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.

Farhoudi, R.[1]; Hosseini, S.H.[2]; Siavashi, M.R.[1*]; Abolhassani, M.[3]; Shayan, P.[2] and Nejadmoghadam, A.[3]

[1]Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran. [2]Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. [2]Department of Immunology, Pasteur Institute of Iran, Tehran, Iran.

Key Words:
Echinococcus granulosus; laminated layer; Western blot; monoclonal antibody.

Correspondence
Siavashi, M.R.,
Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran.
Tel: +98(21)66969297
Fax: +98(21)66467132
Email: M_siavashi@hotmail.com

Received: 15 July 2009
Accepted: 12 June 2010

Abstract

Cystic echinococcosis (CE) is an infection caused by the larval stage of *Echinococcus granulosus*. This is widely distributed through Iran, where a variety of animals act as intermediate host. The immunogenic antigens (Ag) of different compartments of the hydatid cyst have been already determined. One of these compartments is the laminated layer (LL). We have extracted a protein with the MW of 24 kDa from a lysate prepared from the LL and produced a monoclonal antibody (mAb) against this protein. Five mAb named P_3F_{6s}, P_2H_{4s}, P_1A_{6s}, P_1C_{3s}, and P_1F_{7s} have been produced. The isotype analysis showed that P_3F_{6s} is IgG_1 and the rest are IgM. P_3F_{6s} was purified from the ascitic fluid of mice injected with P_3F_{6s} hybridoma intraperitoneally. Western blot and ELISA analysis showed that this mAb could recognize the purified 24 kDa prepared from lysate of LL. Since a 24 kDa protein has been shown to be an immunogenic Ag, this protein can be used as a candidate for the development of diagnostic tests and vaccine strategies. For these aims, P_3F_{6s} can be used for the purification of this 24 kDa protein.

Introduction

Cystic echinococcosis (CE) of humans is an infection which is caused by larval stage (the metacestod) of Echinococcus granulosus and may result in a range of illness, from asymptomatic infection to severe disease that may be fatal (Pawlowski, 1997). The detection of space occupying lesions is the common primary approach in the detection of human CE (Caremani *et al*., 1997; Gharbi *et al*., 1981). Diagnosis in humans is usually through different radiographic and other techniques, which will are confirmed by serological tests. Immunodiagnostic procedures for serum antibody detection are used for etiological confirmation of imaging structures suggestive of CE or for the diagnosis or differential diagnosis of uncharacteristic imaging findings (WHO/OIE). The clinical signs and symptoms of hepatic cysts resemble those of hepatic carcinoma, cirrhosis or other liver disease and is not reliable for the diagnosis of CE (Lightowlers and Gottstein, 1995). Accurate immunodiagnosis of the infection requires highly specific and sensitive antigens for immunodiagnostic assays. The choice of an appropriate source of antigenic material is a crucial point in the improvement of the diagnostic features of tests and this must be based on the developmental stage of the parasite and the host. The most common antigenic sources used for the immunodiagnosis of CE are hydatid cyst fluid, somatic extracts and excretory-secretory products from protoscoleces or adults of *E. granulosus* (Carmena *et al*., 2006). Hydatid cyst fluid is the most common component of the parasite utilized in the serological diagnostic tests of human hydatidosis. Various antigenic molecules of cyst fluid have been used in identifying anti-parasite antibodies. The best known are AgB and Ag5 (Lightowlers *et al*., 1998; Shepherd and McManus, 1987). Although AgB and Ag5 have proved to be diagnostically valuable, there are difficulties related to their lack of sensitivity and specificity and problems with the standardization of their use (Babba *et al*., 1994). Somatic extracts have been widely used in the serodiagnosis of *E. granulosus* infection in dogs and ruminant intermediate hosts (Andrade *et al*., 2004), although in the last few years the detection of excretory-secretory products of the worm in feces (coproantigens) have become the most reliable method for the detection of the parasite in the definitive host (Gasser *et al*., 1992). In addition to cyst fluid, other less studied components, such as the laminated layer (LL) due to its acellular nature which is in reach of the immune system, can be of value for immunological investigations. Since the LL of hydatid cysts represents a considerable amount of parasitic material and, due to its extensive quantity and carbohydrate composition, it could also be important in

influencing the immune response in CE (Taherkhani *et al*., 2007). Therefore, we use this hybridoma technique to isolate antibody against 24 kDa molecule of LL for diagnostic purposes against hydatidosis. Recent studies have indicated that glycoproteins associated with parasite antigens are important in inducing a Th2 type immune response in chronic infections (Taherkhani *et al*., 2007). Carbohydrate components have also been implicated in bringing about immunosuppression via macrophages and other accessory cells. The significance of the carbohydrate-rich LL in the immune response to *E. granulosus* is therefore important (Taherkhani *et al*., 2007).

