Short Paper

Establishment of primary cystic echinococcosis in laboratory mice: our results in the Balb/c strain

Sohraby, Sh. A.[1]; Moazeni, M.[2*] and Rakhshandehroo, E.[2]

[1]Ph.D. Student in Parasitology, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *M. Moazeni, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: moazeni@shirazu.ac.ir*

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Abstract

Background: Establishment of experimental cystic echinococcosis (CE) in laboratory mice is required for the study of CE. Experimental CE may be established in primary or secondary forms; however, the primary form is more reliable for the study of CE. **Aims:** The aim of the study was to assess the possibility of establishment of primary CE in Balb/c mice via the oral administration of *Echinococcus granulosus s.l.* eggs. **Methods:** *E. granulosus s.s.* eggs were obtained from the gravid segments of the adult worms collected from an experimentally infected dog. Thirty-four female Balb/c mice were allocated into 4 groups and were orally infected with *E. granulosus s.s.* eggs as follows: 1) the mice with normal immunity, a single dose of 1000 eggs, 2) the mice with normal immunity, 3 doses of 500 eggs, 3) immunosuppressed mice, a single dose of 1000 eggs, and 4) immunosuppressed mice, 3 doses of 500 eggs. After 6.5 months, all the mice were opened and their internal organs were examined for the presence of CE cysts. The livers of infected mice were also examined for the presence of *E. granulosus s.l.* compartments by the PCR method. **Results:** There was no developed or developing CE cyst in the abdominal cavities or on the internal organs of all the mice in four groups. In addition, in the molecular study, all the examined liver samples were negative for the parasite material. **Conclusion:** The results of the present study revealed that Balb/c mice are not a suitable host for establishment of primary CE following the oral administration of *E. granulosus s.s.* eggs.

Key words: Balb/c, *Echinococcus granulosus s.l.*, Laboratory mice, Primary cystic echinococcosis

Introduction

Cystic echinococcosis (CE) is a complex zoonotic disease with chronic nature all around the world. The disease is caused by the larval stage of *Echinococcus granulosus sensu lato* (*s.l.*), a small tapeworm that lives in the small intestine of dogs and other carnivores (McManus *et al*., 2012). A wide range of mammalians (46 species belonging to 6 orders) including sheep, horses and cattle act as the intermediate hosts. Humans are occasionally or accidentally infected through the ingestion of the parasite's eggs (Yamashita *et al*., 1956; Gómez *et al*., 2016; Salih *et al*., 2017).

Establishment of experimental CE in the intermediate hosts of *E. granulosus s.l.* is seriously required for the study of various aspects of the disease. These issues include biological and immunological behavior of the parasite, evaluation of drug/vaccine efficiency as well as assessment of diagnostic tools for the early detection of the infection in intermediate hosts, particularly in human beings (Ahmadnia *et al*., 2014).

Both primary and secondary experimental infections have been done for development of the larval form of *E. granulosus s.l.* in its intermediate hosts (Yamashita, 1968). Different animal species including sheep, monkeys, baboons, rabbits, gerbils, and mice and various infection routes such as intragastric injection of eggs, intravenous, subcutaneous or intraperitoneal inoculation of activated oncospheres and intraperitoneal injection of protoscoleces have been used for the establishment of experimental CE (Zak and Sande, 1999).

Most of our information on biology, immunology, treatment, vaccine candidates and diagnostic tools of the larval form of *E. granulosus s.l.* have been obtained from the secondary CE in laboratory mice (Colli and Schantz, 1974). This form of infection could be established via the intraperitoneal inoculation of viable protoscoleces of *E. granulosus s.l.* into the mice (Mourglia-Ettlin *et al*., 2016a, b).

The lack of an appropriate laboratory host for the larval stage of *E. granulosus s.l.*, is considered as one of the limitations of research on echinococcosis in the past (Schwabe, 1968). White laboratory mice are suitable intermediate hosts to establish experimental CE, because they are not expensive and their feeding, as well as their housing, is not difficult (Barnet, 1936).

