

## Evaluation and Comparison of Immune Response in Laboratory Model to Low Antigen of Fluid and Protoscolex in Hydatid Cyst

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**Abstract:** *Hydatidosis* is a well-established cyclozoonotic disease, which is cosmopolitan in distribution. The aim of the present study was to evaluate cyst fluid and protoscolex as an alternate source of antigen in serodiagnosis of cystic echinococcosis. Hydatid cyst fluid and protoscolex was aseptically aspirated from individual fertile cysts, clarified by centrifugation at  $5000 \times g$  for 15 min at 4°C and concentrated and dialyzed against PBS using an Amicon filter (USA). Twenty seven mice were randomly divided into 3 groups of 9 mice (two immunized and one control group). Results showed that the level of antibody production was different by use of two different antigens and in all of these groups. Level of antibody in case groups was significantly higher ( $P < 0.05$ ) than control group. In our study, we found that the protective immunity in vaccinated mice was 100%. A mouse model has been developed to evaluate potential protective antigens which could render intermediate hosts resistant to a challenge infection with *Echinococcus granulosus* protoscolex in experimental infection.

**Key words:** *Hydatidosis* • Cyclozoonotic • Mice • Amicon filter • Vaccine

### INTRODUCTION

*Hydatidosis* is a well-established cyclozoonotic disease, which is cosmopolitan in distribution. A number of species of animals suffer from this infection. Available literature reveals that at least 10–60% of animals in south-east Asia harbour the metacestode stage of this parasite [1]. In humans and animals, the diagnosis of hydatid cysts is based on the clinical symptoms as well as the radiographic and ultrasonographic findings and serological test results. In spite of successful control programs in some countries or regions, the parasite still has a very wide geographical distribution. There is clear evidence for the emergence or re-emergence of human cystic *Echinococcosis* in parts of China, central Asia, Eastern Europe and Israel; However, there is a clear need for new advances in the prevention of echinococcosis [2]. However, it is still antibody detection which is most widely used for confirmation of clinical diagnosis and in epidemiological surveys [3].

Experimental infections of mice with eggs or oncospheres of *E. granulosus* showed that susceptibility varies with different strains of mice. *In vitro* experiments have also shown that neutrophils, in association with antibodies, can bring about the killing of *E. granulosus* oncospheres, suggesting a possible role for antibody-dependent cell-mediated cytotoxicity reactions [4]. At the early stages of disease, there is a marked activation of cell mediated immunity to the parasite [5]. The purpose of this study was to determine whether immunization of mice with hydatid fluid and protoscolices antigens might induce the humoral immune response and antibody production against the two antigens.

### MATERIAL AND METHOD

*E. granulosus* hydatid cysts were obtained from the livers of sheep (2-3 years old animals) slaughtered at abattoirs in Amol, Mazandaran, Iran. Hydatid cyst fluid was aseptically aspirated from fertile cysts, centrifuged at  $5000 \times g$  for 15 min at 4°C and concentrated and dialyzed

against PBS using an Amicon Ultra-30, 30 000 MWCO centrifugal filter device (Millipore, USA).

Protoscolices were washed 3-times with PBS and Hank's salt solution (Sigma, St. Louis, USA) containing 100 U/ml of penicillin G and 100 mg/ml of streptomycin sulfate. Samples were freeze-thawed 3 times and mixed with four volumes of PBS, pH 7.4, containing sodium azide at 0.1 mg/ml. Samples were then sonicated at 110 V, 170 W ultrasonic disintegrator (Hielscher, Germany), for 3-15 seconds on ice. The prepared solution was then left on ice for one hour and centrifuged for 30 min at 5000 g and then filtered by Amicon Ultra. Protein concentrations were measured by Bradford method and kept at -20°C until used.

