NEGATIVE - ELISA USING NATIVE AND FILTRATED CYSTIC FLUID ANTIGENS TO RULE OUT CYSTIC ECHINOCOCCOSIS

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Abstract. An increasing number of cases of echinococcosis in Thailand have been imported, probably native infections and medical transfers. Serodiagnosis is one diagnostic choice for interpreting infections before a further step is done. Due to limited antigen, indirect ELISA has been used as a negative screening test for IgG-detection to rule out echinococcosis. Native hydatid cystic fluid (HCF) antigen from Belgium was used for such testing, in which the ODs-ELISA of samples were compared with those of two positive controls. Subsequently, hydatid cyst fluid from a Thai patient was obtained and the filtrated cyst fluid antigen [(<30)-(>10) kDa, HCF30.10] was prepared to develop negative screening results for the serum samples. By using HCF, three of twenty-four samples resulted in higher ODs-ELISA than the controls. In an attempt to observe the cross-reactivity of this native antigen, IgG-antibodies from many helminthiases cross-reacted and showed high ODs-ELISA. The HCF30.10 Ag was used to develop the test and analyze IgG-antibodies from 5 positive controls (2 parasite-confirmed and 3 positive-serodiagnosed), 183 heterologous cases of 29 diseases and 50 healthy control sera. At a cut-off value of 0.484, the test had 100% sensitivity and 42% specificity. Only Malayan filariasis, onchocercosis, fascioliasis, amebiasis, giardiasis and blastocystosis gave true negatives. Antibodies from nematodiases strongly cross-reacted with HCF30.10 Ag. Nine of fifty (18%) healthy serum controls produced higher OD-values than the cut-off. The routine ELISA uses the HCF30.10 Ag to produce a negative result to echinococcosis, because limited cystic fluid antigen (Thai patient) for test improvement, a lot of cross-reactions and only two protoscolex-positive controls are available.

INTRODUCTION

Four species of *Echinococcus* are known to occur in humans, namely *Echinococcus granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli,* the two latter occurring very rarely. The most important species for human hydatidosis are *E. granulosus*, which causes cystic echinococcosis or unilocular echinococcosis or hydatid disease, and *E. multilocularis,* which causes multilocular or alveolar echinococcus. *E. granulosus* is found worldwide, in Europe and the Russian Federation and adjacent countries of Central Asia, Southern Asian and occasionally in other Asian countries, Africa, New Zealand and Australia, North America, South and Central America, and Caribbean countries. *E. multilocularis* is somewhat more restricted in distribution, common in regions of the Northern Hemisphere. Thailand is not an endemic area for these parasites. Humans accidentally ingest *E. granulosus* eggs from contaminated food materials, and perhaps inhalation and/or swallow while working with sheep wool, etc are causes of cystic echinococcosis, which is one of the major zoonoses. The oncosphere develops further to become a cyst in the organs. In nature, the parasite requires dogs and other carnivores as the definitive host, and sheep and humans and other herbivores are intermediate hosts containing hydatid cysts, which develop in the internal organs, such as the liver, lungs, kidneys, or brain (Beaver *et al*, 1984; Thanakitcharu *et al*, 1992; Lymbery and Thompson, 1996). The signs and symptoms of echinococcosis are variable and might be confused with other diseases involving those internal organs. However, diagnostic aids are available to predict both cystic and alveolar echinococcosis such as radioisotopic, ultrasonic scanning, and computerized axial tomographic methods (Von Sinner, 1991; McManus *et al*, 2003). These techniques are unable to make a precise diagnosis as to the nature of infection. Closed aspiration of cysts should be avoided because of the danger of leakage of protoscoleces and the development of secondary cysts or anaphylaxis. Another or complementary, method to detect human hydatidosis is serodiagnosis of a suspected cystic echinococcosis case. Several antigens have been produced by basic and advanced preparations of immunological techniques (Schantz and Kagan, 1980; Di Felice *et al*, 1986; Shepherd and McManus, 1987; Maddison *et al*, 1989; Verastegui *et al*, 1992; Ito *et al*,

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1993, 1999; Nasrieh and Abdel-Hafez, 2004) through molecular biological techniques (Vogel *et al*, 1988; Gottstein, 1992; Ortona *et al*, 2000; Mamuti *et al*, 2002; 2004; Li *et al*, 2004).