The 24 kDa antigen of LL reacted strongly with serum of cystic hydatid disease (CHD) patients in Western blotting. This antigen appears to have benefit in diagnosis of this disease and act as a target for control strategy. Since monoclonal antibodies (mAbs) are valuable tools for isolating and characterizing epitopes of antigenic molecules and there are no similar studies on the antigens of the LL of *E. granulosus* in Iran, we used this technique for the isolation and characterization of the 24 kDa protein. The goal is to identify this specific antigen in order to use it for immunization and diagnostic purposes against hydatidosis.

Material and Methods

Preparation of laminated layer extract from hydatid cyst

Hydatid cysts were obtained from the livers and lungs of naturally infected sheep slaughtered at the local abattoir (Tehran, Iran). 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 by peeling with forceps and scraping with a scalpel under a dissection microscope. The separated tissue were washed several times in PBS (pH 7.4) and examined by microscopy to confirm the absence of traces of the GL and any protoscolexes. Samples were then incubated in 1 M NaCl at 4°C for 30 min. The freeze/thaw process was performed on LL extracts and then 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) (Taherkhani, 2001). The preparation was then disrupted by sonication in a 150-W ultrasonic disintegrator (10 s on, 5 s off) on ice for 15 min. The main part of the sonicated material (milky suspension) was kept at -20◦C to be used for the next stage (Taherkhani, 2001).

Elution of 24 kDa protein from the SDS-PAGE

The LL suspension was run on 12% PAGE according to Laemmli under reducing conditions (Laemmli, 1970). Briefly, different concentrations of LL suspension (6, 4, 2 and 1 µg) was added to 10 µl of sample buffer containing 3% 2 ME and boiled for 4 min. The preparation was then centrifuged in a microfuge at 1500×g for 5 min and 20 µl was added per lane. Gel was run at 2.5 mA/well and stained with reverse staining (Ortiz *et al*., 1992) and silver staining. A selected protein band (24 kDa) was excised from the gel and crushed with a mortar and pestle, kept in 50 mM ammonium carbonate buffer and then dialysed against PBS at 4°C overnight. Protein was eluted from the gel by an Electro-eluter (Bio-Rad - Model 422) (Castellanos *et al*., 1997).

Production of monoclonal antibodies (mAbs)

Six-week-old female BALB/c mice were immunized intraperitoneally (ip) by 50 µg of 24 kDa antigen of LL in complete Freund's adjuvant (Sigma). Three weeks later, the procedure was repeated with incomplete Freund's adjuvant. Ten days later, mice sera were checked with ELISA; mice with a higher serum Ab titer were selected for fusion. Three days before fusion, the same amount of Ag in saline was injected in the tail and then spleen cells were fused with SP2/0 myeloma cells (in a 5:1 ratio) using a 50% polyethylene glycol (PEG) 4000 (Sigma) (De StGroth and Scheidegger, 1980). Ten to 15 days after fusion, the supernatant fractions from wells containing hybridomas were screened by ELISA. Hybrid cells with antibody production against LL were cloned by limited dilution.

Screening obtained hybridomas with ELISA assay

For mAb screening and isotype determination, indirect ELISA was performed as follows: flat-bottomed 96-well polyvinyl chloride plates (Nunc, Denmark) were coated with 10 µg/ml of LL antigen (100 µl/well) in 0.05 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and left overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T). Blocking was performed with 1% bovine serum albumin (BSA) in PBS (200 µl/well) for 2 h. After three washes with PBS-T hybridoma culture supernatants (100 µl/well) were added and incubated for 1 h. The plates were further washed and bound antibodies were detected after the addition of 100 µl of a commercial horseradish peroxidase conjugated goat anti-mouse IgG (Sigma) for 1 h, and then TMB (Sigma) for 15 min (100 µl/well). The reaction was stopped with 50 µl of 4 NH_2SO_4 and the plates were read at 450 nm. Unless otherwise stated, all procedures were carried out at room temperature (RT). Isotype determination was done by Sigma isotyping kit (Casaravilla *et al*., 2005).

