

Immunological aspects of cystic echinococcosis in Erbil

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Hadi M. A. Al-Sakee *

Abstract

Background and objectives: *Echinococcus granulosus* exists as a complex of different strains that differ in a wide variety of criteria that impact on the epidemiology, immunology, pathology and control of hydatid disease. This study was undertaken to investigate both humoral and cellular immune responses that are developed against hydatid cysts in Erbil.

Methods: Thirty patients (9 males and 21 female) with surgically confirmed cystic echinococcosis and 10 apparently healthy individuals were included in this study. IgG ELISA was performed to assess humoral immune responses. CD4/ CD8 ratio, eosinophil count and lymphocyte transformation response were done to assess the cellular immune responses. The level of IFN- γ and TNF- α was also assayed.

Results: The sensitivity of ELISA to detect anti-hydatid antibodies was shown to be 83.33%. CD4 / CD8 ratio was significantly ($P < 0.001$) decreased in patients with cystic echinococcosis as compared to normal control group, while eosinophil count ($P < 0.001$), lymphocyte transformation response ($P < 0.001$) and IFN- γ level ($P < 0.01$) were significantly increased. In contrast the level of TNF α was non- significantly changed in echinococcosis patients.

Conclusion: The current study showed that the local strain of *Echinococcus granulosus* induces both cellular and humoral immune responses, and the number of peripheral blood CD8 T cells was significantly increased in cystic echinococcosis patients. However, hyporesponsiveness to hydatid specific antigens has not been induced.

Key words: Echinococcosis, Immune response, CD4/CD8 ratio, ELISA

Introduction

Human cystic echinococcosis is a chronic zoonotic disease that results from infection with the larval stage of the dog tapeworm *Echinococcus granulosus*¹. The disease has a global distribution especially in areas where sheep-rearing exists and it has been reported as an important public health problem in most countries of the world². A feature of infections by helminthes is that polarize the immune response toward TH2 pattern³. This response, however, has not been correlated with resistance in all

cases, and its role in the host- parasite interaction remains unknown in many infections⁴. Epidemiological data concerning cystic echinococcosis have shown that most infections do not develop to the disease and it is likely that immune response play a pivotal role in limiting cystic larval development⁵. Very little is known about the factors affecting innate susceptibility to hydatid cyst infection, following ingestion of the infective egg stage and establishment of the cyst^{5,6}. In the early stage of the infections, the oncosphere is transported to

*Department of Basic Science, College of Medicine, Hawler Medical University, Erbil, Iraq.

a host organ such as liver or lung ...etc, where it develops into a hydatid cyst. The immature cyst may overcome host defense mechanisms^{7,8,9}. About 8-10 weeks post infection a complex of echinococcal antigens are released from the cyst and stimulate complex immune responses, which include polarized TH2 response balanced with TH1^{10,11}.

Numerous studies have provided evidence that *Echinococcus granulosus* exists as a complex of different strains that differ in a wide variety of criteria that impact on the epidemiology, immunology, pathology and control of hydatid disease^{12,13,14,15}. Cellular and humoral immune responses in humans, in contrast to those in experimentally infected animals, can vary enormously, as evidenced by the different patterns of parasite antigens in different patients and courses of the disease. These disparities are likely related to the strain variation of the parasite and/ or genetic differences between host populations^{16,17}. In Kurdistan region very little information is known about the immune responses that are induced by the local strain of *Echinococcus granulosus*. Therefore, this study was undertaken to investigate both humoral and cellular immune responses that are developed as a result of host defense mechanism against hydatid cysts in Erbil.

Methods

Patients:

Thirty patients (9 males and 21 females) with surgically confirmed cystic echinococcosis were included in this study. The patients had an age range of 9-63 years old. They had either liver cyst (n: 21), Lung cyst (n: 5), Muscle cyst (anterior abdominal wall muscle: n. 2, Psoas major muscle: n. 1) and spleen cyst (n: 1), an additional 10 apparently healthy individuals were also included and used as control group. Blood samples were collected from the patients by venepuncture at surgery in the surgery theater of Rizgary Teaching Hospital in Erbil.