Since a test has not been developed to detect echinococcosis, and only negative results are reported at the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, this research aimed to develop an indirect ELISA with molecular weight cut-off antigen from cystic fluid. This test may be useful in diagnosing echinococcosis.

MATERIALS AND METHODS

Antigens

There were two sources of cystic fluid (HCF) antigen, Belgium and a Thai patient who was possibly infected with an *E. granulosus* metacestode from a Middle East country (either Iraq in 1980-1982 or Saudi Arabia in 1983-1987). The HCF antigen from Belgium was the lyophilized form and was dissolved with distilled water and prepared at 1:100 dilution with carbonated buffer, pH 9.6. The final concentration was 1 μg/ml. The HCF antigen from a Thai patient's cystic liver was centrifuged at 10,000 rpm, 4˚C for 30 minutes, and the human contents were checked while the cyst had been developed in the liver. It was found that this native antigen showed high ODs-ELISA with sera of healthy controls. Therefore, the antigen was pre-tested by immunoblot to compare the antigen-antibody patterns of the sera of the echinococcosis and healthy controls. Sera from the healthy controls strongly reacted with antigens > 30 kDa, but echinococcosis sera reacted with antigen < 30 kDa (Fig1). The native antigen was modified by Ultrafree-CL (30 K, Amicon) and concentrated by stirred Ultrafiltration Cell (PM10, Amicon), and then designed as HCF30.10 Ag. All antigens were determined for protein contents by Coomassie blue test kit (Pierce, USA).

Human sera

Homologous serum samples were 5 cystic echinococcosis cases [2 protoscoleces-confirmed Thai patients and 3 sero-positive cases by 8 kDa of immunoblot test, at the Asahikawa Medical College, Hokkaido, Japan (of these 3 cases, 2 were previously from those carrying higher ODs-ELISA than the controls while the other case had OD between the ODs of the controls of the routine diagnosis at the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Fig 2)]. Twenty-four cases were suspected cystic echinococcosis from our routine diagnosis. Heterologous serum samples (n = 183) were 29 diseases, a) cestodiasis group (27 cases); - taeniasis (n = 10), neurocysticercosis (10), sparganosis (3) and hymenolepiasis nana (4), b) trematodiasis group (38 cases); - paragonimiasis heterotremus (10), paragonimiasis westermani (2), opisthorchiasis (10), schistosomiasis (6) and fascioliasis (5), minute intestinal fluke infections (5), c) nematodiasis group (106 cases); - gnathostomiasis (10), angiostrongyliasis (15), capillariasis (3), hookworm infections (10), strongyloidiasis (10), trichinellosis (10), toxocariasis (8), ascariasis (7), bancroftian filariasis (10), brugian filariasis (10), onchocercosis (3), trichuriasis (7), and enterobiasis (3), and d) other infections were creeping eruption (3), protozoan infections (3 entamebiasis histolytica cases, 3 giardiasis cases, each 1 case of toxoplasmosis and *Blastocystis hominis* infection), lung infection (1, undetected paragonimiasis and toxocariasis by serodiagnosis). Healthy serum controls were 50 samples.

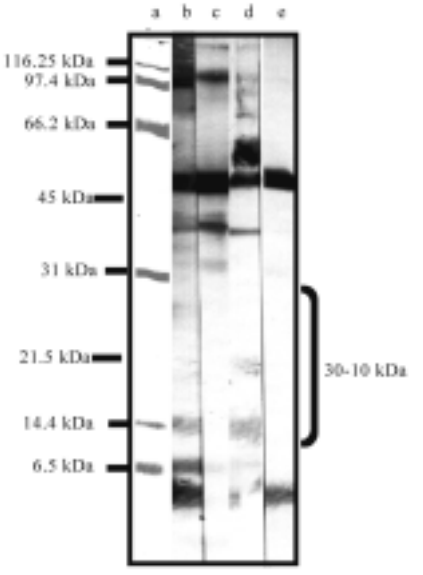

Fig 1- The HCF antigen (Thai patient) was separated into 12-20% gradient gel electrophoresis and transferred onto nitrocellulose membrane, blocked with skimmed-PBS. Strips of blot were reacted with diluted sera of echinococcosis and healthy controls. Then, antigen-antibody complexes reacted with peroxidase-linked rabbit anti-human IgG. The reactions were visualized by substrate (2, 6- dichlorophenol indophenol) and reaction stopped by excess distilled water.

