

A NOVEL METHOD OF PURIFICATION OF SPECIFIC HYDATID CYST ANTIGEN

Z. ZAMANI*, Ph.D., M. ASSMAR, Ph.D., N. PYAZAK, Ph.D.,
K. WAFATIE, M. ASSADIAN, AND A. AMIRKHANI, Ph.D.

From the Departments of Parasitology and Biochemistry, Pasteur Institute of Iran, Tehran, I.R. Iran.

ABSTRACT

Hydatid cyst fluid has been used as a source of antigen for the serodiagnosis of *Echinococcus granulosus* infection. Due to cross-reactions with antigens shared by other helminthes, the specific antigen from hydatid cyst fluid was purified by many workers. We used a relatively simple technique for purification of specific antigen from sheep hydatid cyst fluid. Isopycnic ultracentrifugation with 30% KBr was used followed by SDS-PAGE to check the purity of the antigen. The antigen was of 48 kDa and used in ELISA and IHA with high sensitivity and specificity.

MJIRI, Vol. 15, No. 1, 49-53, 2001.

Keywords: *Echinococcus granulosus*, specific antigen, isopycnic ultracentrifugation.

INTRODUCTION

Echinococcosis is caused by the larval stage of various cestode species of the genus *Echinococcus spp.*^{1,2} Two of these parasite species are widely prevalent and may cause severe disease in humans: *E. granulosus*, the causative agent of cystic echinococcosis and *E. multilocularis* which may proceed to alveolar echinococcosis.³ As diagnosis by clinical symptoms and scanning alone is often difficult and confusing, a battery of serological tests are required to corroborate the evidence reached.^{4,5,6} The cyst fluid has been used as a source of antigen for serodiagnosis, but due to cross-reactivity with antigens shared by other helminthes^{7,8} the serological results have not been very reliable.

Many attempts have been made to purify the specific antigen from hydatid cyst fluid.⁹ In 1971, Oriol used affinity chromatography and gel filtration to purify 2 major antigens 4 and 5.¹⁰ In 1977 Piantelli et al. reported that antigen 4 was comprised of two subunits of 67 kDa and antigen 5 of 3 subunits below 20 kDa.¹¹ In 1989, Fadwa et al. using hydrophobic gel chromatography, anion exchange and gel filtration chromatography purified two glycoproteins of 20 kDa and 48 kDa.¹² As purification of specific antigens of *E. granulosus* involved a

number of steps with different types of chromatography, we decided to use a novel and simple method for its purification to increase the sensitivity and specificity of the serodiagnostic tests in use.

MATERIAL AND METHODS

Preparation of crude antigen

Sheep hydatid cyst fluid was collected from lung and liver cysts from infected sheep at a slaughterhouse (Tehran, Iran). The fluid from the organs was pooled and filtered through 0.45 μm and 0.22 μm filters (Millipore) successively and stored at -70°C .

Hydatid sera

Fifty-three serum samples from surgically proven cases of *E. granulosus* and fifty-three serum samples from healthy Iranian students as controls were used. Seven serum samples from patients with high titers of fascioliasis and seven samples from toxoplasmosis patients were used for cross-reactivity studies. All these heterologous sera had high titers as determined by enzyme-linked immunosorbent assay (ELISA).²¹

Purification of antigen

Isopycnic ultracentrifugation¹⁵ was carried out on 200 mL of filtered sheep hydatid fluid containing 660 μg /mL of protein as estimated by Lowry's method.¹⁶ This

*Correspondence: Dr. M. Assmar, Biochemistry Dept., Pasteur Institute of Iran, Tehran, I.R. Iran. Tel: 6468761-4 Fax: 6465132

Hydatid Cyst Antigen Purification

Table I. Specificity and sensitivity of ELISA using specific antigen against hydatid cyst.