Immunological parameters

I- Serology

a- Hydatid antigens: Hydatid cysts were collected from infected livers of sheep slaughtered in Erbil slaughterhouse and processed according to Baz *et al*¹⁸. The protein concentration was determined according to the method of Lowry *et al*¹⁹.

b-Enzyme-linked immunosorbent assay

(ELISA): Antibody responses were analyzed by ELISA according to the procedure described by Lorenzo *et al*²⁰ with slight modification. Briefly, the wells of polystyrene microplates were coated with 100 µl of 20 µg/ml of hydatid antigens in 0.1M sodium carbonate/ bicarbonate buffer pH 9.2 and incubated overnight at 4°C. After the coating buffer was discarded, the plate was blocked for 60 minutes at 37°C with 1% bovine serum albumin in phosphate buffer saline (PBS), pH 7.4 and washed with PBS-Tween 20 solution (0.05 %). The serum samples were diluted 1:200 in PBS-Tween 20 solution containing 0.5% bovine serum albumin. After 90 minutes of incubation at 37°C the plates were washed thrice with PBS-Tween 20 solution. One hundred µl of peroxidase-conjugate anti-human IgG were added to each well and incubated 60 minutes at 37°C. After washing (3 X), a volume of 100 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature (18-22 °C). After the last incubation, the reaction was stopped with 100 µl of 1N HCL. Optical density was measured at 450 nm. The cut-off value of ELISA, which differentiates positive from negative reactions, was established by the mean value of normal serum group plus three standard deviations.

II- CD4 / CD8 ratio

T cell populations were quantified by direct immunofluorescent staining using commercially available anti-human CD3-FITC/ CD8-PE and anti-human CD3-FITC/ CD4-PE (Sigma, UK). Human CD4 and CD8 T lymphocyte populations were stained and assessed as recommended by the leaflets provided by the manufacturer. Helper suppressor (H/S) ratio was established as

CD4/CD8 ratio.

III- Eosinophil count

Peripheral blood eosinophils were quantified by automatic full blood count coulter (Beckman coulter, France).

IV- Lymphocyte transformation response (LTR)

Lymphocyte transformation response (LTR) was performed as described by Shubbar and Allak ²¹

V- Cytokine assay

The levels of serum IFN- γ (Interferon-gamma) and TNF- α (Tumor necrosis factor - alpha) were assayed using commercially available ELISA kits (USBiological, USA) as recommended by the manufacturer.

Statistical analysis

The results were statistically analyzed by student t-test and single factor ANOVA using Microsoft office Excel 2003. P value ≤ 0.05 was considered statistically significant.

Results

Table (1) shows the results of ELISA on the sera from 30 surgically confirmed cystic echinococcosis patients. The highest sensitivity obtained was in patients with spleen (100%) and liver (90.48%) cysts, while patients with lung infestation exhibited the lowest sensitivity (60%). Helper / suppressor ratio as established by CD4 / CD8 ratio, was highly significantly decreased ($P < 0.001$) in patients with cystic echinococcosis (1.0154 ± 0.2) when compared with that in normal control group (2.008 ± 0.37) (Figure.1). However, no significant ($P > 0.05$) differences were observed among patients with different organs involvement (Table 2).

As shown in Figure (2), eosinophil count was highly significantly ($P < 0.001$) increased in echinococcosis patients (522.8 ± 292.41 cells/cmm) when compared with that in normal control group (165.2 ± 84.65 cells/cmm). Statistical analysis also showed non- significant variation ($P > 0.05$) among patients with different infected

organs (Table 2).