**Immunization and Challenge:** Twenty seven mice (Wistar strain, male, 2-3 month old) were randomly divided into 3 groups of 9 mice (two immunized and one control group). The mice in groups one and two received 100 µg (100 µl) of less than 30 kDa *E. granulosus* antigens of hydatid cyst fluid and Protoscolex plus 100 µl of Freund's complete adjuvant, respectively. Mice in the control group were immunized with adjuvant in PBS. For second immunization after two weeks mice were treated with the same solution plus Freund's incomplete adjuvant. Two weeks after the second immunization, each mouse was challenged with 1000 protoscolices intraperitoneally as described previously. Mice were sacrificed by CO<sub>2</sub> five months post challenge. Blood samples were collected before each immunization from mice and sera were separated by centrifugation at 5000 g for 3 min after placing the samples at 4°C overnight. Sera were stored at -20°C until used.

**ELISA:** To screen the activity of antibody against two types of protein antigens (hydatid fluid and protoscolices antigens), ELISA was carried out as described by Wen and Craig [6]. Assays were carried out in 96-well micro-titer plates. 100 µl of the protein containing mixture (less than 30 kDa proteins) were used per well, except the blocking solution (BSA 3%) which 300 µl of it was used. The wells were washed four times with PBS (pH 7.2) containing 0.1% v/v of Tween-20. Appropriate wells were coated with 5 µg of hydatid fluid and Protoscolex of *E. granulosus* proteins in 100 µl of 0.1 M NaHCO<sub>3</sub> and the plates were then left exposed to air overnight at room temperature to allow the solution to dry. The following day, after washing, wells were blocked with 300 µl of 3% (w/v) bovine serum albumin (BSA) in PBS and then incubated for 2 h at room temperature to block any

remaining unblocked attachment sites on the wells. After washing the wells, diluted sera of mice were added and then the plates were incubated 1 h at room temperature. The plate was washed and then the second antibody, sheep anti-mouse IgG HRP phosphatase (Sigma, USA) was added at 1:5000 dilutions into all the wells and was incubated for 1 h at room temperature. The plate was washed as described above to remove the excess conjugates. For color development, 100 µl of TMB was added to each well as a substrate and the reaction was terminated after 15 min by adding 100 µl of 1M HCL solution to each well. The absorbance at 490 nm was measured in ELISA reader (Bio-Rad, California, USA).

**Dot Blot Protocol:** Have nitrocellulose membrane ready, draw grid by pencil to indicate the region you are going to blot. Then using narrow-mouth pipet tip, spot 2 µl of samples onto the nitrocellulose membrane at the center of the grid. Minimize the area that the solution penetrates (usually 3-4 mm diam.) by applying it slowly. Let the membrane dry. Then Block non-specific sites by soaking in 5% BSA in TBS-T (0.5-1 hr, RT). Use 10cm Petri Dish for reaction chamber. Incubate with primary antibody (0.1-10 µg/ml for purified antibody, 1:100 dilutions for Anti sera) dissolved in BSA/TBS-T for 30 min at RT. Wash three times with TBS-T (3-5 min). Then Incubate with secondary antibody conjugated with HRP (for optimum dilution, follow the manufacturer's recommendation) for 30 min at RT. Wash three times with TBS-T (15 min, 1, 5 min 2), then once with TBS (5 min). Then Incubate with ECL reagent for 1 min, cover with Saran-wrap (remove excessive solution from the surface) and expose X-ray film in the dark room. Try several different lengths of exposure. Finally Compare the signal from your unknown sample to that of standard and estimate the concentration.

## RESULTS

Results showed that the level of antibody production by using these two antigens was different and in all of these groups (mice which were immunized with hydatid cyst fluid and protoscolex of *E. granulosus* less than 30 kDa proteins) level of antibody was significantly higher ( $P < 0.05$ ) than the control group. The mice immunized with hydatid cyst fluid produced higher antibody ( $P < 0.05$ ) than mice immunized with Protoscolex on day 28 (four weeks after the first immunization). Level of antibody on day 56 (8 weeks after the second immunization) in mice immunized with hydatid cyst fluid was higher than in Protoscolex group ( $P < 0.05$ ).

