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Recombinant ferritin protects mice against challenge with *Echinococcus granulosus* ?

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Abstract

Ferritin is well known as the main intracellular iron storage protein in both prokaryotes and eukaryotes, keeping it in a soluble and non-toxic form, though the role of ferritin as a vaccine candidate in echinococcosis has not yet been delineated. Through our study, ferritin was cloned from *Echinococcus granulosus* and expressed in *Escherichia coli*. The recombinant *E. granulosus* ferritin (rEgferritin) has a molecular weight of 19 kDa and could be recognized by anti-mice serum in Western blotting. The specific antibody was detected by enzyme-linked immunosorbent assay. Mice vaccinated with rEgferritin and challenged intraperitoneally with *E. granulosus* protoscoleces revealed significant protective efficacy up to 85.6%, compared with the control group. Thus, rEgferritin could be a promising candidate as an effective vaccine to prevent the infection of echinococcosis.

Keywords

Echinococcus granulosus, rEgferritin, immunoprotection, vaccine

Introduction

Echinococcosis, also known as hydatid disease, is a zoonotic parasitic disease that is distributed widely around the world such as in sub-Saharan Africa, Central Asia, South America and the Mediterranean region (Craig *et al*. 1991, Eckert *et al*. 2001). It can cause substantial human morbidity and mortality, particularly in developing countries, so cystic echinococcosis control programs have been implemented in many parts of the world (Achussler *et al*. 1995). These programs are based on health education, restriction of livestock offal disposal practices, drug treatment and control of dog population. Despite substantial efforts to reduce the transmission of *E. granulosus* from dogs to human, human echinococcosis still persists with high incidence in many parts of the world and in some areas where it is a reemerging problem (Lightowlers *et al*. 2000).

Livestock vaccines may provide an additional approach to the control of echinococcosis. Sheep, cattle, pigs and humans have been infected as intermediate hosts by the metacestode stage of *E. granulosus*. If we can find a vaccine to protect the intermediate host from infection, the incidence of hydatid disease could be reduced due to the definitive host not eating organs with protoscoleces (PSC). Thus vaccines in intermediate hosts could be a very effective and convenient approach, which can stimulate the production of a high level of natural immunity in the intermediate host. Many trials of vaccination with *Echinococccus granulosus* 95 (Eg95) confirmed a high degree of protection against challenge infection with *E. granulosus* (Lightowlers *et al*. 1999, Heath *et al*. 2003). *E. granulosus* has many stages in its life cycle including oncosphere, protoscoleces, adult worm and egg, and only one vaccine cannot protect against so many stages of *E. granulosus*, so here we are trying to screen some new recombinant antigen candidate which has high immunoprotective function in order to contribute to making the potential cocktail vaccine.

In recent years, ferritin as a vaccine candidate has drawn more and more attention. *Schistosoma mansoni* ferritin (Dietzel *et al*. 1992), *Taenia saginata* ferritin (Benitez *et al*. 1996) and *E. granulosus* ferritin (Ersfeld and Craig 1995) have been cloned and expressed. Ferritin of the Chinese strain of *E. granulosus* was also cloned and sequenced (Wang *et al*. 2005) in our laboratory, and already registered on NCBI and the GenBank number is DQ678103. On this basis, we tried in this study to further isolate and identify the rEgferritin and study its immunoprotective efficacy against *E. granulosus* in an attempt to identify it as a vaccine candidate.

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Materials and methods

Ferritin/pGEM-T/JM109 bacterial strain was made by our laboratory. pET-28a expression plasmid was purchased from Novagen. *E. coli* BL21(DE3)pLysS was provided by Dr. Xiao Wei (Saskatchewan University, Saskatoon, Canada).

Collection of PSC

Brood capsules were collected aseptically from fertile *E. granulosus* cysts from livers of infected human patients who were hospitalizing in the liver surgery department of the affiliated Hospital of Ningxia Medical University. The collected PSCs were washed in phosphate-buffered saline (1% PBS) and Hanks' balanced salt solution (Sigma, St. Louis, USA) containing 100 U/ml of penicillin G and 100 U/ml of streptomycin sulfate. The viability of PSCs was determined by trypan blue exclusion assay. Only those batches containing more than 90% viable PSCs were used for mice infection.