Western blotting

LL extract were separated in 10% SDS-PAGE under reducing conditions, and blotted onto a

nitrocellulose membrane using a semi-dry horizontal electrotransfer system. The efficiency of transfer was verified by membrane staining with a ponceau S Red solution (0.3% W/V in 3% trichloroacetic acid). Blocking was performed with 1% BSA in PBS at 4°C overnight. The strips were then probed with hybridoma culture supernatant for 1 h at RT, and then incubated with horseradish peroxidase conjugated goat anti-mouse IgG/IgM (Sigma) diluted at 1/4000 in 0.1% BSA in PBS-T for 1 h at RT. Peroxidase reaction was visualized with 0.06% (w/v) diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl (pH 7.6) and 0.03% (v/v) H2O2. The reaction was stopped after 5 min with distilled water (Casaravilla *et al*., 2005). After the detection of positive hybridomas (hybridomas that recognized LL 24 kDa antigen), hybridomas that reacted strongly to this antigen were selected and their supernatant was analyzed by Western blotting. For the production of Ab *in vivo*, 0.5 ml pristine (Sigma, USA) was injected to BALB/c mice peritoneum. Four weeks before hybridoma injection hybridoma cells (10^6 cells) were injected into the peritoneum and 10 days after tumor formation, the peritoneal fluid was extracted by syringe, centrifuged and frozen until use.

Affinity purification of antigen

The P_3F_{6s} mAb was purified from ascitic fluid on a protein G-sepharose (Pharmacia, Uppsala, Sweden) following the procedure as recommended by the manufacturer. Purified mAb were coupled to CNBr-activated sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 5 mg of antibody per 1 ml of swelling activated sepharose 4B in 0.25 M sodium bicarbonate buffer (pH 9.0) containing 0.5 M sodium chloride for 2 h at RT. The gel was then reacted with 1 M ethanolamone for 2 h at RT before washing alternately with 0.1 M sodium acetate (pH 4.0) containing 0.5 M sodium chloride and coupling buffer for 4 cycles. The washed gel was then stored in 0.15 M PBS (pH 7.2) containing 0.1% sodium azide at 4°C. Culture medium supernatants and LL antigen were dialyzed against Tris buffer with 0.5 M NaCl (pH 8.6) and passed through the affinity column separately at a flow rate of approximately 15 ml/h. The majority of unbound materials and non-specifically bound antigens were washed out of the column by 50 mM Tris-HCl with 0.5M NaCl (pH 8.6). The bound antigen was eluted with 3 M sodium thiocyanate, dialyzed against 10 mM Tris-HCl with 0.1 M NaCl pH 7.5 and kept at -20°C (Khabiri *et al*., 2005). Purified monoclonal antibody (P_3F_{6s}) was analyzed by Western blotting. SDS-PAGE with affinity purified LL 24 kDa antigen and immunoblotting of this antigen with purified monoclonal antibody (P_3F_{6s}) were carried out as described previously.

Affinity determination

Measurement of mAb affinity was performed using ELISA based on the protocols of Beatty *et al*. and Hajighasemi *et al*. (Beatty *et al*., 1987; Hajighasemi *et al*., 2004). Measurements were based upon the Law of Mass Action, which determines the binding strength between a mAb and its immobilized antigen by comparing the OD 50 of two sigmoid curves of antibody serial dilution on plates coated with two different antigen concentrations. All reactions were performed in a sealed microtiter plate (Nunc, Denmark) with reaction volumes of 0.2 ml. Plate washing was performed three times after each incubation with PBS (0.15 M, pH 7.2) containing 0.05% Tween 20 (PBS/T) for 1.5 hr at 37°C. Optical density (OD) was measured by a multiscan ELISA reader at 450 nm.

Results

Isolation of 24 kDa protein of LL

The crude extract of sheep laminated layer was electrophoresed and, after silver staining, the band of 24 kDa was clear (Figure 1). To isolate 24 kDa protein, a preparative gel was run and the gel was stained with reverse staining. A 24 kDa protein was eluted from that section of gel. The eluted protein was run again on SDS-PAGE and only 24 kDa protein was detected with Coomassie blue staining (Figure 2).