As seen in Figure (3), peripheral blood lymphocytes from cystic echinococcosis patients were highly significantly ($P < 0.001$) responded ($22.58\% \pm 4.8\%$) to hydatid antigens when compared to normal control group ($5.27\% \pm 1.6\%$). The levels of IFN- γ and TNF- α in cystic echinococcosis patients were found to be 121.03 ± 32.63 pg/ml and 163.6 ± 101.49 pg/ml, respectively (Figure 5). The level of IFN- γ was significantly ($P < 0.0079$) elevated in echinococcosis patients when compared with its level in normal control group (83.04 ± 20.68 pg/ml). In contrast, the level of TNF- α was non - significantly changed in echinococcosis patients when compared with normal control group (159.72 ± 110.86 pg/ml). As seen in Table.2 there were no significant differences in lymphocyte transformation response and cytokine levels among patients with different organs involvement.

Table 1: Detection of anti-hydatid antibodies in 30 patients with surgically confirmed cystic echinococcosis.

Organ involved	No. of cases	No. of positive (%)	Cut-off value
Liver	21	19 (90.48)	
Lung	5	3 (60)	
Muscle *	3	2 (66.66)	1.23
Spleen	1	1 (100)	
Total	30	25 (83.33)	

Table 2: H/ S ratio, eosinophil count, LTR, IFN- γ and TNF- α in 30 echinococcosis patients according to infected organs.

Organ involved	H/S ratio	Eosinophil count (cells/ cmm)	LTR (%)	IFN- γ (pg/ ml)	TNF- α (pg/ml)
Liver	1 \pm 0.19	533.24 \pm 325.56	23.36 \pm 4.04	118.68 \pm 32.1	159.63 \pm 107.75
Lung	1.1 \pm 0.1	370 \pm 188.8	19.58 \pm 7.27	134.97 \pm 42.13	211.62 \pm 107.72
Muscle	0.87 \pm 0.22	618 \pm 48.01	22.07 \pm 5.96	122.44 \pm 27.17	116.57 \pm 25.62
Spleen	1.42- \pm 0.0	780 \pm 0.0	22.8 \pm 0.0	96.61 \pm 0.0	148.1 \pm 0.0
	F crit- 2.97 F- 2.59; df- 29 P > 0.05	F crit- 2.97 F- 0.81; df- 29 P > 0.05	F crit- 2.97 F- 0.83; df- 29 P > 0.05	F crit- 2.97 F- 0.5; df- 29 P > 0.05	F crit- 2.97 F- 0.58; df- 29 P > 0.05

H/S ratio: Helper / suppressor ratio; LTR: Lymphocyte transformation response
 IFN- γ : Interferon-gamma; TNF- α : Tumor necrosis factor alpha