Expression and purification of rEgferritin

Plasmids of Eg.ferritin/pGEM-T and pET-28a were digested by restriction enzyme NotI and BamHI, then the digested fragments of Eg.ferritin (about 560 bp) and pET-28a were purified and ligated by T4 ligase at 4°C for 16 h. The recombinant plasmid Eg.ferritin/pET-28a was transformed into the bacterial competent *E. coli* strain of BL21(DE3)pLysS cells. rEgferritin was expressed by the induction of 0.05 mM/L isopropyl-β-D-thiogalactoside (IPTG, Promega) at 37°C overnight. The recombinant protein with six His-tag was an inclusion body. It was then dissolved in urea and purified by nickel affinity chromatography column (Novagen). Then urea was removed through dialysis. The purified rEgferritin was analyzed on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The protein concentration was determined by the Bradford method (Bradford 1976).

Animal immunization protocols

Twenty male ICR mice (6 to 8 weeks old; obtained from the Experimental Animal Center of Ningxia Medical University, Yinchuan, China) were randomly divided into two groups. In the first group, mice were immunized subcutaneously in the back with 2-week intervals of 50 μg of rEgferritin in 100 μl PBS emulsified in Freund's complete adjuvant (Sigma) followed by two boosters of injection with the antigen in Freund's incomplete adjuvant. In the control group, mice were immunized only with adjuvant in PBS. The mice were bled via caudal vein at 0, 2, 4, 6 and 8 weeks after immunization, and sera were stored individually at –20°C until analysis.

Detection of specific immunoglobulin (*Ig*) *by ELISA*

The serum antibody responses including IgG and its subclasses after immunization with rEgferritin and control (PBS) were quantified by ELISA as described. 96-well polystyrene microplates (Sino-American Biotechnology Company) were coated with rEgferritin (5μg/100μl/well) overnight at 4°C. Then the coated plates were washed three times with PBS, and saturated for 2 h at room temperature (RT) with 100 μl blocking buffer (PBS with 5% dried skim milk). Then the wells were washed with PBS-T (PBS with 0.05% Tween-20) three times. Serum samples were diluted 1:100 in dilution buffer PBS-T and loaded to microwells and incubated at 37°C for 1 h, and the serum from immunized PBS mice was used as a negative control. After three times washed in PBS-T, bound antibody was added by using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Novagen) and IgG1, IgG2a, IgG2b, and IgG3 (Sino-American Biotechnology Company, Beijing, China) at a 1:1000 dilution in PBS-T at 37°C for 1 h. Serum antibody responses were tested in duplicate. The plates were washed three times and developed for 10 min in the dark with 40 mg/ml TMB (3,3´5,5´-tetramethyl-benzidine) in citrate buffer (0.05 M Na_2HPO_4, 0.025 M citric acid) with pH 4.0 to monitor the peroxidase activity. The reaction was stopped by the addition of 2 M H_2SO_4 to each well. The optical density (OD) values were determined at 490 nm. The antibody titers were indicated using an ELISA microplate reader (Bio-Rad, California, USA).

Western blot analysis

SDS-PAGE and Western blot were carried out as the method of Laemmli (1970). rEgferritin, cystic fluid and PSC from *E. gra-nulosus* as native antigen were separated in a 12% polyacryl-amide gel and transferred into nitrocellulose membranes (Millipore, Tokyo, Japan). Membranes were blocked in 5% dried skim milk at 37°C for 2 h and washed with PBS-T three times. Membranes were cut into strips and incubated with diluted sera of immunized and control mice (1:100) at 37°C for 1.5 h. Membranes were then washed three times with PBS-T, and bound antibody was detected by HRP-conjugated goat anti-mouse IgG (Novagen) (1:1000) at 37°C for 1.5 h. Membranes were washed three times with PBS-T and incubated in the coloration liquid (6 mg 4-chloro-1-naphtol, 2 ml methanol, 10 ml TBS) (Sino-American Biotechnology Company) for 15 min at RT. The distilled water was added to stop the reaction.