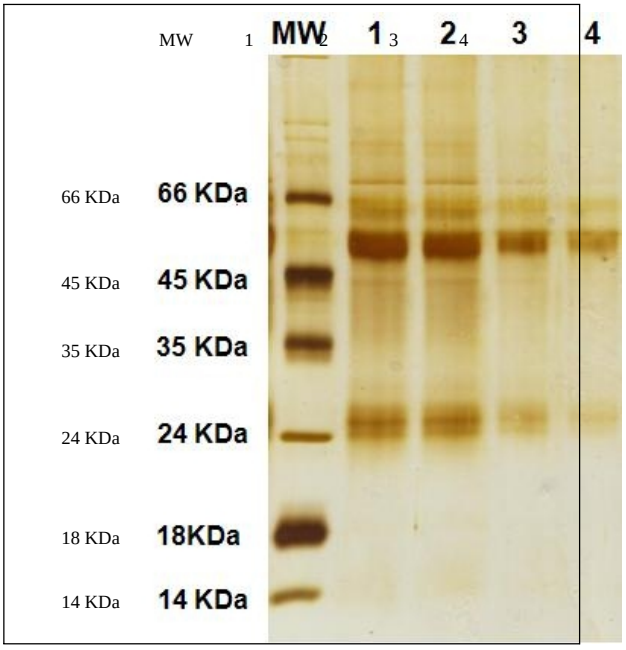


Figure 1: SDS-PAGE analysis of different concentrations of laminated layer suspension stained with silver. Lanes: 1 to 4 represent 6, 4, 2 and 1 µg LL suspension, respectively.

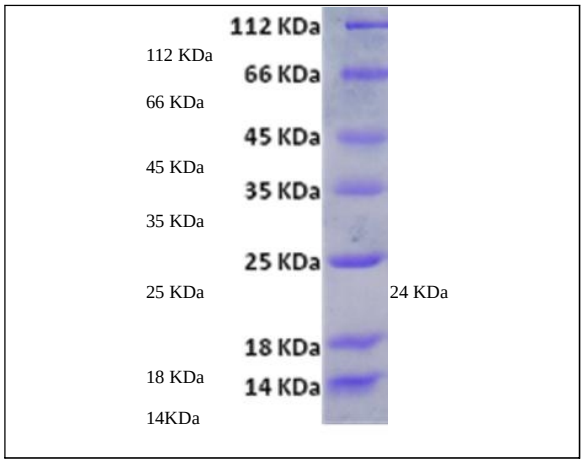
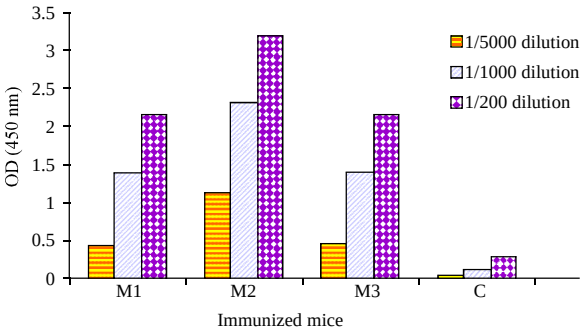


Figure 2: SDS-PAGE profile of eluted 24 kDa protein. Protein was removed by gel elution and confirmed 24 kDa protein by 12% SDS-PAGE and Coomassie staining.

Figure 3: Antibody titer of three mice immunized with 24 kDa antigen of LL. Mice were bled from the eye and sera were checked against 24 kDa protein by ELISA. The spleen cells of mouse M_2 was used for fusion. M_1: mouse (1); M_2: mouse (2); M_3: mouse (3); C: control



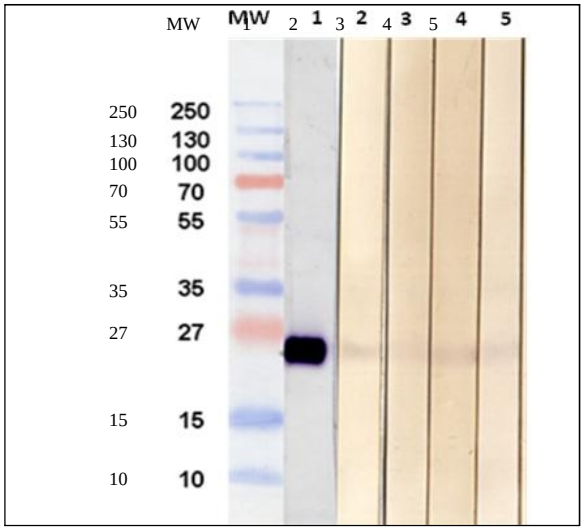


Figure 4: Immunoblotting of LL antigens probed with monoclonal antibodies. Detection of 24 kDa protein by immunoblotting using different mAbs; Lanes: (1) P_3F_{6s}; (2) P_2H_{4s}; (3) P_1A_{6s}; (4) P_1C_{3s}; and (5) P_1F_{7s}. MW: molecular weight. The 24 kDa protein is arrowed.