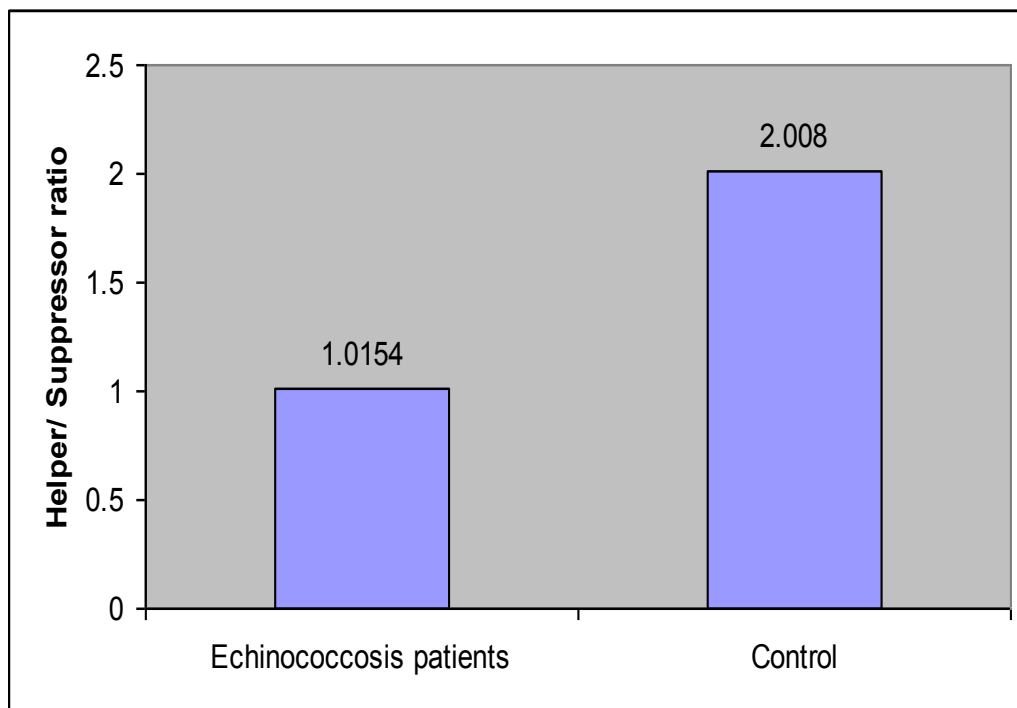


Figure 1: H/S ratio in 30 patients with cystic echinococcosis and 10 uninfected healthy individuals.

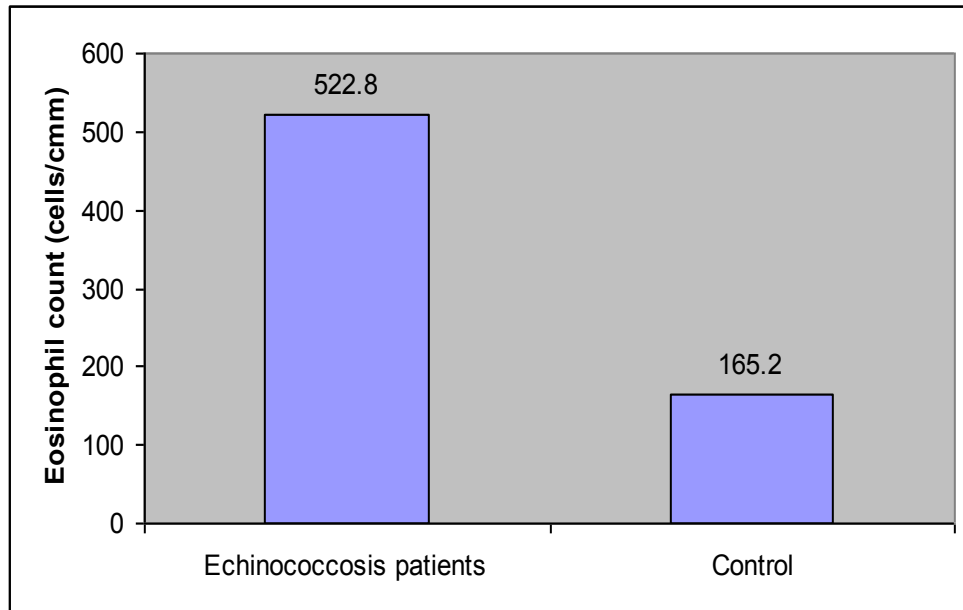


Figure 2: Peripheral blood eosinophil count in 30 echinococcosis patients and 10 uninfected healthy individuals.