Cystic echinococcosis (CE) is a zoonotic infection that needs to be more studied. Experimental CE may be established in primary or secondary forms. Secondary CE is not an ideal model for animals and human infections, because of the differences in infection route, growth rate and the developmental features of the parasite in the host. Moreover, the cysts are restricted to the peritoneal cavity. Hence the primary form (oral ingestion of the parasite eggs) is more reliable in this regard. Since the natural route of the infection to CE in the intermediate hosts of *E. granulosus s.l.* is the oral ingestion of the parasite eggs, this project was designed to investigate the possibility of establishment of experimental CE in laboratory mice via the oral administration of the parasite eggs. However, because of the negative results, almost all the reasons that may affect the consequences of efforts for establishment of primary CE in Balb/c mice are discussed.

Materials and Methods

Infection of dog

A female dog of mixed breed with 1-1.5 months of age and weighing about 2 kg was used in this study for the preparation of *E. granulosus s.s*. (*sensu stricto*) eggs. Heavily infected sheep livers naturally infected with CE were obtained from Shiraz slaughterhouses and transported to the laboratory under cool conditions. The dog was fed with freshly collected infected livers for 3 consecutive days (Fig. 1). Afterwards, the infected dog was housed in a locked kennel under natural lighting and temperature conditions and fed with remaining meals from the college restaurant. The animal had full access to drinking water.

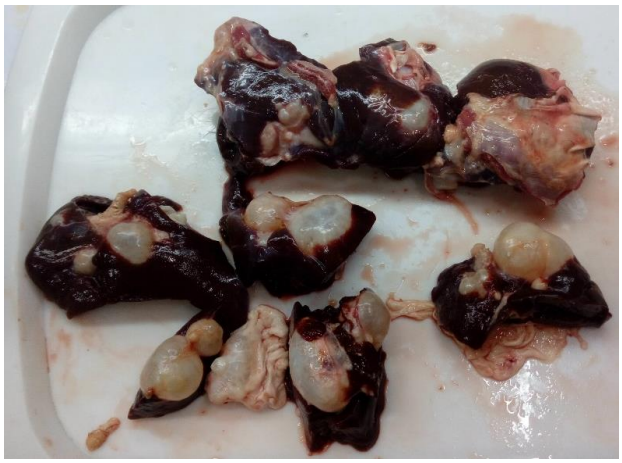

Fig. 1: Sheep livers infected with CE cysts that were fed to the dog, to be experimentally infected with *Echinococcus granulosus sensu lato*

Collection of *E. granulosus s.s.* eggs

The eggs of *E. granulosus s.s.* were collected based on the methods described by Dempster *et al*. (1991) and Zhang *et al*. (2001) with some modifications. Three months after infection, the infected dog was not fed but had access to drinking water one day before being euthanized. The dog was humanly killed by an overdose of intravenous sodium pentobarbital (200 mg/kg). Subsequently, the peritoneal cavity was opened and the small intestine was removed. The small intestine was then opened longitudinally, washed well with tap water to eliminate the intestinal contents, then the mucosal surface was examined for the adult stage of *E. granulosus s.s*. The infected parts of the small intestine were cut into 10 cm pieces and placed in a beaker containing 1X phosphate buffered saline (PBS) and incubated at 28°C. The parasites were released from the intestinal mucosa within 2 h and settled at the bottom of the beaker. PBS was then removed and the parasites were collected in Petri dishes. Subsequently, the gravid proglottids were cut from the collected worms. Recovered gravid segments were then washed three times in warm saline and two times in warm 2% sodium bicarbonate (w/v) to melt the remaining mucus. Finally, the segments were washed two times in PBS (pH = 7.2). For activation of the eggs, gravid segments were incubated at 37°C for 7 days. To release the eggs, gravid proglottids were homogenized in an electric blender, then normal saline was added to the parasite materials (with a ratio of three to one), after mixing, the egg suspension was sieved using a 132-µm sieve. The passed eggs through the sieve were then again washed with normal saline and retained on a 20-µm nylon mesh. Finally, the eggs were collected in small glass capped dishes containing PBS, benzyl penicillin (1000 IU/ml) and streptomycin sulfate (1000 µg/ml) and stored at 4°C before use.

The mice

Forty female Balb/c mice of 4-6 weeks age weighing 20-22 g were used in this study. The mice were inbred and purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran). The mice were housed in controlled conditions (22-25°C, light-cycle of 12 h light/12 h dark) with full access to food and water.

