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. 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 uniocular echinococcosis or hydatid disease, and E. multilocularis, which causes multilocular or alveolar echinococcosis. 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,
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 (Fig 1). 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 5 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), trichinelliosis (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.
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 cestodiases 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,
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-
Cestode infections: A = cystic echinococcosis, B = cysticercosis, C = taeniasis, D = sparganosis, E = hymenolepiasis nana.
Nematode infections: F = gnathostomiasis, G = strongyloidiasis, H = hookworm infection, I = trichinellosis, J = capillariasis,
K = toxocariasis, L = angiostrongyliasis, M = ascariasis, N = trichuriasis, O = Bancroftian filariasis, P = Mayalan filariasis,
Q = onchocercosis, R = enterobiasis.
Trematode infections: S = paragonimiasis heterotremus, T = paragonimiasis westermani, U = opisthorchiasis, V = minute intestinal fluke,
W = schistosomiasis, X = fascioliasis.
Other infections: Y = creeping eruption, Z = lung infection, AB = protozoan infections; - E. histolytica, - toxoplasma, - Giardia lamblia,
- Blastocystis hominis.
Negative serum: AC = healthy controls.

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

Echinococcosis 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 nematodiasis and cestodiasis. 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.

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REFERENCES


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