Indirect ELISA

The HCF antigen (Belgium) was directly used following the recommendation and serum and conjugate dilutions were pre-determined by us at 1:400 and 1:1,000, respectively. The conditions for HCF30.10 antigen were determined by checkerboard titration. The proper conditions were HCF30.10 antigen concentration, 2 μg/ml, primary antibody dilution, 1:400 and secondary antibody dilution (rabbit anti-human IgG) at 1:1,000. Briefly, indirect ELISA consisted of coating with 50 μl of diluted antigen in carbonated buffer, pH 9.6, incubation at 37˚C and then an overnight at 4˚C, washing with Tween 20-PBS, pH 7.4 (T-PBS), blocking step with 1% bovine serum albumin, reaction with diluted primary antibody in T-PBS, combination with 50 μl of diluted secondary antibody in T-PBS (peroxidase-conjugated rabbit anti-human IgG, gamma chain, Dakopatts), visualizing with substrate [ABTS, 2,2-azino-di-(3-ethyl-benzthiazoline sulfate) Sigma], termination of reaction with 1%SDS, and measurement of ODs at 405 nm.

RESULTS

Using HCF (Belgium), 3 of 24 suspected echinococcosis samples resulted in higher ODs-ELISA than the controls. One non-Thai patient and 2 Thai patients presented the following ODs-ELISA, 2.397, 2.341, and 2.004, respectively. The OD of 1 Thai patient, 1.778, was between the positive controls. This case presented a hepatic cyst from working in a Middle East country. Most IgG-antibodies of the hydatidosis-suspected cases produced ODs-ELISA slightly lower and higher than the ODs of the healthy control sera (Fig 2). IgG-antibodies of many helminthiases cross-reacted and showed high ODs-ELISA to this antigen (Fig 3). There were 16 of 98 cases of nematodiases, belonging to 5 diseases. In the group of trematodiases (31 cases), 30 cases had OD-values lower than the controls. Only one case of schistosomiasis had an OD-value greater than OD of the positive control (1.374). All 27 cestodiasis cases had low IgG-antibodies against this antigen and produced lower OD-values than the controls.

The HCF30.10 Ag was used to develop the test and analyze antibodies from 5 positive controls and other serum samples. At a cut-off value of 0.484, the efficacy of the test was calculated by 100% ELISA-sensitivity and 42% ELISA-specificity. All 25 cases of Malayan filariasis, onchocercosis, fascioliasis, amebiasis, giardiasis and blastocystosis gave true negatives. IgG-antibodies from nematodiases and cestodiases strongly cross-reacted with the HCF30.10 Ag. Serum antibodies of all 80 cases of taeniasis,