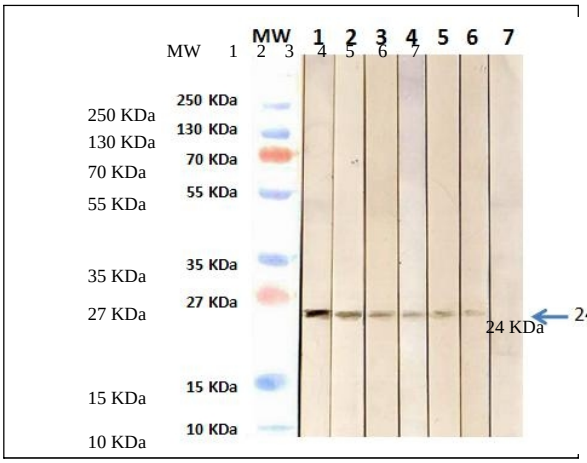


Figure 5: Western blot analysis of P_3F_{6s} monoclonal Ab against different concentrations of 24 kDa antigen of LL. After 12% gel electrophoresis, the gel was blotted onto nitrocellulose and incubated with P_3F_{6s} culture supernatant. Lanes 1-7 represent 4, 2, 1, 0.1, 0.01, 0.001 and 0.0001 µg of 24 kDa protein. The Ab could detect approximately 1 ng of 24 kDa protein. The 24 kDa protein is arrowed. MW: molecular weight.

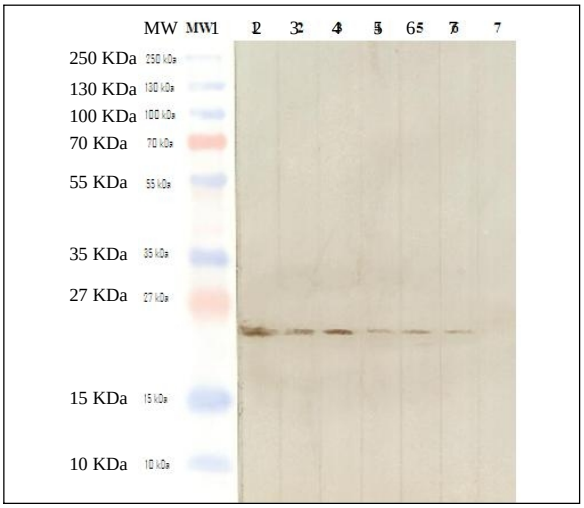


Figure 6: Western blot analysis of ascites fluid containing monoclonal antibody against different concentrations of 24 kDa antigen of LL. Lanes 1-7 represent 2, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µg of 24 kDa protein. The Ab could detect approximately 0.1 ng of 24 kDa protein. The 24 kDa protein is arrowed. MW: molecular weight.

Monoclonal antibody production

Sera of immunized mice were used to test the antibody against 24 kDa antigen of LL by ELISA (Figure 3). Fusion of SP2/0 myeloma cells with splenic cells of BALB/c mice immunized with 24 kDa antigen of LL of hydatid cysts yielded a series of hybridomas. Only five of these, designated as P_3F_{6s}, P_2H_{4s}, P_1A_{6s}, P_1C_{3s} and P1F7s, recognized the 24 kDa antigen of LL in a Western blot assay (Figure 4). P_3F_{6s} secreted IgG_1 and the others produced IgM. P_3F_{6s} hybridoma cells supernatant was analyzed by Western blots using different concentrations of Ag (Figure 5). P_3F_{6s} hybridoma cells were injected ip into BALB/c mice

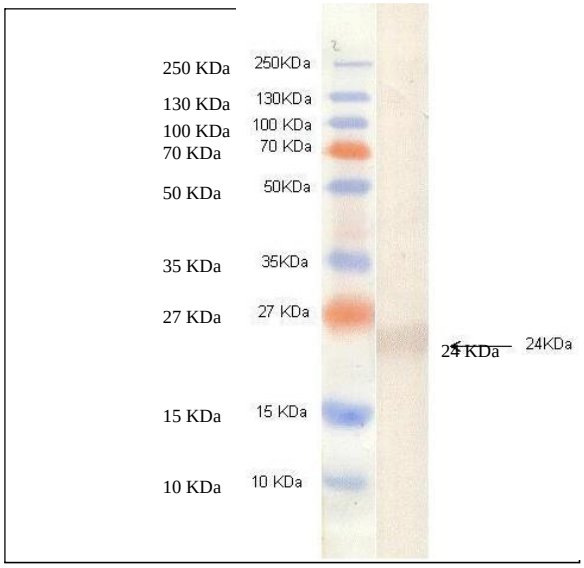


Figure 7: Immunoblotting result of the LL 24kDa antigen purified by protein G column using P_3F_{6s} mAb. SDS-PAGE was performed on 12% gel under reducing conditions.