Challenge infection

Two weeks after the final vaccination, each mouse from the two groups was challenged with 2000 viable PSC intraperitoneally. Five months post-challenge, mice were killed and blood was collected retroorbitally for serum preparation. The carcasses were dissected and examined for visible hydatid cysts. Protective immunity can be easily evaluated in this model by counting the numbers or determining the size of cysts growing in the peritoneal cavities of immunized or control mice. The percentage of protection in mice was determined by the method described by Dempster *et al*. (1995): Protective im-

munity in vaccinated animals (%) = (1-average of cysts in test group/average of cysts in control group) × 100%.

Statistical analysis

All data were tested for significance by using one-way analysis of variance (ANOVA); P values of <0.05 were considered significant.

Results

Expression and purification rEgferritin

The recombinant plasmid Egferrin/pET-28a was constructed and transformed into *E. coli* BL21(DE3)plysS. The plasmids extracted from transformed *E. coli* BL21(DE3) plysS were digested by the restriction enzymes BamHI and NotI. The DNA

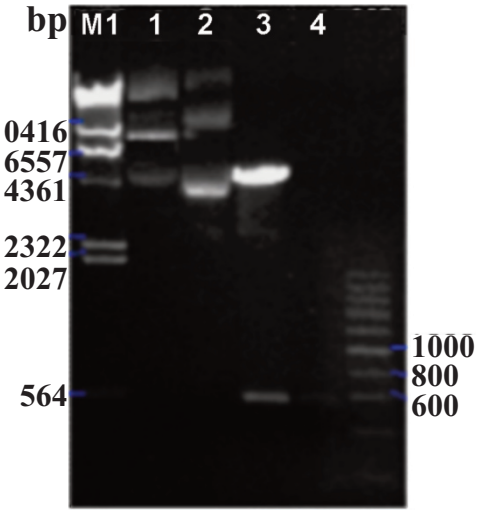

Fig. 1. The restriction enzyme digestion of recombinant expressed plasmid Egferritin/pET-28a by BamHI and NotI. M1 Lander DNA HindIII marker. Lane 1 – recombinant plasmid Egferritin/pET-28a; lane 2 – plasmid pET-28a; lane 3 – recombinant plasmid Egferritin/pET-28a was digested by BamHI and NotI; lane 4 – amplification product; M2 – 200 bp DNA marker

size of the digested products separated by agarose gel electrophoresis is similar to the production of polymerase chain reaction (PCR) at about 560 bp (Fig. 1). The image confirmed that the recombinant expression plasmid Egferritin/pET-28a was constructed successfully; the 6 His-labeled recombinant protein was purified with Ni^{2+} chelating column. Its molecular weight is about 19 kDa (Fig. 2).

Identification of rEgferritin and PSC and cystic fluid

Immunizing mice by rEgferritin, 4 weeks post-immunization, obtaining sera from caudal vein of mice, using the anti-mouse

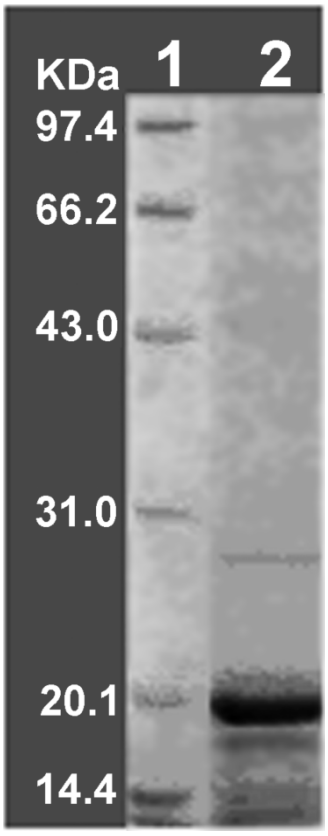

Fig. 2. SDS-PAGE analysis of purified rEgferritin. Lane 1 – molecular mass marker; lane 2 – purified protein

serum to identify rEgferritin and native antigen PSC and cystic fluid (Fig. 3). It showed that anti-mouse serum could recognize rEgferritin and native antigen PSC and cystic fluid. Their bands were the same size, i.e., about 19 kDa.