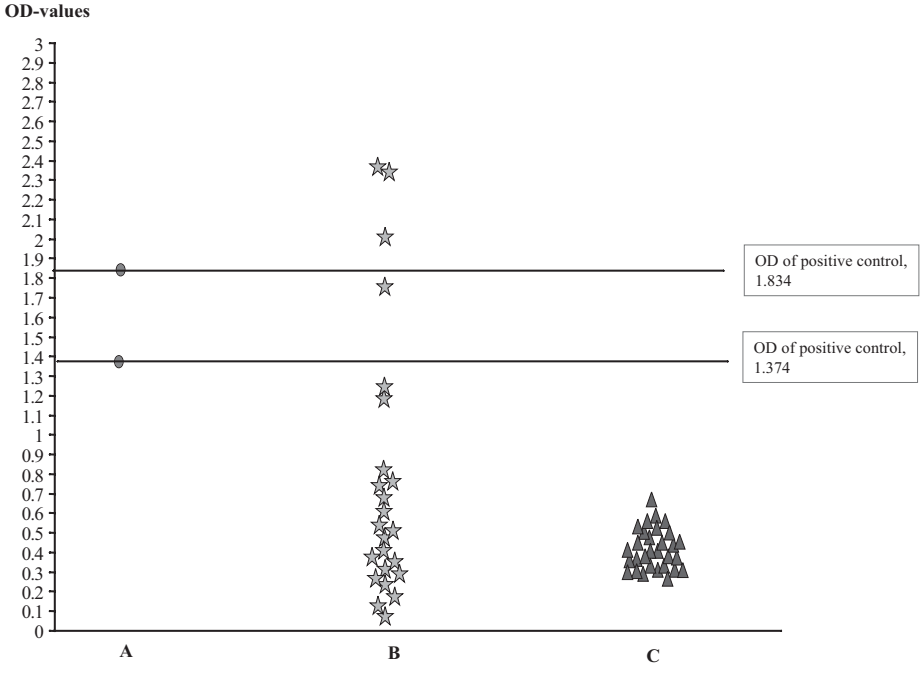

Fig 2- ODs-ELISA resulted from reactions between native cystic fluid antigen (Belgium) of hydatid cyst and cystic echinococcosis controls (A), suspected echinococcosis cases (B) and healthy controls (C).

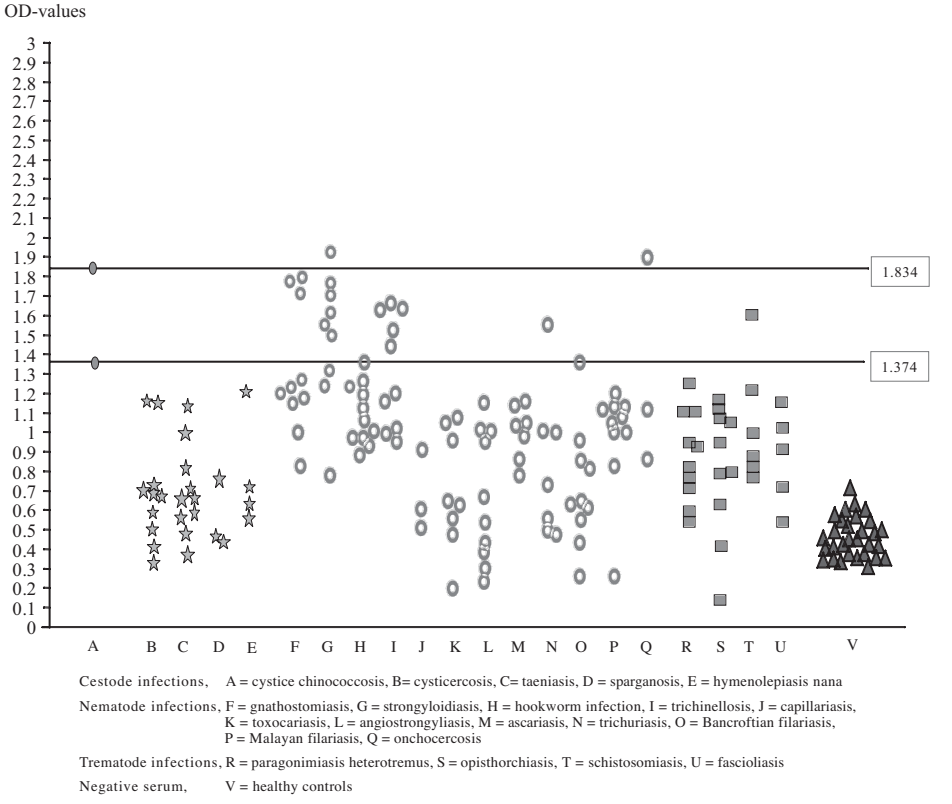

Fig 3- OD-ELISA by reactions between native cystic fluid antigen (Belgium) and serum samples.

paragonimiasis heterotremus and paragonimiasis westermani were false-positive, including gnathostomiasis, hookworm infection, strongyloidiasis, trichinellosis, capillariasis, ascariasis, trichuriasis, and lung infection. Forty-five of 77 sera of other parasitic infections gave OD-values > 0.484. Nine of fifty (18%) healthy serum controls produced higher OD-values than the cut-off value (Fig 4).