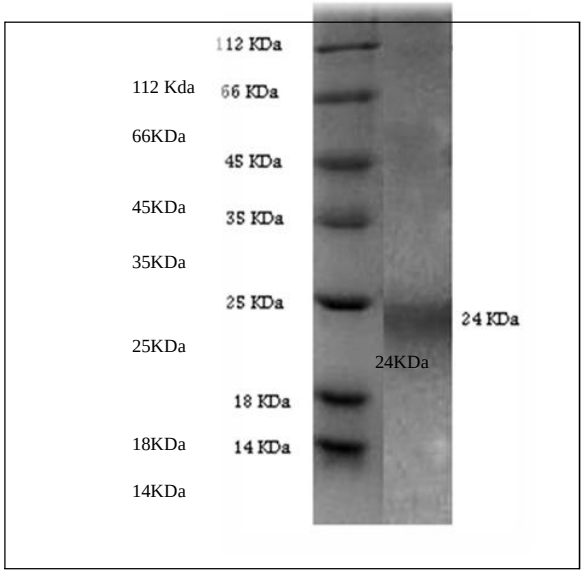


Figure 8: SDS-PAGE of affinity-purified LL 24 kDa antigen.

and the ascitic fluids of tumor-induced mice were analyzed by Western blots using different concentrations of Ag (Figure 6). After purification of the P_3F_{6s} mAb from ascitic fluid, the purified 24 kDa LL antigen was produced on a protein G-sepharose Western blot (Figure 7). The LL 24 kDa antigen was purified by affinity purification and its integrity and molecular weight confirmed by SDS-PAGE (Figure 8). ELISA plates were coated with 5 or 10 µg/ml of purified 24 kDa antigen of LL and ELISA test with different concentration (1, 5, 10, 20, 50 and 100 nM) of purified mAb (P_3F_{6s}) were performed. The binding percentage diagram according to our results was drawn. Then, the affinity constant (Kaff) of the antibody with regards to the formula below was calculated:

$K_{aff} = n\text{-}1/2(n[Ab^{'}]\text{-}[Ab]$

N = ratio of two antigen concentration; Ab = antibody concentration in nM scale.

$K_{aff} = 2\text{-}1/\ 2(1[11.28\times 10^{-9}] - [8.71\times 10^{-9}]) = 1.5\ .14\times 10^{-9} = 0.19 \times 10^{9} \times Mol^{-1}$

Discussion

In the present study, we raised several mAb against a 24 kDa antigen of LL. One antibody was of the IgG1 subclass and four were of the IgM class. These mAb isotypes could recognize 24 kDa Ag in both ELISA and Western blot analyses. These isotypes, and P_3F_{6s} in particular, were purified from ascitic fluid and were used to purify the 24 kDa antigen from crude antigens of LL. The 24 kDa Ag is important in diagnostic process of human hydatidosis since it is presented by the LL of the hydatid cyst (outer layer) and could influence the immune response in CE. In contrast to *E. multilocularis*, until recent years, there was no accurate report on the LL antigens of *E. granulosus* by SDS-PAGE or immunoblotting. In a limited study, only the common antigens present on the GL, LL and protoscolex of *E. granulosus* by the double diffusion test and immunoelectrophoresis has been reported (Capron *et al*., 1967; Valera and Torres, 1998). In these studies, the membrane was used as a whole, and there was no evidence of antigenic or immunogenic properties of the LL (Taherkhani and Rogan, 2000). Taherkhani (Taherkhani, 2001; Taherkhani, 1998) used SDS-PAGE, immunoblotting and mAb against hydatid cyst components, and studied the antigens on the LL of cysts in various hosts, including sheep, human, horse, mouse and gerbils. The antigens of protoscolex extract, cyst fluid and sheep serum were also studied. Electrophoretic analysis of the LL of sheep hydatid cyst extract showed MW from 13 kDa to 250 kDa. The electrophoresis of LL extracts obtained from various hosts that showed different polypeptide bands, could be from common or host molecules. Interestingly, electrophoretic bands of the LL obtained from sheep compared to other hosts under study were more numerous and stronger, which was assumed to be the result of *E. granulosus* intraspecies differences, parasite genomes and host-parasite interactions. In order to differentiate between LL antigens and other parasitic elements (protoscolex, cyst fluid and sheep serum antigens), Lightowlers and Gottstein (Lightowlers and Gottstein, 1995), used SDS-PAGE analysis and found specific Ab against the LL. Both 27 kDa and 20 kDa bands were seen in the LL, protoscolex and cyst fluid, and it is believed that their origins are the same (Taherkhani and Rogan, 2000). Gottestein used immune depletion method to isolate a semi pure antigen from the laminated layer of an alveolar cyst (an immature stage of