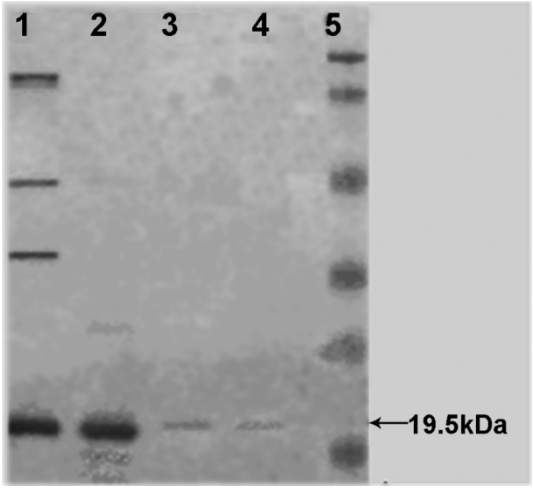

Fig. 3. Anti-mice sera recognized rEgferritin and PSC and cystic fluid. Lane 1 – expressed rEgferritin; lane 2 – purified rEgferritin; lane 3 – cystic fluid; lane 4 – protoscoleces; lane 5 – mark of protein

Table I. OD values of IgG were detected on 0, 2, 4, 6 and 8 weeks

Groups	0 weeks	2 weeks	4 weeks	6 weeks	8 weeks
Immunized group	0.126 ± 0.095	0.194 ± 0.080	0.423 ± 0.1109*	0.570 ± 0.1986*	0.9364 ± 0.3282*
Control group	0.118 ± 0.070	0.111 ± 0.022	0.181 ± 0.1209	0.2612 ± 0.129	0.3133 ± 0.1019

Data are presented as mean ± SE of 10 mice per group. *P<0.01 compared with IgG antibody response of control mice.

Table II. Number of hydatid cysts and protective immunity in vaccinated and control mice

Groups	No. of mice	Mice with hydatid cysts	No. of cysts (mean ± SE)	Size of hydatid cysts (mean ± SE) in mm	Immunoprotection (%)
Immunized group	10	3	0.56 ± 0.26*	0.27 ± 0.16*	85.6%*
Control group	9	7	3.89 ± 1.01	2.12 ± 0.47	–

Data were expressed as mean ± SE. *P<0.01 compare with control group.

Detection of IgG and subclasses

Purified rEgferritin was coated onto the microplate. Detected titer of IgG of anti-serum from pre-immune and post-immune 2, 4, 6 and 8 weeks by ELISA are shown on Table I. The titers of IgG antibody increased with time after immunization. The serum collected from immunized mice at 4 weeks after immunization contained much higher titer of IgG antibody than that of the control mice (P<0.01). Four subclasses of IgG against rEgferritin were induced in ICR mice, the OD values of IgG1, IgG2a, IgG2b in the immunized group were all higher than that of the control group (P<0.05), OD values of IgG3 and IgE did not significantly differ between the immunized group and the control group (Fig. 4).

Protective immunity in mice

Apart from one mouse in the control group, that died when challenged with PSC, all other 19 mice were dissected 5 months after the challenge of PSC of *E. granulosus*, and all organs were examined carefully to find hydatid cysts. It showed that the mice vaccinated with rEgferritin had a lower number of visible hydatid cysts compared to the control group (Table II), and also that mean size of hydatid cysts of the control group was 2.12 ± 0.47 mm, i.e. much larger than the 0.27 ± 0.16 mm size observed in the vaccinated mice (P<0.01). Based on a formula described previously, the protective immunity induced by rEgferritin was 85.6%.

