

Circulating Antigen Detection in Patient Serum for Diagnosis of Cystic Echinococcosis

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Hydatidosis is a major public health problem with a worldwide distribution in humans. The purpose of this study is to investigate the circulating antigen in the sera of CE patients. This study was performed on 188 sera which were taken from 181 patients who attended the various departments of Ege University of Medical Faculty and to the laboratory of Parasitology department with the suspicion of Cystic Echinococcosis (CE). Sera were tested with IHA and ELISA for Circulating antibodies and with Circulating Antigen-ELISA (CAg-ELISA) test for circulating antigen. All the patients were questioned personally and clinical data were obtained from the departments where they operated to confirm the diagnosis of CE and 53 serum specimens were shown to be taken from CE proven cases. Hydatid Cyst Fluid (HCF) harvested from the fertile cysts in the liver of the infected sheep was used both in preparing the ELISA and IHA tests for detecting antibodies and for immunizing the rabbits to obtain immune serum. Specific polyvalent immunoglobulin's required for CAg-ELISA test in detecting the circulating antigen for CE in sera, were obtained from the rabbits which were immunized against hydatid cyst antigen and used after being purified with ammonium sulphat (NH_4)₂So₄ precipitation and gel filtration column chromatography performed in the molecular biology department. In 82 specimens with ELISA and in 81 specimens with IHA presence of antibodies, over the cut-off values, in varying titrations were shown out of 188 specimens. In 2(1.1%) patients who were found to negative by both IHA and ELISA, presence of CE was shown after the surgical operation. The specificity (79.26%) and sensitivity (88.68%) of IHA and specificity (78.52%) and sensitivity (90.57%) of ELISA tests were found. In 24(45.6%) specimen with CAg-ELISA test circulating antigen were detected in values over the cut-off level. All patients with positive CAg-ELISA are also confirmed with the personal data obtained from either personally or from the clinics where they were operated. These results indicated that it will be great help to assess antigen detecting tests in additional to antibody detecting tests in the serodiagnosis of patients with suspicion of CE.

Key Words: Hydatidosis, Cystic echinococcosis, CAg- ELISA, IHA, Circulating antigen.

Hydatidosis (echinococcosis), caused by the dog tapeworm *Echinococcus granulosus* is primarily in a domestic cycle that involves dogs definitive host and others domestic animals as intermediate hosts. The disease is well known in sheep herding areas of Asia, Africa, south and Central America¹. This disease leads to medical, veterinary, economic problems and constitutes a major public issue². Various surveys in Turkey and Iran either have found hydatid cysts in sheep,

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camels, cattle and goats³⁻⁶. Serodiagnosis of hydatid disease caused by *Echinococcus granulosus* in man has been one of the useful methods for clinicians since no parasitological diagnosis is possible by non-invasive procedures. The clinical signs of the disease are non-specific however parasitic antigens are present in the hydatid fluid (HF) and the somatic tissues of the metacestode by Two denominated antigens⁵ and antigen B⁷.

Diagnosis of human CE commonly involves serological confirmation based on specific antibody detection by indirect haemagglutination test (IHA) or Enzyme-linked immunosorbent assays (ELISA). Both tests have sensitivity but variable specificity with crude hydatid cyst fluid antigens, which are usually derived from sheep or cattle cysts⁸. In order to improve specificity for immunodiagnosis of human CE, hydatid cyst fluid antigens has been used as antigen for immunodiagnosis of human cystic echinococcosis and follow up of patients after surgical operation⁹.

Antibody detection assay is used when it is not possible to distinguish between recent and past infections and cannot be used for assessment of the efficacy of treatments¹⁰. Circulating antigen in serum and other body fluids has been described in a variety of parasitic infections, including CE. The detection of circulating hydatid antigen in serum in active infections may be more useful than the detection of circulating serum antibodies in the serum for the diagnosis of CE¹¹. Circulating immune complexes and circulating antigen have been demonstrated in sera from hydatidosis patients in several studies, Gottstein 1984¹², Craig 1986¹³, Shariff & Parija 1993¹⁴ and Barbieri *et al.*, 1994¹⁵, but it has not been possible to achieve 100% results. Therefore, the detection of circulating hydatid antigen in serum may be more useful than the detection of circulating serum antibodies in the diagnosis of the active or past CE. In this study, we did the detection of circulating antigen in the sera of CE patients of Ege University Hospital.