E. multilocularis parasite). This antigen became the base for detecting alveolar echinococcosis in Em_2 ELISA (Gottstein, 1985; Gottstein *et al*., 1983). Sero-epidemiologic studies in Alaska showed that Em_2 ELISA not only diagnosed asymptomatic cases that are not detectable by other serological methods, but it is also possible to identify the lesion caused at the initial stages by metacestode (Lightowlers and Gottstein, 1995). The 24 kDa antigen obtained in this report by mAb could have the similar effects in *E. granulosus* because it has a high immunogenicity and is detectable in low titers of antibody showing significant reaction with sera of hydatid cyst patients after immunoblotting (unpublished data).

Siavashi *et al*. compared extracts of crude protoscolex and LL for the identification of various electrophoretic bands with SDS-PAGE (unpublished data). Probes of antibodies against host immunoglobulins were used for immunoblotting. Besides the variety of molecules and their differences, the intensity of protein-anti-sera reactions varied greatly. Some peptides reacted strongly against one serum, whereas the same protein reacted weakly with another serum. One explanation for this is the slow growth rate of cysts in the host body (Thompson, 2001) which could take several years (Pawlowsky *et al*., 2001; Thompson and McManus, 2001); this rate is influenced by parasitic species, hosts, and the extent of infections (Thompson, 2001). Therefore, considering the long period of cyst growth, it is evident that cysts in different stages elicit a variety of immune responses. Thus, the differences seen in the results of serum immunoblotting can be explained.

According to the results of our study, a 24 kDa protein antigen of the hydatid cyst laminated layer is a unique protein extracted only from this layer and represents a new protein for future studies of the immunization of livestock against hydatidosis. It may also be useful as a highly sensitive and specific diagnostic test for human cystic echinococcosis.

Acknowledgments

This project was supported with a grant from the Pasteur Institute of Iran and the Immunopathology Central Excellence of the University of Tehran.

References

1. Pawlowski, Z. (1997) Terminology related to Echinococcus and echinococcosis. Acta. Trop. 67: 1-5.
2. Caremani, M.; Benci, A.; Maestrini, R.; Accorci, A.; Caremani, D. and Lapini, L. (1997) Ultrasound imaging in cystic echinococcosis. Proposal of a new sonographic classification. Acta. Trop. 67: 92-105
3. Gharbi, H.A.; Hassine, W.; Brauner, M.W. and Dupuch, K. (1981) Ultrasound examination of hydatid liver. Radiology 139: 459-463.
4. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. www.oie.int.
5. Lightowlers, M.W.; Gottstein, B. (1995) Echinococcosis/hydatidosis: antigen, immunological and molecular diagnosis. pp. 355-410. In, The biology of *Echinococcus* and hydatid disease. R.C.A. Thompson and A.J. Lymbery (Ed.), CAB International, Wallingford, United Kingdom.
6. Carmena, D.; Benito, A. and Eraso, E. (2006) Antigens for the immunodiagnosis of Echinococcus granulosus infection. Acta. Trop. 98: 74-86.
7. Lightowlers, M.W.; Liu, D.; Haralambus, A. (1998) Subunit composition and specificity of the major cyst fluid antigens of Echinococcus granulosus. Mol. Biochem. Parasitol. 37: 171-182.
8. Shepherd, J.C.; McManus, D.P. (1987) Specific and cross–reactive antigens of *Echinococcus granulosus* hydatid cyst fluid. Mol. Biochem. Parasitol. 25: 143-154.
9. Babba, H.; Messedi, A.; Masmoudi, S.; Zribi, M.; Grillot, R.; Ambriose- Thomas, P.; Beyrouti, I. and Sahnoun, Y. (1994) Diagnosis of human hydatidosis: comparison between imagery and six serologic techniques. Am. J. Trop. Med. Hyg. 50: 64-68.
10. Andrade, M.A.; Siles-Lucas, M.; Espinosa, E.; Arellano, J.L.P.; Gottstein, B. and Muro, A. (2004) *Echinococcus multilocularis* laminated layer components and the E14t 14-3-3 recombinant protein decrease NO production by activated rat macrophage *in vitro*. *Nitric Oxide* 10: 150-155.
11. Gasser, R.B.; Jenkins, D.J.; Health, D.D. and Lawrence, S.B. (1992) Use of *Echinococcus granulosus* worm antigens for immunodiagnosis of *Echinococcus granulosus* infection in dogs. Vet. Parasitol. 45: 89-100.
12. Taherkhani, H.; Zeyhle, E. and Rogan, M.T. (2007) Antibody responses in human cystic hydatid disease to the laminated layer of *Echinococcus granulosus*. Parasitol. Res. 101: 647-52.
13. Taherkhani, H. (2001) Analysis of Echinococcus granulosus Laminated Layer Carbohydrates by Lectin Blotting. Iran. Biomed. J. 5: 47-51.
14. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
15. Ortiz, M.L.; Calero, M.; Patron, C.F.; Castellanos, L. and endez, E. (1992) Imidazole-SDS-Zn reverse staining of proteins in gels containing or not SDS and microsequence of individual unmodified electroblotted proteins. FEBS Lett. 296: 300-4.
16. Castellanos, LR.; Patron, C.; Hardy, E.; Santana,