Type of test	Patient serum				Normal serum				Specificity	Sensitivity
	Total No.	No. Pos.	No. Neg.	%Pos.	Total No.	No. Neg.	No. Pos.	% Pos		
IFA	35	32	3	91.4	53	49	4	92.5	92.5	91.4
ELISA	35	34	1	97.1	53	52	1	98.1	98.1	97.1
IHA	35	32	3	91.4	53	50	3	94.3	91.4	94.3

was mixed with 30% potassium bromide (KBr) and ultracentrifugation was carried out in a Beckman centrifuge (XL-90) at 100,000g for 20h at 10°C. 10 mL fractions were collected and numbered from higher density to lower density and the protein content of each was estimated at 280 nm by a spectrophotometer (Perkin-Elmer 5505). The antigen content was estimated by counter current immunoelectrophoresis (CCIE).¹⁷ Fractions containing the antigen were mixed together and isopycnic ultracentrifugation carried out as described before. Fractions were collected and tested as detailed and the ones containing the antigen were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).¹⁸

Counter current immunoelectrophoresis (CCIE)

CCIE was performed with 1% agarose prepared in veronal acetate buffer, pH 8.2. Staining was done in 0.5%

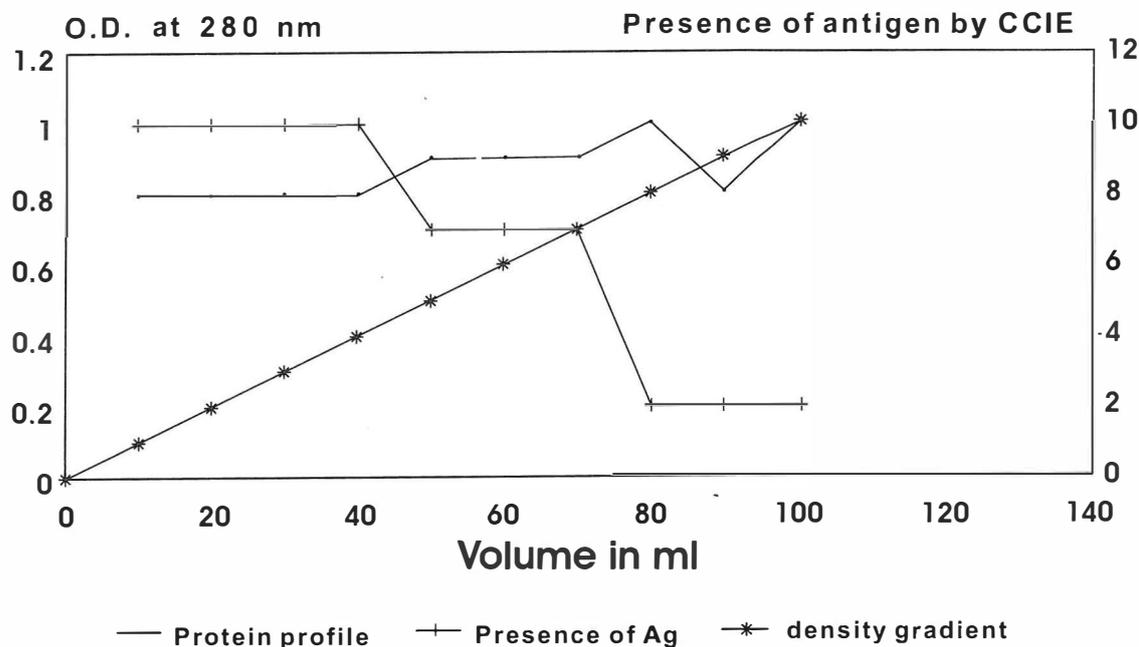
amido black.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out under reducing conditions by the method of Laemmli,¹⁸ with 2.5% gel for stacking and 10% resolving gel with Tris-HCl in a discontinuous buffer system using standard molecular weight markers. The run was performed in Tris-glycine pH 8.3 with 250 V for 4h. The gel was fixed and proteins detected by silver¹⁹ and glycoproteins by the method of periodic acid Schiff's staining.²⁰