Immunosuppression in mice

To suppress the immune system, the mice received intraperitoneally two doses of cyclophosphamide (25 mg/kg), 24 and 48 h before infection.

Immunosuppression test

To ensure the occurrence of immunosuppression after cyclophosphamide injection, blood samples were taken from 3 mice with normal immunity (as the control group) and from 3 immunosuppressed mice 24 h after the second cyclophosphamide injection. The blood samples were collected in heparinized tubes. Hematological tests were performed using an automatic blood cell counter (Exigo, Stockholm, Sweden) and total WBC, lymphocytes and neutrophils were counted and recorded for the mice in two groups.

Infection of the mice

Thirty-four mice were randomly divided into 4 groups as follows: 1) the mice with normal immunity

that were orally infected with a single dose of 1000 *E. granulosus s.s.* eggs, 2) the mice with normal immunity that were orally infected with 3 doses of 500 eggs in three consecutive days, 3) the immunosuppressed mice that were orally infected with a single dose of 1000 eggs, and 4) the immunosuppressed mice that were orally infected with 3 doses of 500 eggs in three consecutive days.

Necropsy of mice

One hundred ninety-five days (6.5 months) after the start of infection, all the mice in different groups were humanely euthanized and necropsied. Subsequently, all the internal organs were carefully examined for the presence of CE cysts.

Molecular study

Molecular examination was done for genotype identification of the adult form of the used parasite. In addition, liver tissues of the infected mice were traced for the presence of parasite compartments by the PCR method. Five adult parasites and the livers of three animals in each group were homogenized and used for molecular analysis. The lysis and DNA extraction of homogenates was done on about 40 to 50 mg of each sample using a commercial kit (Sinaclon, Iran), according to the recommended instructions. A partial fragment of cytochrome c oxidase subunit 1 gene (cox1) (approximately 430 bp) was amplified using universal primers JB3 (5´-TTT TTT GGG CAT CCT GAG GTT TAT-3´) and JB4.5 (5´-TAA AGA AAG AAC ATA ATG AAA ATG-3´) (Bowles *et al*., 1992). The PCR reactions were adjusted in a final volume of 25 µL, including 12.5 µL of PCR premix (Ampliqon, Denmark, Cat. No. A180301), 10 pmol of each primer, 6.5 µL H_2O and 4 µL of extracted DNA as a template. Cycling conditions included an initial DNA denaturation at 94°C for 5 min, followed by 40 cycles, denaturation at 94°C for 45 s, annealing at 48°C for 1 min, and a final extension at 72°C for 1 min. The parasite sample and the sterile water were considered as the positive and negative controls, respectively.

The presence and the size of the expected amplicons were assessed by electrophoresis on each reaction product in 1% (*w/v*) Tris-acetate/EDTA agarose gel and visualized under ultraviolet illumination. The PCR products obtained for the parasite sample were sequenced (Bioneer, Korea) and compared with other reference sequences for *E. granulosus s.l.* available in the GenBank using the BLAST search.

Ethical approval

All experiments involving the mice were approved by the local ethics committee of the School of Veterinary Medicine, Shiraz University. The trials were in accordance with the principles of laboratory animal care. Unnecessary animal suffering was avoided during the study.

Statistical analysis

The results of immunosuppression tests were analyzed in SPSS software (version 22), using independent samples t-test. P-value of <0.05 was considered significant.

Results

When the dog was fed with heavily infected livers for 3 consecutive days, three months later, gravid segments of *E. granulosus s.s.* were discharged with the dog's feces. After necropsy of the animal, a large number of adult forms of the parasite were attached to the mucosal surface of its small intestine. The worms were carefully collected and all the required eggs for infection of the mice were obtained from gravid segments of collected adult worms.

After intraperitoneal injection of two doses of cyclophosphamide (25 mg/kg with 24 h interval), suppression of mice's immune system occurred. The results of immunosuppression tests are presented in Table 1.

Oral administration of a single dose of 1000 eggs of *E. granulosus s.s.*, not only in non-immunosuppressed but also in immunosuppressed Balb/c mice, did not result in primary CE in the infected animals. When both groups of non-immunosuppressed and immunosuppressed Balb/c mice were infected by oral administration of three doses of 500 eggs on three consecutive days, the results were again negative (Table 2).