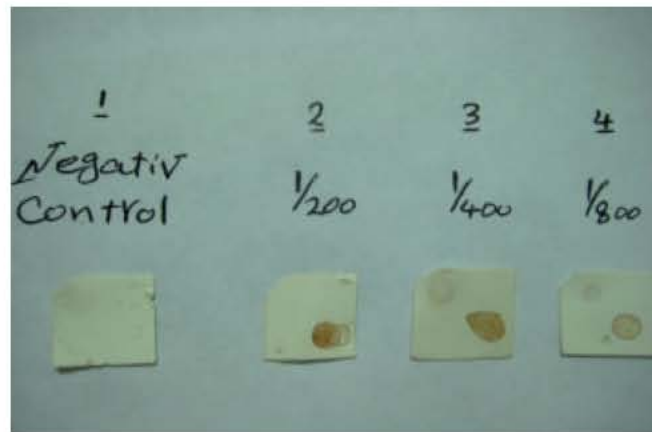


Fig. 1: Dot blot on infection mice with Hydatid cyst and comparison with control group

Table 1: Mean absorbance of test and control groups antibody detection against hydatid cyst fluid and protoscolex on day 0, 28 and 56 and before killing

Groups Day	Fluid	Protoscolex	Control
Pre immunity	0.16	0.16	0.11
4 week after injection	1.35	1.21	0.35
8 week after injection	2.19	2.01	0.72
Before killing	2.98	2.05	0.83

Results of dot blot test also showed that 1/400 diluents of serum in comparison with other dilutions was the best titer for causing immunization (Figure 1).

## DISCUSSION

Immunodiagnosis is an important tool in the diagnosis of an *E. granulosus* infection. Primary screening tests such as the EgHF-ELISA usually exhibit some lack of specificity. This was clearly confirmed by the 95% cross-reactivity observed with AE sera and the high level (58%) of cysticercosis cross-reactivity. There was also a marked cross-reactivity with sera from patients with schistosomiasis, fascioliasis and several nematode infections [7]. In our study, we found that the protective immunity in vaccinated mice was 100%. A mouse model has been developed to evaluate potential protective antigens which could render intermediate hosts resistant to a challenge infection with *Echinococcus granulosus* protoscolex in experimental study [8].

Hashemi tabar in Mashhad show that none of the vaccinated mice with the whole body of *E. granulosus* had cysts that indicate 100% protective immunity and also showed that in all of the control mice there were a lot of cysts in internal organs after 8 months. Also, he indicated

that the level of antibody in mice which were immunized with adult worms of *E. granulosus* on day 28 was seven times higher than before immunization and was higher than hydatid cyst fluid and protoscolex. Level of antibody in mice immunized with adult worms of *E. granulosus* was also higher than in hydatid cyst fluid and protoscolex groups at day 49 [9, 10].

In our study, the levels of antibody production were evaluated in mice against two groups of less than 30 kDa antigens (fluid and protoscolex). Mice immunized with Protoscolex did not produce high level of antibody four weeks after the first immunization in comparison to those immunized by fluid. Antibody produced by hydatid cyst fluid of *E. granulosus* was 4 to 5 times more than before immunization. Lin *et al.*, reported that the specific IgG was induced during the 3rd week and continued to increase until week 10 [10]. Although the level of antibody on day 56 and before killing was very higher than controls, but the level of antibody in mice immunized with fluid antigen of *E. granulosus* was only slightly higher than in Protoscolex groups.

Zhang showed that mice produced lower levels of antibodies than of a secondary challenge infection given three weeks later by a different route (intraperitoneal, subcutaneous or intravenous injection). Most mice did not evoke significant antibody responses against oncospherical antigens until five weeks post-infection [11]. Antigens derived from cyst fluid [12] and protoscolices [13] have been used against *E. granulosus* and mice produced a higher level of antibody against both antigens. Although two type of peptide antigens described in this paper can be used for ELISA test, but it is necessary to study and compare the specificity and sensitivity of these antigens in diagnosis of hydatid cyst.

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