Methodology

In this research, from 181 patients who were affected by hydatid cyst, 188 blood serums were taken. These patients were referred to the medical school affiliated hospital of Ege, with the first diagnosis of hydatid cyst; these patients'

samples were sent to the serological laboratory of parasitology department of medical school. 5cc blood was taken from all of the patients and the serum of the blood were separated and stored in -70⁰c.

Specific diagnosis of hydatidosis is based on immunodiagnostic methods, in this research serums were examined for their antibody titer by IHA and ELISA¹⁶. To find CAg in the serums, CAg-ELISA test was applied. All the information about the patient's sonography, CT scan, MRI and radiography were gathered.

Antigen preparation

To use in serological tests and to immunize the laboratory animals, the liver of the sheep which had hydatid cyst were gathered¹⁷. Fertile hydatid cysts were aspirated by syringe. After gathering in different test tube, then the supernant of the tubes were aspirated .all of samples were centrifuged for 30 minutes with a centrifuged in 5000 rpm and -4⁰c. The supernant of the tubes were aspirated again and they were passed through a 5um filter. The liquid fraction stored at -20⁰ c until use. The prepared antigen was measured according to their protein density by spectrophotometer (Bausch and Lomb spect 21) with 260 nm and 280 nm wave length¹⁸.

Antibody preparation

To measure Circulating Antigen (CAg) in the patients who were affected by hydatid cyst, polyvalent antibody was necessary, to prepare the antibody; the antigen which was obtained by hydatid cyst was injected into the rabbits¹⁹. These antigens were injected with 0.5cc freund's complete adjuvant. After first times, they were re injected sub-cutaneously with 0.5cc Freud's incomplete adjuvant mixed with an equal volume of the same antigen. The injection was done 5 times every week. Each time, before injection, the rabbits were blood sampled by the amount of 1cc. The serums were separated then by using IHA and ELISA tests, antibody titer measured²⁰. After the fifth injection, it was recognized that antibody titer reach to its maximum titer which doesn't show any change comparing to the forth injection. The rabbits were anesthetized temporally to obtain 5cc blood. The serum of the blood was separated and stored -20⁰ c until use.

Purification of the antibodies

To separate the antibody from other

proteins, which exist in the serums of the immunized rabbits, $(\text{NH}_4)_2\text{SO}_4$, gel filtration and column chromatography methods were used²¹. All of serums were immunized by $(\text{NH}_4)_2\text{SO}_4$, then to obtain pure antibody the mentioned suspension was purified by gel filtration (G-200, for gel filtration, Lot No. 6434, particle size 40-120um, Pharmacia Uppsala – Sweden) and column chromatography²².
ELISA

Briefly, ELISA was described by Farragut et al, the optimal concentration of rabbit anti-HCF IgG (5 µg/ml) was diluted in PBS and 100 µl of solution was placed into a 96-well ELISA plate followed by incubation at 37° for 3 hours. Wells of plate were washed 5 times with PBS plus 0.1% Tween 20. All serums from surgically confirmed CE patients (diluted 1/10 with 50 mM bicarbonate buffer pH 9.6) were added into the wells and the plate was incubated at 37° for 2 hours. After two hours incubation the plates were removed by washing 5 times in PBS. After washing, 100 µl/well of peroxidase – conjugated rabbit anti-HCF antibodies were added. 100 µl of blocking buffer (1% BSA/PBS) was added and the plate was incubated for one hour at 37°. After one hour incubation the plates were washed 5 times with PBS. After washing, 200 µl of peroxidase/substrate

solution were added and incubated for thirty minutes at room temperature, finally one drop of H_2SO_4 (2 M) was added to each well and the absorbance measured in micro plate reader at 490 nm by method of Farragut, *et al.*, 1998²³.