H. and Huerta,V. (1997) High Yield Elution of Proteins from Sodium Dodecyl Sulfate Polyacrylamide Gels at the Low-Picomole Level. Application to N-Terminal Sequencing of a Scarce Protein and to In-Solution Biological Activity Analysis of On-Gel Renatured Proteins. J. Protein Chem. 16: 415-19.
17. De StGroth, S.F.; Scheidegger, D. (1980) Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods 35: 1-21.
18. Casaravilla, C.; Malgor, R.; Rossi, A.; Sakai, H.; Nonaka, N.; Kamiya, M. and Carmona, C. (2005) Production and characterization of monoclonal antibodies against excretory/secretory product of adult *Echinococcus granulosus*, and their application to coproantigen detection. Parasitol. Int. 54: 43-49.
19. Khabiri, A.K.; Bagheri, F.; Alimohammadian, M.H.; Assmar, M. and Nadaf, S.R. (2005) Leishmanin skin test in guinea pig with a single purified protein of *Leishmania major*. Exp. Parasitol. 111: 239-243.
20. Beatty, J.D.; Beatty, B.G. and Vlahos, W.G. (1987) Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. J. Immunol. Meth. 100: 173-179.
21. Hajighasemi, F.; Saboor-Yaraghi, A. and Shokri, F. (2004) Measurement of Affinity Constant of Anti–human IgG Monoclonal Antibody by an ELISA- based Method. Irn. J. Immunol. 1: 1-8.
22. Capron, A.; Vernes, A. and Biguet, J. (1967) Le diagnostic immune electrophoretique de l ´hydatidose. In: Le Kyste Hydatique du Foie. Simep, Lyon, pp: 27-40.
23. Valera, V.M.; Torres, J.M. (1998) Antigenic characterization of *Echinococcus granulosus* cysts. Boll Ist Sieroter Milan. 56: 303-309.
24. Taherkhani, H.; Rogan, M.T. (2000) General characterization of laminated layer of *Echinococcus granulosus*. Irn. J. Med. Sci. 25: 95-104.
25. Taherkhani, H. (1998) Studies on the antigenicity of laminated layer of *Echinococcus granulosus*. PhD thesis, University of Salford, UK.
26. Gottstein, B. (1985) Purification and characterization of a specific antigen from *Ecchinoccocus multilocularis*. Parasitol Immunol.7: 201-212.
27. Gottstein, B.; Eckert, J. and Fey, H. (1983) Serological differentiation between *Echinococcus granulosus* and *Ecchinoccocus multilocularis* infections in man. Z. Parasitenkd. 69: 347-356.
28. Thompson, R.C.A. (2001) Echinococcosis. In: Principle and practice of clinical parasitology. Gillespie S.H. & Pearson R.D. John Willey & Sons Ltd.
29. Pawlowsky, Z.S.; Eckert, J. and Vuitton, D.A. (2001) Echinococcosis in human: clinical aspects, diagnosis and treatment. In: WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. Eckert J. (Ed) pp: 20-71.
30. Thompson, R.C.A.; McManus, D.P. (2001) Aetiology: parasites and life- cycles. In, WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. Eckert J. (Eds), pp: 1-19.