In molecular study, the expected cox1 region was

Table 1: The results of immunosuppression test in immunosuppressed mice

Mice	Total WBC ($\times 10^3/mm^3$)		Neutrophils (%)		Lymphocytes (%)	
	Test	Control	Test	Control	Test	Control
1	4.50	6.80	48.00	58.00	39.00	65.00
2	4.67	6.40	46.80	56.00	39.20	64.00
3	4.56	6.70	47.60	53.00	39.40	69.00
Mean±SE	4.57 ± 0.08	6.63 ± 0.20	47.46 ± 0.61	55.66 ± 2.51	39.20 ± 0.20	66.00 ± 2.64
P-value	0.001		0.007		0.003	

Table 2: The results of experimental oral administration of *Echinococcus granulosus sensu strico* eggs to establish primary cystic echinococcosis in Balb/c mice

Group	Number of mice	Immunity	Number of eggs eaten	Infection
1	8	Normal	A single dose of 1000 eggs	Negative
2	9	Normal	Three doses of 500 eggs in 3 consecutive days	Negative
3	8	Suppressed	A single dose of 1000 eggs	Negative
4	9	Suppressed	Three doses of 500 eggs in 3 consecutive days	Negative

successfully amplified for the sampled worms (as positive control) showing bands about 450 bp in size. Based on analysis on partial COX1 sequence (GenBank accession number: MW351789), the isolate was identified as genotype 1(G1) in sheep. However, all the examined liver samples obtained from the mice of four groups were negative for *E. granulosus s.s.* material.

Discussion

Laboratory mice may be used in different studies on *E. granulosus* such as investigation of the efficacy of ovicides, relationships between the parasite and its hosts, immunity induced by protoscoleces and regulation of the immune system (Dempster *et al*., 1991). In suitable intermediate hosts of *E. granulosus*, the cysts grow rapidly and contain a large number of protoscoleces, conversely, in non-suitable species, the cysts' growth is slow and the cysts have little or no protoscoleces (Romig and Bilger, 1999). Yamashita *et al*. (1956b) stated that few white mice become infected, with the larval form of *E. granulosus* and the growth rate is slow and most of the cysts are acephalic. However, they believed that some mice strains are more susceptible for establishment of secondary CE than others (Yamashita *et al*., 1957, 1958).

No satisfactory infection rates have been obtained after oral administration of *E. granulosus* eggs in various strains of *Mus musculus* including the laboratory white mice (Yamashita, 1968; Colli and Schantz, 1974; Romig and Bilger, 1999). Nevertheless, more satisfactory results may be obtained after intraperitoneal or intravenous injection of artificially hatched and activated oncospheres (Romig and Bilger, 1999). However, *in vitro* activation of oncospheres is a hard and tedious task. Hence, in the intraperitoneal injection of activated oncospheres, less than 1.7% of the used eggs may develop to the cysts (Dempster *et al*., 1991).

The results of attempts to infect the laboratory mice to primary CE via oral administration of *E. granulosus* eggs are highly contradictory. Various mice strains may demonstrate different susceptibility/resistance to *E. granulosus* eggs, hatched eggs, or activated oncospheres (Ahmadnia *et al*., 2014). The establishment of primary CE following the oral administration of *E. granulosus* eggs has been reported in dd-strain (Yamashita *et al*., 1956), CF1 strain (Coli and Williams, 1972; Colli and Schantz, 1974; Dempster *et al*., 1991) and DBA/2J, CBA/J, and Balb/cJ strains (Dempster *et al*., 1991).

The result of infection to the larval stage of *E. granulosus* depends on the parasite load and frequency of infection (Conchedda *et al*., 2004). In the present study, we tried both single (1000 eggs) and repeated (three doses of 500 eggs in three consecutive days) routes of infection, however, in two different trials, the results were the same and no infection occurred.

The formation of the cysts after infection depends on the balance of host immunity and parasite avoidance strategies (Conchedda *et al*., 2004). The control of initial inflammatory responses in Balb/c mice could help the formation and survival of CE cysts (Breijo *et al*., 2008; Zhang *et al*., 2008). However, in this study, we experimentally suppressed the immunity of the mice, but immunosuppression did not alter the result of infection.