RESULTS

In this research, from all 181 patients who were hospitalized because of HC, 188 blood samples were taken. To diagnosing of presence of antibodies, they were examined by using IHA and ELISA tests, further more, to diagnose the presence of parasite antibody, the samples examined by using CAg-ELISA. Out of the total number of patients, complete data of 128 cases were received; 53 people had an operation. The presence of HC was approved by parasitology department. In all the 188 under study cases, by using ELISA test, 82 (43.6%) people were proved to have above cut-off level antibody but by using IHA test 81 (43%) samples were recognized to have above cut-off level antibody titer, only two samples were recognized with no considerable antibody level. However, later surgical operations proved the presence of HC.

Table 1. Circulations Antigen and Antibody detection in human sera

Test name	Specificity(%)	Sensitivity (%)
ELISA(Ab)	78.52	90.57
IHA(Ab)	79.26	88.68
IHA+ELISA(Ab)	78.89	84.62
CAg-ELISA(CAg)	100	45.28
ELISA + CAg-ELISA (Ab +CAg)	89.26	67.92
CAg-ELISA+IHA(CAg + Ab)	89.63	66.98
CAg-ELISA+ELISA+IHACAg + Ab +Ab))	85.93	74.84

To know the presence of circulating antigen all 53 samples were tested by CAg-ELISA test. 24 (45.6%) people proved to have CAg. In one case the presence of antigen was not proved by using CAg-ELISA. After operation it was recognized that the patient does not have HC. After the operation, it was found out that all of the cases were affected by HC. The serums were analyzed serologically. The sensitivity of ELISA test was

90.57% and 78.12%. The specification and sensitivity of IHA was 79.66% and 88.88% respectively. So there is a correlation between two tests.

In this research, the serums were examined by CAg-ELISA tests to detect circulating antigen. The specification and sensitivity of CAg-ELISA test were 100% and 45.6% respectively.

DISCUSSION

Diagnosis of hydatid disease relies on demonstrating the hydatid cyst by radiology, ultrasonography, computed tomography, or magnetic resonance imaging and on serology (1). The variable sensitivity and poor specificity of most of the available serological tests limit their diagnostic value in suspected cases of hydatidosis for this reason; several assay systems generally have been used simultaneously to increase accuracy, sensitivity and specificity (24). The use of conventional antibody detection methods for specific hydatid disease diagnosis in humans has often been compared by the cross-reaction between hydatid antigen and antibody in sera of patients infected with other parasites, especially *Taenia solium*, cysticercosis, filariasis and fascioliasis (25). On the other hand, the antibody is not raised in some of the hydatidosis patients or the titer is low especially in old persons and infants. Also in cerebral, ocular, and calcified cysts, the antibody titer is low and cannot be easily detected (24). Attempts to affinity-deplete the non-specific components in the hydatid antigen preparation enhances in the hydatid antigen preparation enhances the specificity of the diagnostic test to some extent, but the process is often laborious and extent, and results in less of sensitivity.

Craig (25) found that the sensitivity of CAg-ELISA for Turkana patients was 90% and for Uk patients 50%. The enhanced antigen detection for Turkana patients in CAg-ELISA was possibly due to the fact that hydatid cysts of Turkana patients tended to fast growing, highly fertile and active, this producing larges amounts of CAg (25).

Analysis of the antigen detection data in relation to the serum antibody titers showed no correlation between antibody titers in ELISA and absorbance values in the MAb-based CAg assay. Nevertheless the potential of the CAg-ELISA using MAb was limited by the fact that it had a relatively low sensitivity, compared with those using polyclonal antiserum. Naturally polyclonal antisera tend to provide better sensitivity than MAb because they can react with multiple epitopes of thy antigen. Another major reason for

the low sensitivity observed in this study was probably due to interfering effect of normal human serum components on the binding of MAb or NMIgG with SHCF(26). Indeed the in reference with immunoassays by human serum factors has been noted previously.