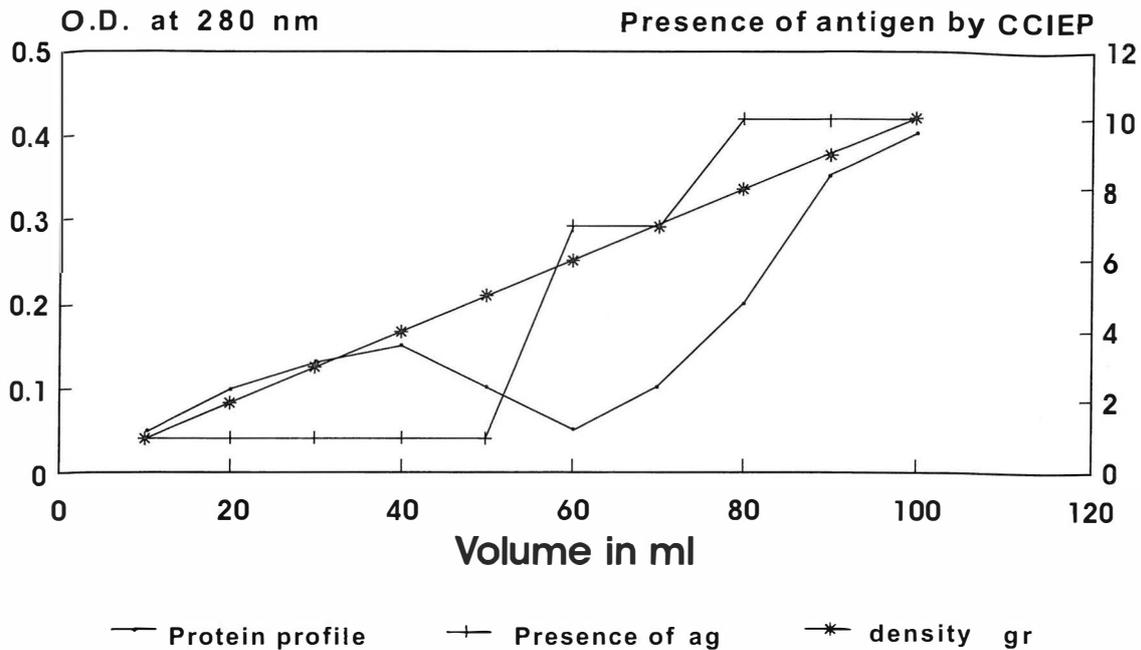
Enzyme linked immunosorbent assay

ELISA was done by the method of Hira et al.²¹ 5.0 and 2.5 µg/mL of purified antigen was coated overnight at 4°C at the bottom of a microtiter polyacetylene plate (NUNC) in 0.5 M carbonate-bicarbonate buffer, pH 9.6. After washing with PBS (pH 7.2) containing tween 20,



Presence of ag. 0-2-ve,4-6+ve,8-10+++ve

Fig. 1. First step of KBr purification of specific antigen of *E. granulosus*.



Presence of ag., 0-2-ve,4-6+ve,8-10+++ve

Fig. 2. Second step of KBr purification of specific antigen of *E. granulosus*.

100 μ L of patients' and normal sera diluted to 1:101 and 1:201 were poured in the coated wells and incubated at 37°C for 1h. 100 μ L peroxidase conjugated anti-human IgG was poured after washing the wells and then substrate was added and absorbance readings taken at 492 nm. Cutoff was calculated by addition of negative mean with standard deviations of negative means. Positives were those whose absorbance was more than 10% above cutoff value.

Indirect hemagglutination test (IHA)

19 mL of 1: 20,000 tannic acid was added to 1 mL packed Alsevier's sheep red blood cells (SRBC). After washing with PBS, to one volume of packed SRBC, five times the volume of optimized antigen and 4 mL PBS was added and incubated at RT for 30 min. Red cells were washed with 1% normal rabbit serum (NRS) and then a suspension of 2.0-5% SRBC was prepared in 1% NRS. In each V-shaped microtiter plate well 25 μ L inactivated patient's serum and 25 μ L cell suspension were added. Results were seen after incubation for 2h at RT.²³

RESULTS

Purification of the antigen

After the first ultracentrifuge it was seen that the last four fractions with the lightest density contained the specific antigen of hydatid cyst as detected by CCIE (Fig.

1). The ultracentrifuge was repeated once more and the last four fractions with the least density were seen to contain the antigen required by CCIE (Fig. 2). The fractions were pooled and purity of the antigen checked by SDS-PAGE. Two bands of approximately 48 kDa and 35 kDa were detected by silver staining method (Fig. 3). The antigen was a glycoprotein as shown by PAS staining. This antigen was deemed as a specific antigen for hydatid cyst and used in ELISA for detection of hydatid antigen in patient antiserum.

From 35 patient serum samples 34 were proven positive and 52 out of 53 samples of controls turned out to be negative (Table I). No cross-reactivity was seen with fasciola, *Treponema pallidum*, *Entamoeba coli*, *Giardia lamblia* or toxoplasma positive samples. As can be seen, specificity of the antigen in ELISA is 98.1% and sensitivity is 97.1% (Table I).