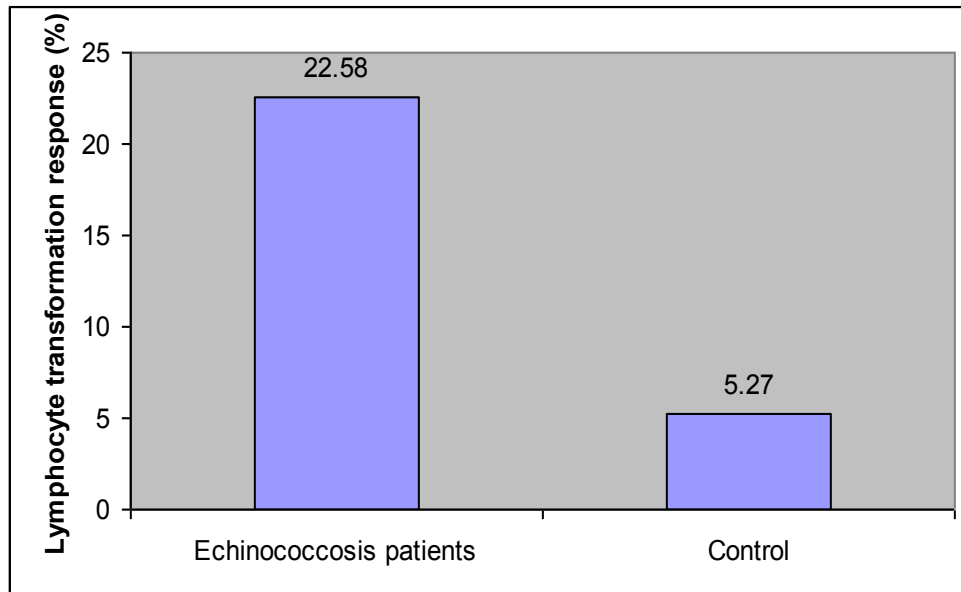


Figure 3: Lymphocyte transformation response in 30 echinococcosis patients and 10 uninfected healthy individuals.

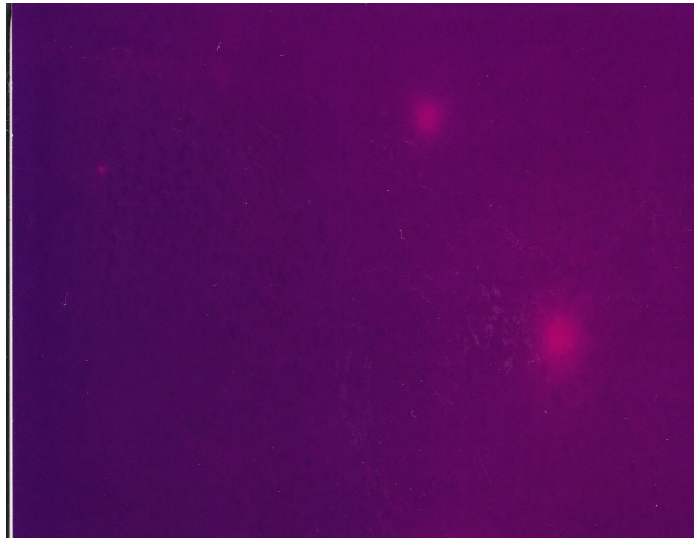


Figure 4: CD8 T lymphocytes coated with anti- human CD8- Phytoerythren (200 X).

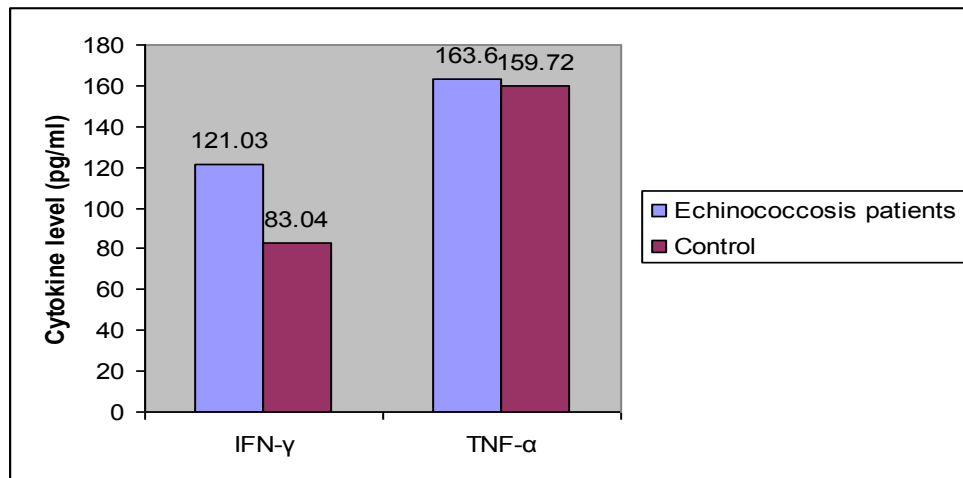


Figure 5: Interferon-gamma (IFN- γ) and tumor necrosis factor- alpha (TNF- α) levels in 30 patients with cystic echinococcosis and 10 uninfected healthy individuals.