DISCUSSION

In our routine diagnosis, 20 of 24 suspected cases presented OD-values lower than the cut-off value (1.374). It is possible that a few of these suspected cases may carry low antibodies against hydatid cysts in patients' bodies. This causes low OD-values, and the disadvantages our routine test are a) only 2 protoscoleces-confirmed cases could be used in OD-ELISA comparison, b) the antigen contents of the hydatid fluids, because antigens from the different strains of intermediate hosts also respond to a variety of sero-tests (Shambesh *et al*, 1995; Poretti *et al*, 1999). In addition to the above reasons, the results of serodiagnostic tests might depend on immunological regulatory or effector mechanisms in patients, antigen preparation, the clinical features of the patients, including the number, size, location, integrity and morphology of the cysts (Gonzales *et al*, 1996; Rigano *et al*, 1998; Ortono *et al*, 2000). Subsequently, this native antigen was used to detect cross-reactions with sera of many parasitic infections. A few serum samples gave such reactions, especially the nematodiasis group. Although, the researchers discussed the cross-reactions in sero-diagnostic work using cystic hydatid fluid antigen a lot, we could not improve the limited antigen from Belgium. ELISA was therefore used as a tool for negative screening results. Using the antigen from Belgium, the majority of heterologous sera produced lower ODs-ELISA than the controls.

Later, we obtained cystic fluid antigen from a Thai patient and used ELISA with this native antigen. High cross-reactions presented (not shown) and pre-observation of immunoblot showed detected bands for sera of two healthy controls with high molecular weights of antigens, and also echinococcosis sera. Echino-

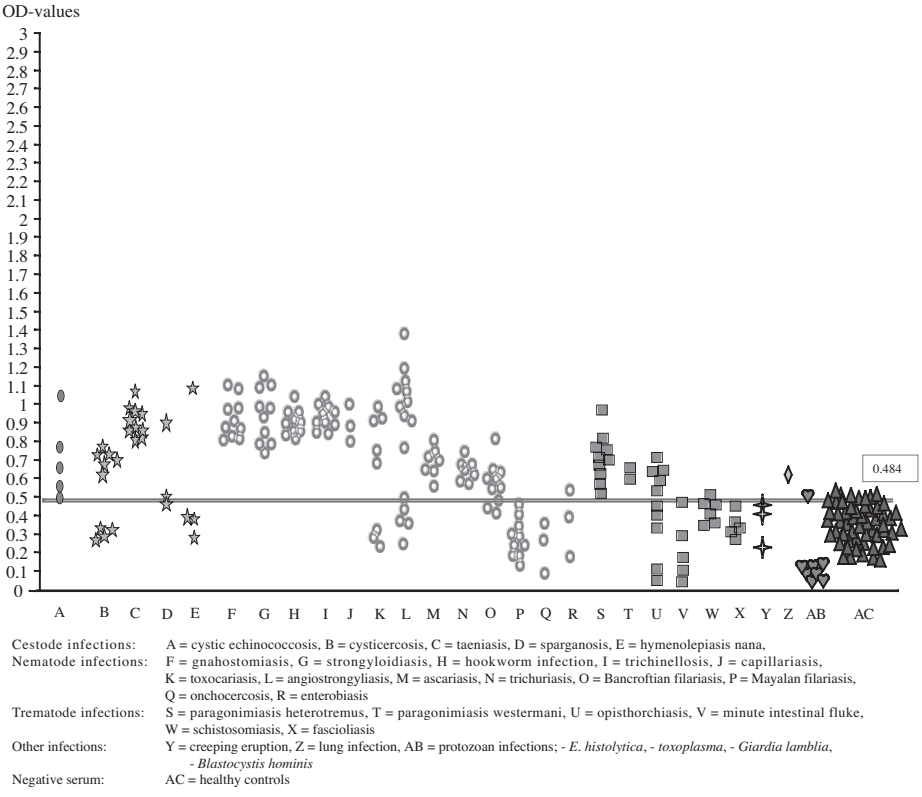

Fig 4- OD-ELISA by reactions between cystic fluid (30.10 kDa) antigen and serum samples.