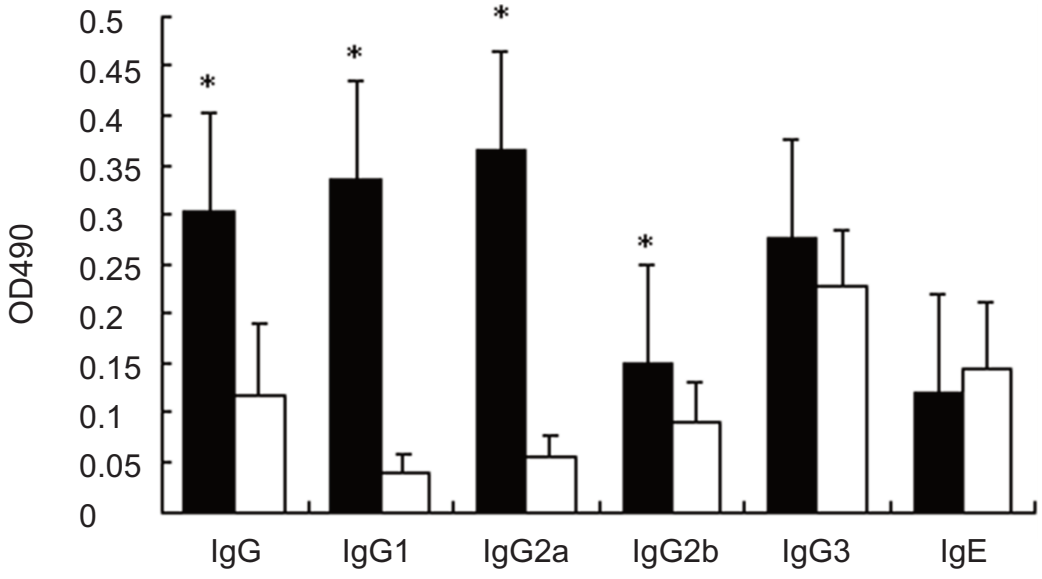

Fig. 4. Isotype of IgG and IgE distribution of the antibody response against rEgferritin as detected by ELISA. Four subclasses of IgG against rEgferritin were induced in ICR mice. IgG1, IgG2a and IgG2b were the prevailing subclasses. Data were expressed as mean ± SE. *P<0.05 compared with IgG1, IgG2a and IgG2b antibody responses of control mice. Black rectangle – immunized group, white rectangle – control group

Discussion

Iron, which plays a role in DNA synthesis, electron transfer and oxygen activation, is required for animals, plants, and microorganisms. It is so insoluble under physiological conditions that special iron-binding and storage proteins, i.e., ferritins, need to maintain iron in a soluble form and also protect against the toxic effects of excess iron. Although little is known about its immunological feature, ferritin as a prospective vaccine in the future has drawn more and more attention. Some parasite ferritins were cloned and expressed. Evidence from several studies (Clemens and Basch 1989, Achussler 1995) has indicated that ferritin plays an important role in early growth and development of schistosomula and is essential for embryonic development of *Shistosoma mansoni*. *E. granulosus* ferritin was highly immunogenic and thus a useful diagnostic antigen for detection of the disease (Ersfeld and Craig 1995). So ferritin may be a potential vaccine to prevent echinococcosis. Through our study, we constructed recombinant expression plasmid rEgferritin/pET-28a, and expressed, purified and characterized the rEgferritin by identifying rEgferritin and native antigen PSC and cystic fluid by anti-serum from immunized mice. They were recognized on same location of the bands at about 19 kDa. It revealed that the recombinant antigen has the same epitope with native antigens. We hypothesized that if rEgferritin could act a vaccine to immunize mice, that it perhaps could induce the host to produce a specific antibody against *E. granulosus*. The results of the challenge infection study confirmed the presumption that a significant reduction in the parasite burden was obtained by rEgferritin immunization. It induced protective immunity attaining to reach 85.6% in the murine model. Both the numbers and the sizes of cysts in the two groups of mice were significantly different. It indicated that rEgferritin has the potential to be a vaccine against *E. granulosus* and that the feature of this protein requires further studies.