A number of parasites, including metacestodes of *Echinococcus*, produce soluble antigens which are released into host tissues and the blood stream²⁷. Circulating antigen has been detected previously in three experimental larval cestode infection, i.e. mesocestoides corti and *Echinococcus multilocularis* infections of mice CAg and CIC have been detected by ELISA in human hydatid infections and higher levels of both were found in turkana (Kenya) compared to uk cases(25). An immunodiagnostic test based on an ELISA for detection of specific CAg could be very useful particularly as encouraging results were obtained in detecting CAg in false negative sera the specificity of this CAg-ELISA for human hydatidosis needs to be assessed^{13, 28}.

In this study, 188 serums were examined by CAg-ELISA test to find out circulating antigen. Twenty four serums were recognized to have HC. On the other hand only 53 people had a surgical operation. Department of pathology and parasitology approved the presence of HC. In 24(45.6%) cases out of 53 which were proved to have HC the presence of circulating larval stage antigen was recorded by using CAg-ELISA test. The results of this study have a close relationship with the findings of Devi and Craig (17, 25).

Craig (Craig) observed the presence of antigen in about 50% of the patients by using CAg-ELISA test (13). In other study, by Gottstein using the immunoelectrophoresis, circulating antigen could be detected in four of 10 patients (40%) with cystic echinococcosis (12). some works tried to detected hydatid cyst antigens in serum. These tests also resulted in various rates of sensitivity and specificity, Parija 1998(29), Ravinder et al. 2000(24), Devi et al. 2003(17). This study indicated that the antigen detection assay might be a useful method for diagnosis of patients with hydatidosis, although the antibody detection assay has a relatively high sensitivity.