coccosis sera showed many reactive bands at low antigen molecular weights, that only antigens approximately 6.5 kDa and < 6.5 kDa antigens were poorly seen in healthy control sera (Fig 1). However, the HCF30.10 antigen still showed several cross-reactions with IgG-antibodies of other parasitic infections, including the additional serum samples, other than reactions with the native HCF antigen from Belgium. The test was determined at 100% sensitivity, but low specificity, at 42%. The results for the native HCF (Belgium) and the HCF30.10 antigens were different. The majority of the heterologous serum samples reacted with the whole cystic fluid antigen and showed lower ODs than the two controls. This contrasted with antibodies of cestodiasis and nematodiasis, which strongly cross-reacted with the HCF30.10 antigen and gave higher ODs than the cut-off value. For filariasis, Bancroftian filariasis sera (8/10 cases) with the HCF30.10 antigen gave higher ODs than the cut-off value, but all Malayan filariasis sera (10 cases) and onchocercosis (3 cases) showed opposite results. The ODs of both paragonimiasis species gave the same results, over the cut-off value. It seems that antigenic molecules the HCF30.10 antigen are suitable for cross-reaction with antibodies of Bancroftian filariasis, paragonimiasis heterotremus, paragonimiasis westermani and other diseases of nematodiases and cestodiases. Several echinococcosis publications mentioned the potential antigens of HCF at low molecular weights by immunoblot and ELISA. The reactive components of cyst fluid antigens, 8, 16 and 24 kDa are highly antigenic (Lightowlers *et al*, 1989). Two polypeptide bands, 8 and 116 kDa, from hydatid fluids in sheep, goat, pigs and humans are recognized by all hydatidosis sera but not by any sera from patients with other parasitic infections or viral hepatitis, or from healthy controls (Kanwar *et al*, 1992). By isoelectric focusing technique, an antigen B-rich fraction (8 kDa) from *E. granulosus* cyst fluid is useful in positive species-serodetection of cystic and alveolar echinococcosis (Ito *et al*, 1999).

In our diagnostic test, we only tried to use indirect ELISA for a negative screening result because of limited antigen, with only two protoscolex-positive controls and three cases of positive immunoblot, including frequent cross-reactions. A low antibody

response may possibly occur in a hydatidosis case, which causes a false negative. However, indirect ELISA for detecting echinococcosis will use HCF30.10 antigen. In addition, if we could obtain enough cystic fluid antigen, it could be prepared as a potential antigen for ELISA or direct use in an immunoblot test.

ACKNOWLEDGEMENTS

The authors gratefully thank Professor Akira Ito, his staff and Dr Mamuti Wulamu, PhD student, at the Department of Parasitology, Asahikawa Medical College, Hokkaido, Japan for their assistance in confirming serum samples of suspected cystic echinococcosis cases with the potential antigens, which contributed to detecting seropositive echinococcosis cases. The first author personally thank Professor Sornchai Looareesuwan, former Dean of the Faculty of Tropical Medicine, Mahidol University for his partial financial support in practicing echinococcosis serodiagnosis, at Asahikawa Medical College.

REFERENCES

Beaver PC, Jung RC, Cupp EW. Cyclophyllidean tapeworms. In: Clinical parasitology. 9th ed. Philadelphia: Lea and Febiger, 1984:527-43.

Di Felice G, Pini C, Afferni C, Vicari G. Purification and partial characterization of the major antigen of *Echinococcus granulosus* (antigen 5) with monoclonal antibodies. *Mol Biochem Parasitol* 1986;20:133-42.

Gonzales G, Nieto A, Fernandez C, *et al*. Two different 8 kDa monomers are involved in the oligomeric organization of the native *Echinococcus granulosus* antigen B. *Parasite Immunol* 1996;18:587-96.

Gottstein B. Molecular and immunological diagnosis of echinococcosis. *Clin Microbiol Rev* 1992;5:248-65.

Ito A, Wang XG, Liu YH. Differential serodiagnosis of alveolar and cystic hydatid disease in the People's Republic of China. *Am J Trop Med Hyg* 1993;49: 208-13.

Ito A, Ma L, Schantz PM, *et al*. Differential serodiagnosis for cystic and alveolar echinococcosis using fractions of *Echinococcus granulosus* cystic fluid (antigen B) and *Echinococcus multilocularis* protoscolex (EM18). *Am J Trop Med Hyg* 1999;60: 188-92.