Numerous studies have demonstrated that humoral immunity plays a crucial role in the protection against *E. granulosus* (Barriga and Al Khalidi 1986, Haralabidis *et al*. 1995). In experimentally induced secondary infections in mice, intraperitoneally injected PSC are surrounded by considerable cellular infiltration within 3 days, initially by involving activated macrophages and subsequently including neutrophils, eosinophils, and lymphocytes (Richards *et al*. 1983; Riley *et al*. 1985, 1986). Only small amount of IgM, IgG1, IgG2a, and IgG3 could be detected early in serum, and higher levels were detectable later (Pater *et al*. 1998). The production of high levels of IgE is a the common consequence of infection by helminthes (Capron and Dessaint 1992, Bell 1996). The IgE-dependent mast cell reaction has evolved primarily to localize eosinophils near the parasite and then enhance their antiparasitic functions (Bell 1996). In secondary infection by alveolar echinococcosis (AE) in mice, the same results were observed (Emery *et al*. 1997). In our study, we found that six antibodies (IgG, IgG1, IgG2a, IgG2b, IgG3 and IgE) of mice were detected, the OD values of IgG1, IgG2a, IgG2b of immunized group were all higher than in the control group ($P<0.05$), the result was in agreement with the report above. The OD values of IgE were not significaty different between immunized group and control group. We did not detect any change of IgE. We suppose that the time of antiserum collected was not optimal for detecting IgE, because this increased very early, and so we missed the optimal detection time.

Though our data indicated that rEgferritin could induce protective immunity and stimulate the host to generate immune response, the mechanism of the induced protection is still unknown. This requires further study.

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References

Achussler P., Potters E., Winnen T., Bottke W., Kunz W. 1995. An isoform of ferritin as a component of protein yolk platelets in *Schistosoma mansoni*. *Molecular Reproduction and Development*, 41, 325–330. DOI: 10.1002/mrd.1080410307.

Barriga O.O., Al Khalidi N.W. 1986. Humoral immunity in the prepatent primary infection of dogs with *Echinococcus granulosus*. *Veterinary Immunology and Immunopathology*, 11, 375–389. DOI: 10.1016/0165-2427(86)90039-5.

Bell R.G. 1996. IgE, allergies and helminth parasites: a new perspective on an old conundrum. *Immunology and Cell Biology*, 74, 337–345. DOI: 10.1038/icb.1996.60.

Benitez L., Harrison L.J.S., Parkhouse R.M.E., Garate T. 1996. Sequence and immunogenicity of *Taenia saginata* ferritin. *Molecular and Biochemical Parasitology*, 82, 113–116. DOI: 10.1016/0166-6851(96)02713-2.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–254. DOI: 10.1016/0003-2697(76)90527-3.

Capron A., Dessaint J.P. 1992. Immunologic aspects of schistosomiasis. *Annual Review of Medicine*, 43, 209–218. DOI: 10.1146/annurev.me.43.020192.001233.

Clemens L.E., Basch P.F. 1989. *Schistosoma mansoni*: Effect of transferrin and growth factors on development of schistosomula *in vitro*. *Parasitology*, 75, 417–421. DOI: 10.2307/3282599.

Craig P.S., Deshan L., Zhaoxun D. 1991. Hydatid disease in China. *Parasitology Today*, 7, 46–50. DOI: 10.1016/0169-4758(91)90188-T.

Dempster R.P., Harrison G.B., Berridge M.V. 1995. Maternal transfer of protection from *Echinococcus granulosus* infection in sheep. *Research in Veterinary Science*, 58, 197–202. DOI: 10.1016/0034-5288(95)90101-9.

