by Bradford assay. The protein concentration of AgB in the first, second, and third methods was 0.4, 0.1, and 0.7 mg/ml, respectively (Figure 3).

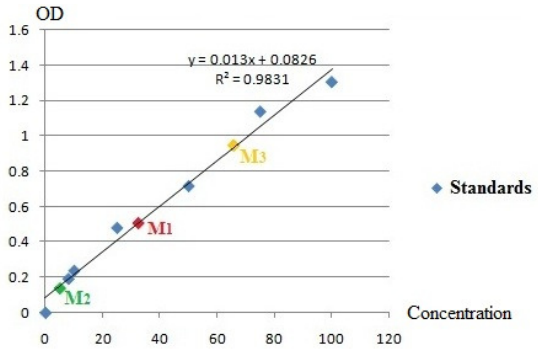

Figure 3. AgB protein concentration measured by three methods using Bradford assay

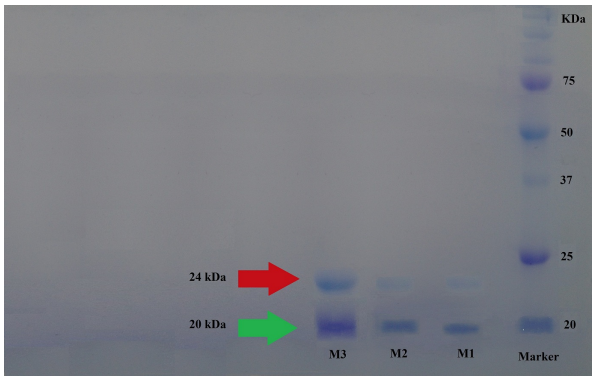

Figure 4. The subunits bands of purified AgB using the first (M1), second (M2), and third (M3) methods by SDS-PAGE assay.

Moreover, specific bands were formed, as presented in Figure 4. SDS-PAGE results showed bands on 20 and 24 KDa subunits of AgB in M1, M2, and M3 solutions, respectively; the M3 bands represented the high concentration of AgB.

DISCUSSION

Hydatidosis, caused by the genus *Echinococcus*, is one of the most important zoonotic diseases with a worldwide distribution. In Iran, 16 isolates of *E. granulosus* have been detected in different animals (Rokni, 2009). This disease is endemic to Middle East and Mediterranean regions such as Turkey, Iraq, Tunisia, and Iran (Eckert et al., 2002). Recent studies have demonstrated the increasing rate of hydatidosis in humans (Hajipirloo et al., 2013). Moreover, the World Health Assembly (WHA) introduced CE as one of the eight neglected zoonotic diseases in 2013 (Mableson et al., 2014). Serological assay is used to confirm radiological diagnosis. Overall, ELISA assay is known as a primary screening test (Moro and Schantz, 2009), and consequently, the used Ag should be specific; it is worth mentioning that AgB is both sensitive and specific, as recommended by WHO. In the first method, we centrifuged and dialyzed HCF twice to make it denser; therefore, AgB could be isolated more successfully in comparison with the second method. In the second method, which was previously adopted by Asghari and Mohammadzadeh, dialysis was performed once and only 0.1 mg/ml of AgB was isolated. However, in the third method, we used PEG 4000 as a thickener for the first time, which produced satisfactory results and AgB could be isolated in higher amounts.

Based on the aforementioned findings, we can conclude that a thickener such as PEG 4000 is more effective than increasing the time of centrifugation and dialysis. Moreover, another advantage of applying PEG 4000 is that it changes HCF (containing a certain amount of protein) into a dense fluid with a greater protein content; therefore, the isolation process could be performed using a lower amount of HCF with more effective results. In the present study, SDS-PAGE was

performed to confirm the accuracy and purity of AgB. AgB is normally used for seroepidemiological survey of hydatidosis in Iran. According to a study by Abdi in 2013, the prevalence of this disease was estimated at 2.25% in Ilam, Iran, based on ELISA assay. Also, use of this Ag for the diagnosis of hydatidosis has been reported in Shiraz, Arak, and some other cities in Iran (Asghari et al., 2013). Considering the high sensitivity and specificity of AgB ELISA assay, prevalent use of this method in seroepidemiological surveys, and its application in research centers, it is important to introduce an optimal method for AgB isolation.

In this study, we aimed to compare the efficacy of three different methods in AgB isolation to propose the most effective method for this purpose. The results showed that AgB concentration in HCF was low and use of PEG 4000 was suggested to concentrate HCF by decreasing the volume of HCF. In this study, the highest concentration of AgB was in result of the third method by PEG 4000.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Asghari, M., Mohebali, M., Kia, E.B., Farahnak, A., Aryaeipour, M., Asadian, S., Rokni, M.B., 2013. Seroepidemiology of Human Hydatidosis Using AgB-ELISA Test in Arak, Central Iran. Iranian journal of public health 42, 391-396.