Kanwar JR, Kaushik SP, Sawhney IM, *et al*. Specific antibodies in serum of patients with hydatidosis recognized by immunoblotting. *J Med Microbiol* 1992;36:46-51.

Li J, Zhang WB, McManus DP. Recombinant antigens for immunodiagnosis of cystic echinococcosis. *Biol Proced Online* 2004;6:67-77.

Lightowlers MW, Liu DY, Haralambous A, Rickard MD. Subunit composition and specificity of the major cyst fluid antigens of *Echinnococcus granulosus. Mol Biochem Parasitol* 1989;37:171-82.

Lymbery AJ, Thompson RCA. Species of *Echinococcus*: pattern and process. *Parasitol Today* 1996;12:486-91.

McManus DP, Zhang WB, Li J, Bartley PB. Echinococcosis. *Lancet* 2003;362:1295-304.

Maddison SE, Slemenda SB, Schantz PM, *et al*. A specific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 Kda. *Am J Trop Med Hyg* 1989;40:377-83.

Mamuti W, Yamasaki H, Sako Y, *et al*. Usefulness of hydatid cyst fluid of *Echinococcus granulosus* developed in mice with secondary infection for serodiagnosis of cystic echinococcosis in humans. *Clin Diagn Lab Immunol* 2002;9:573-6.

Mamuti W, Yamasaki H, Sako Y, *et al*. Molecular cloning, expression, and serological evaluation of an 8-kilodalton subunit of antigen B from *Echinococcus multilocularis*. *J Clin Microbiol* 2004;42:1082-8.

Nasrieh MA, Abdel-Hafez SK. *Echinococcus granulosus* in Jordan: assessment of various antigenic preparations for use in the serodiagnosis of surgically confirmed cases using enzyme immuno assays and the indirect haemagglutination test. *Diagn Microbiol Infect Dis* 2004;48:117-23.

Ortona E, Rigano R, Margutti P, *et al*. Native and recombinant antigens in the immunodiagnosis of human cystic echinococcosis. *Parasite Immunol* 2000;22:553-9.

Poretti D, Felleisen E, Grimm F, *et al*. Differential immunodiagnosis between cystic hydatid disease and other cross-reactive pathologies. *Am J Trop Med Hyg* 1999;60: 193-8.

Rigano R, Profumo E, Ioppolo S, *et al*. Cytokine patterns in seropositive and seronegative patients with *Echinococcus granulosus* infection. *Immunol Lett* 1998;64:5-8.

Schantz PM, Chai J, Craig PS, *et al*. Epidemiology and control of hydatid disease. In: Thompson RC,

Lymbery AJ, eds. Echinococcosis and hydatid disease. London: CAB International, 1995:233-331.

Schantz PM, Kagan IG. Echinococcosis (hydatidosis). In: Houba V, ed. Immunological investigation of tropial parasite diseases. Edinburgh: Churchill Livingstone, 1980:104-29.

Shepherd JC, McManus DP. Specific and cross-reactive antigens of *Echinococcus granulosus* hydatid cyst fluid. *Mol Biochem Parasitol* 1987;25:143-54.

Shambesh MK, Craig PS, Gusbi AM, Echtuish EF, Wen H. Immunoblot evaluation of the 100 and 130 kDa antigens in camel hydatid fluid for the serodiagnosis of human cystic echinococcosis in Libya. *Trans R Soc Trop Med Hyg* 1995;89:276-9.

Thanakitcharu S, Saenghirunvattana S, Tovaranonte P, Rochanawutnon M. Pulmonary hydatid disease: a case report. *Thai J Tuberc Chest Dis* 1992;13: 245-51.

Von Sinner WN. New diagnostic signs in hydatid disease; radiography, ultrasound, CT and MRI correlated to pathology. *Eur J Radiol* 1991;12:150-9.

Verastegui M, Moro P, Guevara A, *et al*. Enzyme-linked immunoelectrotransfer blot test for diagnosis of human hydatid disease. *J Clin Microbiol* 1992;30:1557-61.

Vogel M, Gottstein B, Muller N, Seebeck T. Production of a recombinant antigen of *Echinococcus multilocularis* with high immunodiagnostic sensitivity and specificity. *Biochem Parasitol* 1988;31:117-25.