The pure antigen was used for indirect hemagglutination test on 35 patients' sera out of which 32 patients were proven positive and out of 53 controls 50 were negative (Table II). Hence, the sensitivity of this test is 91.4% and the specificity is 94.3%.

DISCUSSION

Using a relatively simple technique of two-step isopycnic ultracentrifugation, the specific antigen for hydatid cyst was purified.¹⁵ The antigen contained two bands of

Hydatid Cyst Antigen Purification

48 kDa and 35 kDa as detected by silver staining method.¹⁹ The antigens were glycoproteins, the 48 kDa band was proven to be specific for hydatid cyst, and the 35 kDa antigen was a minor impurity. Fadwa et al.⁸ using a three step chromatography system with different ion exchange, hydrophobic and gel filtration column purified two antigens of 48 kDa and 20 kDa. According to Fadwa⁸ the 48 kDa antigen is a glycoprotein and shows 33% cross-reactivity with sera of patients infected with *E. multilocularis*. It is quite likely that our antigen is similar to the one purified by Fadwa et al, considering its nature and molecular weight.

For ELISA, a comparison was carried out between total hydatid cyst fluid and purified antigen as coating antigen on the plate. It was seen that, whereas for the cyst fluid about 20 µg/mL was used, the pure antigen required only 5 µg/mL for detection of antibody in patients' sera. Specificity of ELISA by total fluid is 96% and its sensitivity is 81.1%, while specificity and sensitivity of pure antigen were 98.1% and 97.1% respectively. Hira et al.²¹ used antigen 5, and by using 5 µg/mL as coating antigen in ELISA showed 98.28% and 98.18% specificity and sensitivity.

For IHA, sensitized sheep red cells were coated with purified antigen and out of 35 patients' antisera 32 were positive. Out of 53 healthy controls, 50 were negative; hence, sensitivity and specificity were 91.4% and 94.3% respectively. Hira et al.²² using arc 5 to coat sensitized sheep red blood cells achieved 96.36% sensitivity and 93.1% specificity for IHA. Njeruh et al.²³ used a lipoprotein heat resistant partially purified antigen and achieved 88% sensitivity showing no cross-reactivity to other parasitic and bacterial antigens.

Recently, concavalin A affinity chromatography was used for purification of a 42 kDa band;¹⁴ in fact, the authors have claimed that purification procedures could

affect the diagnostic value of antigens with identical behavior in SDS-PAGE. The sensitivity of the 42 kDa band was 95% for *E. granulosus* and 100% specific for hydatidosis. The 8 kDa band accepted recently for diagnosis by WHO²³ was 91% specific for *E. granulosus* and 100% for hydatidosis. The 48 kDa antigen purified in this study proved to be 98.1% specific and 97.1% sensitive by ELISA for *Echinococcus granulosus* and 100% specific for hydatidosis. It can be concluded that the antigen can be used with high sensitivity for ELISA and IHA test with little probability of cross-reactivity with other parasites.

REFERENCES

1. Craig P: Tapeworms (Cestodes). In: Manson-Bahr PEC, Bell DR, (eds.), Manson's Tropical Diseases. London: Balliere-Tindall, pp. 541-552, 1987.
2. Thompson RCA, Lumbray AJ, Constanine CC: Variations in echinococcus towards a taxonomic revision of the genus. Adv Parasitol 35: 145-176, 1995.
3. Thompson RCA, Eckert J: Observations on *Echinococcus multilocularis* in the definitive host. Zeitschrift fur Parasitenkunde 69: 335-345, 1983.
4. Bhatia BB, Pathak CML: Echinococcus. In: Parija SC, (ed.), Review of Parasitic Zoonoses, New Delhi: AITBS Publishers and distributors, pp. 268-280, 1990.
5. Gottstein B: An immunoassay for circulating antigens in human echinococcus. Am J Trop Med Hyg 33: 1105-1111, 1984.
6. Parija SC, Mishra SR, Rao RS: Sensitized chick cells and indirect haemagglutination test for echinococcus. J Med Microbiol 22: 237-239, 1986.
7. Scharzt PM, Shanks D, Wilson M: Serological cross-reactions with sera from patients with echinococcosis and cysticercosis. Am J Trop Med Hyg 29: 609-612, 1980.
8. Fadwa M, Al Yaman A, Knobloch M: Isolation and partial characterization of species specific and cross reactive antigens of *Echinococcus granulosus* cyst fluid. J Mol Biochem 37: 101-108, 1989.
9. Pozzuli R, Musiani P, Arru E, Piantelli M, Mazzarella M: *Echinococcus granulosus*: Isolation and characterization of sheep hydatid fluid antigens. Exp Parasitol 32, 1: 45-55, 1972.
10. Piantelli M, Pozzuli R, Arru E, Oriol C: *Echinococcus granulosus*: identification of subunits of major antigens. J Immunol 119 (4): 1382-1386, 1971.
11. Musiani P, Piantelli M, Lauriola L, Arru E, Pozzuli R: *Echinococcus granulosus*: identification of subunits of the major antigens. J Clin Pathol 31: 475-478, 1978.
12. Oriol R, Williams JF, Perez Esfandi MV, Oriol CP: Purification of lipoprotein antigens of *Echinococcus granulosus* from sheep hydatid fluid. Am J Trop Med Hyg 20 (4): 596, 1971.