Discussion

Immunodiagnostic testing for serum antibodies or circulating antigens provides supportive evidence of echinococcosis. An enzyme-linked immunosorbent assay or indirect haemagglutination test are commonly used as initial screen for anti-hydatid antibodies²². Moreover, fast detection of the cases in the endemic areas is very important, in order to prevent further spread of the disease to other areas²³. In the present study IgG ELISA employing crude hydatid fluid antigen gave a sensitivity of 83.33%. However, the obtained sensitivity was relatively lower than that obtained by Ghorbanelinezhad *et al*²³. On the other hand Kanwar *et al*²⁴ have shown that, employing purified 8 KDa hydatid specific antigens, the sensitivity of ELISA was found to be 77%. The high number of false negative results may be a result of several factors such as strain variation of the parasite which may change the immune response that impact on the diagnostic results. Moreover, the presence of inhibitors in the serum and the role of immune complex which interfere with normal antigen-antibody interaction^{24,25}. In the present study the sensitivity of ELISA was also affected by the localization of the cysts. ELISA technique has a sensitivity of 80%-100% in detection of liver cysts, while the sensitivity of this test is around 50%-60% in lung cysts²⁶. Liver cysts release more antigenic materials into surrounding host tissues, unlike lung cysts which are usually surrounded by an intact hyaline cyst wall^{27,28}. In helminthes infections not only the innate immune system, but also adaptive immune mechanisms appeared to be involved in the defense against the parasite¹⁷. While parasite-specific antibodies appear not to exhibit a direct restricting role on the growth of hydatid cysts in humans, the immunological effector function may be attributed primarily to T Lymphocytes^{5,6}. In the present study the CD4/CD8 ratio was significantly decreased. In general, it is believed that proteinaceous antigens added

exogenously elicit primarily CD4 T lymphocytes responses, which are MHC class II restricted, however, recent reports have demonstrated cross-priming of CD8 T lymphocytes in infectious diseases and antigen presenting cells can take exogenous antigens and present them to CD8 T cells through the Class I MHC molecules²⁹. In the present study, cystic echinococcosis patients showed significant eosinophilia and this observation is in agreement with previous studies in this regard^{1,30}. In addition to their role as terminal effector cells in helminthes infections through the IgE-dependent cell cytotoxicity³, eosinophils may also serve as specific antigen presenting cells and present antigens to the primed T cells, which were then induced to produce IL-5³¹. In the current study the level of IFN- γ was significantly elevated in echinococcosis patients when compared with normal control group. Crude hydatid fluid contains a mixture of distinct antigens of both host and parasite origin. The two most abundant antigens are antigen 5 and antigen B²⁸. This crude mixture can promote activation of both TH1 and TH2 cells^{9,10,11}. Some studies have shown that at the time of cyst development TH1 response, which associated with increased level of IFN- γ , could be detected^{10,11}, but if TH1 responses begin to damage the parasite, then the parasite starts to dominate TH2 response through release of antigens that induce TH2 promoting cytokines such as IL-4 and IL-13⁷. TNF- α is a pro-inflammatory cytokine that is expressed early during acute inflammatory responses³². Since cystic echinococcosis is a long standing infection, the level of TNF- α could be declined with the development of the cyst. All 30 cystic echinococcosis patients examined, developed a significant lymphocyte transformation response to crude hydatid fluid antigens, while none of the healthy individuals showed any specific lymphocyte transformation response to that antigens, suggesting the presence of efficient specific memory cells in the echinococcosis patients. The data presented in

this study suggest that the lymphocyte transformation response provides a test with increased sensitivity compared with serology. So lymphocyte transformation responses along with serology provide supportive evidence for specific diagnosis of human cystic echinococcosis. However, Kharebov *et al*³³ have reported that, while the specific humoral response decreased gradually after successful treatment (chemotherapy or surgery), the cellular response remained positive for a prolonged period (> 35 years).