REFERENCES

1. Eckert J, Deplazes P, Craig PS, Gemmell MA, Gottstein B, Heath D, Jenkins DJ, Kamiya M, Lightowlers M. Echinococcosis in animals: clinical aspects, diagnosis and treatment. In Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS eds, WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. Paris, France. *World Organization for Animal Health*. 2001, p 72-79.
2. Schantz P. M., J. Chai, P. S. Craig, J. Eckert, D. J. Jenkins, C. N. L. Macpherson, and A. Thakur. 1995. Epidemiology and control of hydatid disease. In. Echinococcosis and hydatid disease R.C.A. Thompson and A.J. Lymbery (eds.). CAB International, Wallingford, U.K., p. 233–331.
3. Sarkari B, Sadjjadi SM, Beheshtian MM, Aghae M, Sedaghat F, Human cystic Echinococcosis in Yasuj district in southwest of Iran an epidemiological study of seroprevalence and surgical cases over a ten-year period. *Zoonoses Public Health* 2010; **57**(2): 146-150
4. Sadjjadi SM, Abidi H, Sarkari B, Izadpanah A, Kazemian S. Evaluation of enzyme-linked immunosorbent assay, utilizing native antigen B for serodiagnosis of human hydatidosis. *Iran J Immunol* 2007; **4**: 167-172.
5. Altintas N. Past to present : echinococcosis in Turkey. *Acta Trop* , 2003; **85**: 105-112.
6. Cetinkaya Z, Ciftci IH, Demirel R, Altindis M, Ayaz E. A seroepidemiologic study on cystic echinococcosis in Midwestern region of Turkey. *Saudi Med J* 2005; **26**: 350-351.
7. Pozzuoli R, Piantelli M, Perruci C, Arru E, Musiani P. Isolation of the most immunoreactive antigens of *Echinococcus granulosus* from sheep hydatid fluid. *J Immunol* 1975; **115**: 1459-1463.
8. Eckert J, Deplazes P. Biological, Epidemiological and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clint. Microbiol. Rev.* 2004, **17**: 107-135.
9. Ortona E, Rigano S R, Buttari B, Delunardo F, Ioppolo S, Margutti P, Profumo E, Teggi A, Vaccari S, Siracusano A. An update on immunodiagnosis of cystic echinococcosis. *Acta Trop* 2003; **85**: 165- 171.
10. Doiz O, Benito R, Sbihi Y, Osuna A, Clavel A, Gomez-Lus R. Western blot applied to the diagnosis and post-treatment monitoring of human hydatidosis. *Diag Microbiol Infect Dis* 2001; **41**: 139-142.
11. Ravinder PT, Parija SC, Rao KS. Evaluation of human hydatid disease before and after surgery and chemotherapy by demonstration of hydatid antigens and antibodies in serum. *J Med Microbiol*, 1997; **47**: 859-864.
12. Gottstein B. An immunoassay for the detection of circulating antigens in human echinococcosis. *Am J Trop Med Hyg* 1984; **33**: 1185-1191.
13. Craig PS, Nelson GS. The detection of circulating antigen in human hydatid disease. *Ann Trop Med Parasitol* 1984; **78**: 219-227.
14. Shariff, M., Parija, S.C., Co- agglutination, (Co-A) test for circulating antigen in hydatid disease. *J. Med. Microbiol.* 1993; **38**: 391-394.
15. Barbieri, M., Severi, M.A., Pirez, M.I., Battistoni, J., Nieto, A., Use of specific antibody and circulating antigen serum level in the hydatid immunodiagnosis of asymptomatic population. *Int. J. Parasitol.* 1994; **27**: 937-942.
16. Siavashi MR, Taherkhani H, Rezaei KR, Razavi Deligani MR, Assmar M. Comparison of dot-ELISA and sandwich ELISA diagnostic tests in detection of human hydatidosis. *Iran Biomed J* 2005; **9**: 91-94.
17. Devi CS, Parija SC. A new serum hydatid antigen detection test for diagnosis of cystic echinococcosis. *Am. J. Trop. Med. Hyg.* 2003; **69**(5): pp. 525-528.
18. Liu D, Lightowlers MW and Rickard MD. Evaluation of a monoclonal antibody based competition ELISA for the diagnosis of human hydatidosis. *Parasitology* 1992; **104**: 357-361.
19. Shariff, M., Parija, S.C., Co-agglutination (Co-A) test for circulating antigen in hydatid disease. *J. Med. Microbiol.* 1993; **38**: 391-394.
20. Parija SC, Mishra SR, Rao RS, 1986. Sensitized chick cells in the indirect haemagglutination test for echinococcosis. *J Med Microbiol* **22**: 237-239.
21. Hbert GA, Peham PL, Pittman B., Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Applied Microbiol.* 1973; 26-36.
22. Lightowlers MW, Rickard MD, Honey RD, OBendorf DL and Mitchell GF. Serological diagnosis of *Echinococcus granulosus* infection in sheep using cyst fluid antigen processed by antibody affinity chromatography. *Australian Veterinary* 1984; **61**(4): 101-108.
23. Ferragut G, Ljungstrom I, Nieto A. Relevance of circulating antigen detection to follow-up experimental and human cystic hydatid infections. *Parasite Immunology*, 1998; **20**: 541-549.
24. Ravinder PT, Parija SC, Subba Rao KSVK. Urinary Hydatid Antigen by

- Coagglutination, a Cost-Effective and Rapid Test for Diagnosis of Cystic Echinococcosis in a Rural or Field Setting. *Journal of Clinical Microbiology*, Aug, 2000, p. 2972-2974.
25. Craig, P.S., Detection of specific circulating antigen, immune complexes and antibodies in human hydatidosis from turkana (Kenya) and Great Britain, by enzyme-immunoassay. *Para. Immunol.* 1986; **8**: 171-188.
26. Liu D, Rickard MD, and Lightowers MW. Assessments of monoclonal antibodies to Echinococcus granulosus Antigen 5 and Antigen B for detection of human hydatid circulating antigens. *Parasitology*, 1993; **106**: 75-81.
27. Sunita T, Dubey ML, Khurana S, Malla N. Specific antibody detection in serum, urine and saliva samples for the diagnosis of cystic echinococcosis. *Act Trop.* 2007; 101: 187-191.
28. Carmena D, Benito A, Eraso E. Antigens for the immunodiagnosis of Echinococcus granulosus infection: an update. *Acta Trop* 2006; **98**: 74-86.
29. Parija, S.C., A review of some simple immunoassays in the serodiagnosis of cystic hydatid disease. *Acta. Trop.* 1998; **70**: 17-24.