Barnes, T.S., Deplazes, P ,.Gottstein, B., Jenkins, D.J., Mathis, A., Siles-Lucas, M., Torgerson, P.R., Ziadinov, I., Heath, D.D., 2012. Challenges for diagnosis and control of cystic hydatid disease. Acta tropica 123, 1-7.

Carmena, D., Benito, A., Eraso, E., 2006. Antigens for the immunodiagnosis of Echinococcus granulosus infection: An update. Acta tropica 98, 74-86.

Eckert, J., Gemmell, M., Meslin, F., Powloeski, Z., 2002. WHO/OIE Manual of Echinococcosis in Human and Animal. World Organization for Animal Health, 20-40.

Gillespie, S.H., Hawkey, P.M., 1995. Medical Parasitology: A Practical Approach, IRL Press at Oxford University Press.

Gonzalez-Sapienza, G., Cachau, R.E., 2003. Identification of critical residues of an immunodominant region of Echinococcus granulosus antigen B .The Journal of biological chemistry 278, 20179-20184.

Hajipirloo, H.M., Bozorgomid, A., Alinia, T., Tappeh Kh, H., Mahmodlou, R., 2013. Human cystic echinococcosis in west azerbaijan, northwest iran: a retrospective hospital based survey from 2000 to 200 .٩Iranian journal of parasitology 8, 323-326.

Mableson, H.E., Okello, A., Picozzi, K., Welburn, S.C., 2014. Neglected zoonotic diseases-the long and winding road to advocacy. PLoS neglected tropical diseases 8, e2800.

Mamuti, W., Sako, Y., Nakao, M., Xiao, N., Nakaya, K., Ishikawa, Y., Yamasaki, H., Lightowlers, M.W., Ito, A., 2006. Recent advances in characterization of Echinococcus antigen B. Parasitology international 55 Suppl, S57-62.

Mohammadzadeh, T., Sako, Y., Sadjjadi, S.M., Sarkari, B., Ito, A .٢٠١٢ ,.Comparison of the usefulness of hydatid cyst fluid, native antigen B and recombinant antigen B8/1 for serological diagnosis of cystic echinococcosis. Transactions of the Royal Society of Tropical Medicine and Hygiene 106, 371-375.

Moro, P., Schantz ,P.M., 2009. Echinococcosis: a review. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases 13, 125-133.

Rafiei, A., Jahanshahi, A., Talazadeh, A., 2008. Evaluation of Specific IgG Antibody Detection in Diagnosis and Post Surgical Monitoring of Hydatic Echinococcosis. Iranian Journal Parasitology 3, 10-14.

Rahimi, H., Sadjjadi, S., Sarkari, B., 2011. Performance of antigen B isolated from different hosts and cyst locations in diagnosis of cystic echinococcosis. Iranian journal of parasitology 6, 12-19.

Rigano, R., Profumo, E., Bruschi, F., Carulli, G., Azzara, A., Ioppolo, S., Buttari, B., Ortona, E., Margutti, P., Teggi, A., Siracusano, A., 2001. Modulation of human immune response by Echinococcus granulosus antigen B and its possible role in evading host defenses. Infection and immunity 69, 288-296.

Rokni, M., 2009. Echinococcosis /hydatidosis in Iran. Iranian journal of parasitology 4, 1-16.

Sadjjadi, SM., Abidi, H., Sarkari, B ,.Izadpanah, A., Kazemian, S., 2007. Evaluation 0f Enzyme Linked Immunosorbant Assay, Utilizing Native Antigen B for Serodiagnosis of Human Hydatidosis. Iranian Journal of Immunology 4, 167-172.

Sarkari, B., Sadjjadi, SM., Abidi, H., Izadpanah, A., Kazemian, S ,.Rafiei, A., 2007. Application of Western Blotting Using Native Antigen B for Serodiagnosis of Human Cystic Echinococcosis. Iranian Journal of Parasitology 2, 7-12.

Silva, A., 2011. Hydatid cyst/cystic echinococcosis: anatomical and surgical nomenclature and method to quantify the cyst content solidification. Chinese Medical Journal 124, 2806-2812.

Zhang, W., Li, J., Jones, M.K., Zhang, Z., Zhao, L., Blair, D., McManus, D.P., 2010. The Echinococcus granulosus antigen B gene family comprises at least 10 unique genes in five subclasses which are differentially expressed. PLoS neglected tropical diseases 4, e784.