Finally, the current work showed that the local strain of *Echinococcus granulosus* induces both cellular and humoral immune responses, and the number of circulating CD8 T cells was significantly increased in cystic echinococcosis patients, however, hypersponsivness to hydatid specific antigens has not been induced as indicated by lymphocyte transformation response to hydatid antigens. Molecular studies to specify the local strain of *Echinococcus granulosus* in Kurdistan region and investigation of the production of homologous mammalian cytokines by *Echinococcus granulosus* meta-cestode are recommended.

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References

- 1- Deka B, W D J and Dever L L. Disseminated echinococcosis involving the pulmonary artery. *Infections in Medicine*, 2009; 26: 148-51
- 2- WHO. WHO Mediterranean zoonoses control center, information circular: Issue dedicated on cystic echinococcosis and the Mediterranean. No. 57- June 2003.
- 3- Pearce E J, Scott P A and Sher A. Immune regulation in parasitic infection and disease. In Paul W E (ed). *Fundamental immunology*. 4th ed. Philadelphia, Lippincott-Ravean publishers; 1999.
- 4- Siracusano A, Ortona E and Rigano R. Molecular and cellular tools in human cystic echinococcosis: Current Drug Targets Immune, Endocrine and Metabolic Disorders, 2002; 2: 235-45.
- 5- Zhang W, Ross A and MacManus D. Mechanisms of immunity in hydatid disease: Implications for vaccine development. *J Immunol*, 2008; 181: 6679-85.
- 6- Zhang W, Li J and Mcmanus DP. Concepts in immunology and diagnosis of hydatid disease. *Clin-Microbol Rev*, 2003; 16: 18-36.
- 7- Rigano R, Profumo E, Bruschi F, Carulli G, Azara AJ, Ioppo S. *et al*. Modulation of human immune response by *Echinococcus granulosus* antigen B and its possible role in evading host defenses. *Infect. Immun*; 2001; 69: 288-96.
- 8- Virginio V, Taroco L, Ramose A, Ferreira A, Zaha A, Ferreira H and *et al*. Effect of protoscoleces and AgB from *Echinococcus granulosus* on human neutrophils: Possible implications on the parasites immune evasion mechanisms. *Parasitol Res*, 2006; 100: 935-42.
- 9- Rigano R, Buttari B, Profumo E, Ortona E, Delunado F, Margutti P, Mattei V *et al*. *Echinococcus granulosus* antigen B impairs human dendritic cell differentiation and polarizes immature dendritic cell maturation towards a Th2 cell response. *Infect Immun*, 2007; 75: 1667-78.
- 10- Dematteis S, Rottenberg M and Baz A. Cytokine response and outcome of infection depends on the infective dose of parasites in experimental infection by *Echinococcus granulosus*. *Parasite Immunol*, 2003; 25: 189-98.
- 11- Al-Qaoud K and Abdel Hafez S. Humoral and cytokine response during protection of mice against secondary hydatidosis caused by *Echinococcus granulosus*. *Parasitol Res*, 2005; 98: 54-60.
- 12- Obwaller A, Schneider R, Walochnik J, Gollackner B, Deutz A, Janitschke K *et al*. *Echinococcus granulosus* strain differentiation based on sequence heterogeneity in mitochondrial genes of cytochrome c oxidase-1 and NADH dehydrogenase-1. *Parasitology*, 2004; 128: 569-75.
- 13- Thompson RC, Boxell AC, Ralston BJ, Constantine CC, Hobbs R P, Shury T *et al*. Molecular and morphological characterization of *Echinococcus* in cervids from North America. *Parasitol*, 2006; 132: 439-47.
- 14- Roratto PA, Bartholomei-Santos ML, Gutierrez AM, Kamenetzky L, Rosenzvit MC and Zaha A. Detection of genetic polymorphism among and within *Echinococcus granulosus* strains by heteroduplex analysis of a microsatellite from the U1snRNA genes. *Genet Mol Res*, 2006; 5: 542-52.
- 15- De La Rue M L, Dinkel A Mackenstedt U and Roming T. New data on *Echinococcus* spp. In Southern Brazil. *Rev Inst Med Trop S. Paulo*, 2006; 48: 103-4.