Based on the study of Dempster *et al*. (1991) almost 80% of orally administered eggs may be shed intact within 24 h after infection. They stated that the rapid transit time through the mouse intestine is the main reason for this rate of egg expulsion.

Balb/c mice have been used as an experimental model for the study of CE (Rahimi *et al*., 2011; Ahmadnia *et al*., 2014) and among different mice strains, some researchers introduced Balb/c as the strain of choice for studies relating to CE (Dempster *et al*., 1991). However, while Balb/c mice are highly susceptible to secondary CE (Mourglia-Ettlin *et al*., 2016a), our efforts for establishment of primary CE in this strain was unsuccessful. Therefore, negative results of the present study may be attributed to incomplete egg hatching process in the gastrointestinal tract of Balb/c strain. Consistent with this hypothesis, Dempster *et al*. (1991) stated that most of the orally administered eggs may be shed unhatched due to the rapid passage of eggs through the mouse intestine and incapability of the mouse bile to stimulate the oncosphere activation.

Due to the high probability of the presence of the parasite in the liver, in the present study, to trace the parasite, the liver tissue was investigated by molecular method. However, the result was negative and there was a full concordance between the results of molecular study and necropsy findings.

Based on the results of previous studies and accordingly the present study, different factors may affect the results of attempts to establish primary CE in mice following the oral administration of *E. granulosus s.l.* eggs. Variations in egg dosage, discrepancy among the batches of used eggs, differences in mouse strain and possible biological diversity among the strain of *E. granulosus s.l.* may be considered as the probable reasons for varying infection rates or conflicting results (Colli and Schantz, 1974). Furthermore, the parasite virulence (Conchedda *et al*., 2004), may be effective in the results of infection and formation or non-formation of the cysts. Genetic status of the used mice may also alter the result of infection. It is known that the host genetic background influences the consequence of helminth infections (Mourglia-Ettlin *et al*., 2016b). In addition, inbred/outbred Balb/c mice is another issue that may affect the results of infection.

Some researchers believe that the fresh eggs of *E. granulosus s.l.* should be incubated at suitable conditions, to become infective for intermediate hosts (Heath, 1991). The incubation time required may be 6 days or more (Smyth and Howkins, 1966). However, Thevenet *et al*. (2005) inoculated the freshly collected eggs (from the mature worms), into 10 lambs and found the cysts in all 10 infected animals. We used the fresh eggs of *E. granulosus s.s.* after 7 days of incubation at 37°C.

Differences in dietary conditions and immunological

status of the mice and environmental conditions in which the mice were kept could be considered as the other possible reasons for the contradictory results. However, the negative results of the present study showed that the Balb/c mice may be unsuitable for primary CE via oral ingestion of the parasite eggs. In addition, the used eggs may not be properly developed before oral administration. Nevertheless, the unsuccessful results of this study may be due to other unknown reasons.

In conclusion, based on the results of previous studies, various mice strains may demonstrate different susceptibility/resistance to *E. granulosus s.l.* eggs. Balb/c mice are highly susceptible to secondary CE and there are some reports of primary CE in Balb/cj following the oral administration of *E. granulosus s.l.* eggs. However, our efforts for the establishment of primary CE in both non-immunosuppressed and immunosuppressed Balb/c mice were unsuccessful. This may be attributed to incomplete egg hatching process in the gastrointestinal tract of this strain.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Ahmadnia, S; Moazeni, M and Mohammadi-Samani, S (2014). Hydatid cyst formation in male Balb/c mice following the intraperitoneal injection of live protoscoleces and activated oncospheres: a comparative study. J. Parasit. Dis., 38: 77-80.

Barnett, LE (1936). White mice and experimental echinococcosis. A.N.Z. J. Surg., 5: 323.

Breijo, M; Anesetti, G; Martinez, L; Sim, RB and Ferreira, AM (2008). *Echinococcus granulosus*: the establishment of the metacestode is associated with control of complement-mediated early inflammation. Exp. Parasitol., 118: 188-196.

Brunetti, E; Praticò, L; Neumayr, A; Maestri, M and Tamarozzi, F (2016). Update on treatment for cystic echinococcosis of the liver. Curr. Treat. Options Infect. Dis., 8: 153-164.

Colli, CW and Schantz, PM (1974). Growth and development of *Echinococcus granulosus* from embryophores in an abnormal host (Mus musculus). J. Parasitol., 58: 422-426.