Dietzel J., Hirzmann J., Preis D., Symmons P., Kunz W. 1992. Ferritins of *Schistosoma mansoni*: sequence comparison and expression in female and male worms. *Molecular and Biochemical Parasitology*, 50, 245–254. DOI: 10.1016/0166-6851(92)90221-5.

Eckert J., Schantz M.P., Gasser R.B., Torgerson P.R., Bessonov A.S., Movsessian S.O., Thakur A., Grimm F., Nikogossian M.A. 2001. Geographic distribution and prevalence. In: (Eds. J. Eckert, M.A. Gemmell, F.-X. Meslin and Z.S. Pawłowski) *WHO/OLE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern. World Health Organization for Animal Health. Paris, France*, 100–142.

Emery I., Leclerc C., Houin R., Vuitton D.A., Liance M. 1997. Lack of H-2 gene influence on mouse susceptibility to secondary alveolar echinococcosis. *International Journal for Parasitology*, 27, 1433–1436. DOI: 10.1016/S0020-7519(97)00091-X.

Ersfeld K., Craig P.S. 1995. Cloning and immunological characterisation of *Echinococcus granulosus* ferritin. *Parasitology Research*, 81, 382–387. DOI: 10.1007/BF00931498.

Haralabidis S., Karagouni E., Frydas S., Dotsika E. 1995. Immunoglobulin and cytokine profile in murine secondary hydatidosis. *Parasite Immunology*, 17, 625–630. DOI: 10.1111/j.1365-3024.1995.tb01008.x.

Heath D.D., Jensen O., Lightowlers M.W. 2003. Progress in control of hydatidosis using vaccination – a review of formulation and delivery of the vaccine and recommendations for practical use in control programmes. *Acta Tropica*, 85, 133–143. DOI: 10.1016/S0001-706X(02)00219-X.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685. DOI: 10.1038/227680a0.

Lightowlers M.W., Flisser A., Gauci C.G., Heath D.D., Jensen O., Rolfe R. 2000. Vaccination against cysticercosis and hydatid disease. *Parasitology Today*, 16, 191–196. DOI: 10.1016/S0169-4758(99)01633-6.

Lightowlers M.W., Jensen O., Fernandez E., Iriarte J.A., Woollard D.J., Gauci C.G., Jenkins D.J., Heath D.D. 1999. Vaccination trials in Australia and Argentina confirm the effectiveness of the EG95 hydatid vaccine in sheep. *International Journal for Parasitology*, 29, 531–534. DOI: 10.1016/S0020-7519(99)00003-X.

Pater C., Muller V., Harraga S., Liance M., Godot V., Carbillet J.-P., Meillet D., Römig T., Vuitton D.A. 1998. Intestinal and systemic humoral immunological events in the susceptible Balb/c mouse strain after oral administration of *Echinococcus multilocularis* eggs. *Parasite Immunology*, 20, 623–629. DOI: 10.1046/j.1365-3024.1998.00195.x.

Richards K.S., Arme C., Bridges J.F. 1983. *Echinococcus granulosus equinus*: an ultrastructural study of murine tissue response to hydatid cysts. *Parasitology*, 86(Pt3), 407–417. DOI: 10.1017/S0031182000050605.

Riley E.M., Dixon J.B., Jenkins P., Ross G. 1986. *Echinococcus granulosus* infection in mice: host responses during primary and secondary infection. *Parasitology*, 92(Pt2), 391–403. DOI: 10.1017/S0031182000064155.

Riley E.M., Dixon J.B., Kelly D.F., Cox D.A. 1985. The immune response to *Echinococcus granulosus*: sequential histological observations of lymphoreticular and connective tissues during early murine infection. *Journal of Comparative Pathology*, 95, 93–104. DOI: 10.1016/0021-9975(85)90081-7.

Wang Y., Zhao J., Ding S., Wang J., Zhao W. 2005. Cloning and sequence analyzing of the ferritin gene from *Echinococcus granulosus* of China. *Ningxia Medical University Journal*, 27, 253–255. DOI: CNKI:SUN:XNXY.0.2005-04-000.

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