- 16- Gottstein B. Molecular and immunological diagnosis of echinococcosis. Clin Microbiol Rev; 1992; 5:248-61.
- 17- Mehlhorn H. Parasitology. Berlin: Springer-verlag Berlin Heidelberg, 2001.
- 18- Baz A, Hernandez S, Damatteis S, Carol H and Nieto A. Idiotypic modulation of the antibody response of mice to *E. granulosus* antigen. Immunol, 1995; 87:350-4.
- 19- Lowry OH, Rosenbrough NJ, Farr AL and Randall R J. Protein measurement with the folin phenol-reagent. J Bio Chem, 1951; 193: 265-75.
- 20- Lorenzo C, Ferreira H, Monteiro K, Rosenzvit M, Kamenetzky L, Garcia H *et al.* Comparative analysis of the diagnostic performance of six major *Echinococcus granulosus* antigen assessed in a double blind randomized multicenter study. J Clin Microbiol, 2005; 43:2764-70.
- 21- Shubber EK and AL-Allak BM. Spontaneous chromosomal aberration and SCE in human lymphocyte, I effect of culture condition. Nucleus, 1986; 29:92-8.
- 22- Morar R and Feldman C. Pulmonary echinococcosis. Eur Respir J, 2003; 21: 1069-77.
- 23- Ghorbanalinezhad E, Assmar M, Piazak N and Khabiri A. Development of new ELISA kit for the diagnosis of hydatidosis in humans. Iran J Pub Health, 2001; 30: 67-70.
- 24- Kanwar J R, Kanwar R K, Grewal A S and Vinayak V K. Significance of detection of immune-complexed 8 KDa hydatid-specific antigen for immunodiagnosis of hydatidosis. FEMS Immunol Microbiol, 1994; 9: 231-36.
- 25- Chematai A K, Bowry T R and Ahmad Z. Evaluation of five immunodiagnostic technique in echinococcosis patients. Bull World Health Organ, 1981; 59: 767-72.
- 26- Kuroo MS. Hydatid disease: Current status and recent advances. Ann Saudi Med, 2002; 22: 56-4.
- 27- Manson PE and Bell DR. Mansons Tropical Disease, Baillier Tindall, 1987.
- 28- Craig P S. Immunodiagnosis of *Echinococcus granulosus* and a comparison of techniques for diagnosis of canine echinococcosis. In Anderson F L, Ouhelli H and Kachani M (eds). Compendium on cystic echinococcosis in Africa and in Middle Eastern countries with special reference to Morocco. Provo, USA, Brigham Young University. 1997.
- 29- Manfras B J, Reuter S, Wendland T and Kern P. Increased activation and oligoclonality of peripheral CD8+ T cells in the chronic human helminth infection alveolar echinococcosis. Infect Immun, 2002; 70: 1168-74.
- 30- Elshazly A M, Azab M S, ElBeshbishi S N and Elsheikha H M. Hepatic hydatid disease: four case reports. Cases J, 2009; 2: 58.
- 31- Padigl U M, Lee J J, Nolan T J, Schad G A and Braham D. Eosinophils can function as antigen-presenting cells to induce primary and secondary immune responses to *Stroglyoides stercoralis*. Infect Immun, 2006; 74: 3232-38.
- 32- Abbas AK and Lichtman AH. Cellular and molecular immunology. 5th ed. Philadelphia: Saunders, an imprint of Elsevier Science, 2003.
- 33- Kharebov A, Nahmias J and El-On J. Cellular and humoral immune responses of hydatidosis patients to *Echinococcus granulosus* purified antigens. Am J Trop Med Hyg, 1997; 57: 619-25