Colli, CW and Williams, JF (1972). Influence of temperature on infectivity of eggs of *Echinococcus granulosus* in laboratory rodents. J. Parasitol., 58: 422-426.

Conchedda, M; Gabriele, F and Bortoletti, G (2004). Immunobiology of cystic echinococcosis. Parassitologia. 46: 375-380.

Dempster, RP; Berridge, MV; Harrison, GB and Heath, DD (1991). *Echinococcus granulosus*: development of an intermediate host mouse model for use in vaccination studies. Int. J. Parasitol., 21: 549-554.

Gómez, R; Allaoua, Y; Colmenares, R; Gil, S; Roquero, P and Ramia, JM (2016). Hydatid cyst of the gallbaldder: A systematic review of the literature. World J. Hepatol., 8: 1087-1092.

Heath, DD (1991). Daily egg-production of dogs infected with *Echinococcus granulosus*. Arch. Hidatidosis. 30: 321-328.

McManus, DP; Gray, DJ; Zhang, W and Yang, Y (2012). Diagnosis, treatment, and management of echinococcosis. B.M.J., 344: e3866.

Mourglia-Ettlin, G; Cucher, M; Arbildi, P; Rosenzvit, M and Dematteis, S (2016a). Natural and induced antibodies contribute to differential susceptibility to secondary cystic echinococcosis of Balb/c and C57Bl/6 mice. Immunobiology. 221: 103-115.

Mourglia-Ettlin, G; Merlino, A; Capurro, R and Dematteis, S (2016b). Susceptibility and resistance to *Echinococcus granulosus* infection: Associations between mouse strains and early peritoneal immune responses. Immunobiology. 221: 418-426.

Rahimi, HR; Sarkari, B; Mohammadzadeh, T and Sadjjadi, SM (2011). Immune responses to antigens of in vitro reared *Echinococcus granulosus* adult worms in Balb/c mice. Iran J. Immunol., 8: 236-243.

Romig, T and Bilger, B (1999). Animal models for Echinococcosis. In Handbook of animal models of infection. PP: 877-884.

Salih, AM; Kakamad, FH; Hammood, ZD; Yasin, B and Ahmed, DM (2017). Abdominal wall Hydatid cyst: A review a literature with a case report. Int. J. Surg. Case Rep., 37: 154-156.

Schwabe, CW (1968). Larval Echinococcus infection in laboratory animals. Bulletin of the World Health Organization. 39: 126-127.

Smyth, JD and Howkins, AB (1966). An in vitro technique for the production of eggs of *Echinococcus granulosus* by maturation of partly developed strobila. Parasitology. 56: 763-766.

Yamashita, JIRO (1968). Development of Echinococcus in laboratory animals. Bulletin of the World Health Organization. 39: 127-130.

Yamashita, J; Ohbayashi, M; Kitamura, Y; Suzuki, K and Okugi, M (1958). Studies on echinococcosis VIII. Experimental *Echinococcosis multilocularis* in various rodents; especially on the difference of susceptibility among uniform strains of the mouse. JPN. J. Vet. Res., 6: 135-155.

Yamashita, J; Ohbayashi, M and Konno, S (1956). Studies on Echinococcosis IV. Experimental infection of the white mouse. JPN. J. Vet. Res., 4: 123-128.

Yamashita, J; Ohbayashi, M and Konno, S (1957). Studies on Echinococcosis VI: Secondary *Echinococcosis multilocularis* in Mice. JPN. J. Vet. Res., 5: 197-202.

Zak, O and Sande, MA (1999). Hand book of animal models of infection. In: Romig, T and Bilger, B (Eds.), *Animal models for echinococcosis*. (1st Edn.), San Diego, London, Boston, Academic Press. PP: 877-884.

Zhang, W; Ross, AG and McManus, DP (2008). Mechanisms of immunity in hydatid disease: implications for vaccine development. J. Immunol., 181: 6679-6685.

Zhang, W; You, H; Zhang, Z; Turson, G; Hasyet, A and McManus, DP (2001). Further studies on an intermediate host murine model showing that a primary *Echinococcus granulosus* infection is protective against subsequent oncospheral challenge. Parasitol. Int., 50: 279-283.