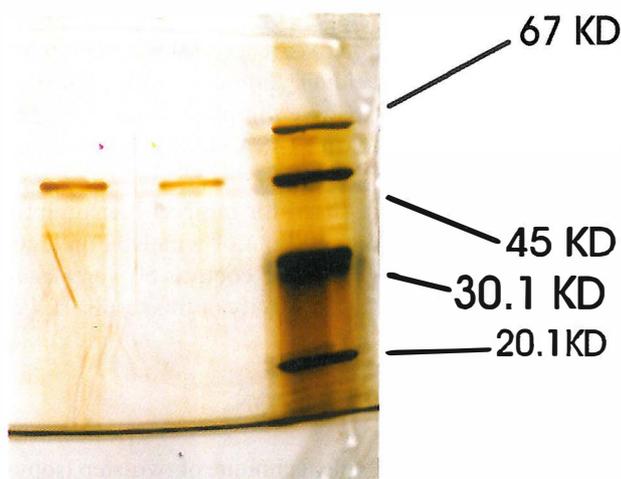


Fig. 3. SDS PAGE of specific hydatid cyst antigen.

13. Maddison SE, Selemenda SB, Schantz PM, Freid JA, et al: A specific diagnostic antigen of *E. granulosus* with an apparent molecular weight of 8 kDa. *Am J Trop Med Hyg* 40 (4): 377-383, 1989.
14. Shibhu Y, Janssen D, Osuna A: Serological recognition of hydatid cyst antigens using different purification methods. *Diagnostic Microb Inf Dis* 24 (4): 205-211, 1996.
15. Tao Qimin T, Wang J, Baifeng F, et al: Further research on hepatitis B vaccine. *Chinese Medical Journal* 94 (5): 329-332, 1981.
16. Lowry OH, Rosenburgh NJ, Farr LA, Randall R: Protein measurement with folin reagent. *J Biol Chem* 193: 265-275, 1951.
17. Johnstone A, Thorpe R: *Immunochemistry in Practice*. London: Blackwell Scientific Publications, pp. 145-146, 1987.
18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 259: 680-8, 1970.
19. Blum H, Bier H, Gros H: Improved staining of plant proteins, RNA and DNA in polyacrylamide gel electrophoresis. *Electrophoresis* 8: 83-90, 1987.
20. Fairbanks G, Steck TL, Wallach DLH: Method to detect glycoproteins in gels. *Biochem* 10: 2606, 1971.
21. Hira PR, Bahr GM, Shweiki HM, Behbahani K: An enzyme linked immunosorbent assay using an arc 5 antigen for diagnosis of cystic hydatid disease. *Ann Trop Med Hyg* 36 (3): 157-162, 1990.
22. Hira PR, Bahr GM, Shweiki JM, Behbahani K: Counterimmunoelectrophoresis using an arc 5 antigen for rapid diagnosis of hydatidosis and comparison with indirect haemagglutination. *Am J Trop Med Hyg* 36 (3): 562-567, 1990.
23. Njeruh FM, Gauthama JM, Okelo GB, Tumboh Orei AG: Diagnosis of human hydatid disease in surgically confirmed cases by the use of indirect haemagglutination test based on a thermostable lipoprotein and an unfractionated hydatid cyst fluid. *Ann Trop Med Parasitol* 83 (3): 299-303, 1989.
24. De Rcke PH, Apt W, Campano S, Thompson RCA: Many facets of echinococcus/hydatidosis. *Parasitol Today* 12 (8): 295-297, 1996.

