

## Comparison of serological and clinical findings in turkish patients with cystic echinococcosis

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### Summary

Cystic echinococcosis (CE), caused by the cestode *Echinococcus granulosus*, is potentially dangerous for humans. The aim of this study was to examine serological and clinical findings regarding cysts localisation and individual responses in 54 patients with CE. The majority of patients in this study were females (63 %) and the average age was 46.3 years. Most of the patients lived in rural areas or kept a dog (46 %) for a long time. The most frequent symptoms were hypochondrial pain (48.9 %), epigastric discomfort (27.7 %), vomiting (21.3 %), minor cough (12.8 %), urticaria (6.3 %), weakness (4.3 %), fever (2.1 %), side- or back-ache (4.3 %). However, 17 % of the patients showed no symptoms. In every case, the ultrasound (USG) and/or computer tomography (CT) investigations were positive. In most cases (53.2 % of the patients) a single cyst was found but 46.8 % of the patients had multiple cyst formations (from 2 to 9 cysts) located in the liver. Sporadic lung, splenic, mesenteric, tibial and cerebral localisations were also found. The patients were individually treated with albendazole (10 – 15 mg/kg) five days prior and six months after the surgical treatment. Serum samples were investigated by the serological techniques: IHAT, ELISA and Western blot using hydatid fluid antigen. In the patient sera, the specific antibody levels were mostly increased after surgery. Different results were obtained only in two patients. In the first case, seroconversion was delayed. In the other case all ELISA results were negative, however, the Western blot analysis and surgery proved the presence of CE. The results suggest that the different antibody response of patients depends on the individual immune response. Multiple localization and various stages of CE cysts demonstrate the necessity of a complex approach for the confirmation of a correct diagnosis.

**Key words:** *Echinococcus granulosus*; cystic echinococcosis; clinical finding; serodiagnosis; Turkey

### Introduction

Cystic hydatid disease is the larval cystic stage of a small tapeworm *Echinococcus granulosus* and is potentially dangerous for humans. It is an important public health problem in many regions of the world, both in endemic areas and in regions where the disease is emerging or re-emerging (Craig *et al.*, 1996). New cases are mostly found in areas where dogs and livestock coexist. In primary echinococcosis, metacestodes develop from oncospheres after per-oral infection with *E. granulosus* eggs. In secondary echinococcosis, larval tissue proliferates after being spread from the primary site of the metacestode. This can occur by trauma such as spontaneous rupture of cyst or during medical interventions. Hydatid cysts have the ability to grow quite large and manifest as cysts especially in the liver (65 %) and lungs (25 %), but also in various organs including the brain, heart, bones, kidney and spleen (Gottstein & Hemphill, 1997). Clinical manifestation depends on the localization and size of the cyst. Imaging techniques (US, CT) and serology provide both useful and complementary information on the character of the cyst that may be relevant for therapeutic intervention (Lightowlers & Gottstein, 1995; Altintas *et al.*, 1999a). Serological tests provide an extremely important information related to the diagnosis and prognosis of disease. Among the serological tests, the IgG-ELISA and IHAT are widely used for diagnosis and for monitoring the disease in the postoperative period (Force *et al.*, 1992; Zarzosa *et al.*, 1999; Rebhandl *et al.*, 1999).

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The aim of this study was to compare serological and clinical findings regarding cysts localisation and individual serological responses in patients with cystic echinococcosis.

## Material and Methods

### *Patient sera*

Patients included in the study were hospitalised in Atatürk Research and Training Hospital Izmir, Turkey, from 2002 to 2004. Sera from a total of 54 patients (48 with hepatic-, 1 with lung-, 1 with tibia-, 1 with cerebral-paraoxipital-, 1 with neck postcervical cystic echinococcosis, 1 patient with both, liver and spleen located cysts and 1 patient with multilocalisation of hydatid cysts in the liver, spleen and mesenterium were analysed. The majority of the patients had single cystic lesion situated in one lobe of the liver (usually in the right lobe). All sera were investigated by indirect hemagglutination test (IHAT) and enzyme linked immunosorbent assay (ELISA). Sera of four patients were examined before and after surgery. Biochemical, haematological and histological examinations of the patients were done by routine clinical laboratory methods. The US and CT examinations were also performed.

### *Antigen*

Sheep hydatid fluid (HF) was collected from fertile cysts obtained from slaughterhouse. After centrifugation at 10 000g for 30 min at 4°C, antigen was concentrated in an Amicon ultra filtration cell with a YM2 membrane (Amicon Corp., Bedford, USA) and kept at -20°C for subsequent use.

### *Determination of Echinococcus-specific antibody production by IHAT*

An indirect hemagglutination test (IHAT) was used according to the method described by Garabedian *et al.* (1957). Serial dilutions, starting at 1/8 were examined and titer of 1/32 or higher was considered positive.

### *Determination of Echinococcus-specific antibody production by ELISA*

Standard indirect ELISA was carried out on polystyrene microtitre plates with 96 wells (F-Form; Maxisorp, Nunc, Roskilde, Denmark) coated with 100 µl/well of hydatid fluid (at a concentration of 5 µg of proteins per well) in carbonate-bicarbonate buffer, pH 9.6, overnight at +4°C. Plates were then washed 3 times in 0.5 % PBS Tween 20 (PBS-T) and blocked for 1 hour with 5 % non-fat milk with PBS-T at 37°C. Then 100 µl of serum samples diluted 1/200 in 5 % non-fat milk with PBS-T were added and incubated for 1h at 37°C. After washing, horseradish peroxidase-conjugated anti-human IgG (Sigma, St.Louis, MO, USA) diluted 1/10000 was applied for 1h. After repeated washing, the chromogen o-phenylenediamine (Sigma) with H<sub>2</sub>O<sub>2</sub> was added as substrate. The reaction was stopped with 0.5 M H<sub>2</sub>SO<sub>4</sub> after 15 min. Optical densities (OD) were read spectrophotometrically at 492 nm (Thermo Labsystems Opsys MR, USA). Cut off values were determined by

taking the average OD of negative control sera plus 3 standard deviations (SD).

### *SDS-PAGE electrophoresis and Western blot analysis*

Sera of patients positive by ELISA were confirmed by the Western-blot technique. Electrophoresis (ELFO-SDS PAGE) was performed using a Bio-Rad Mini Protein Slab Cell (Bio-Rad Laboratories, CA, USA) on a 12 % SDS-polyacrylamide gel and 4 % stacking gel under reducing conditions (Laemmli, 1970). Hydatid fluid antigen from fertile cysts of sheep was electrophoresed at 200V for 1hour at a room temperature. Low molecular weight markers (pre-stained SDS-PAGE standards, Bio-Rad) were included in each electrophoretic run. Following electrophoresis, proteins were transferred to nitrocellulose (NC) membrane in Tris-glycine buffer (pH 8.8) at constant voltage of 250V for 1hour using a Bio-Rad Trans-Blot Cell. After blotting, the NC membrane was cut into 2.5 mm - wide strips and blocked with 5 % bovine serum albumin in PBS (pH 7.2) for 30 min at 37°C. Sera diluted 1/50 with 5 % bovine serum albumin in PBS were incubated for 1 hour at 37°C. The strips were washed three times with PBS-Tween 20 and reacted with horseradish peroxidase conjugated anti human IgG in dilution of 1/500 for 1hour at 37°C with continuous shaking. Subsequently, the strips were washed again three times with PBS-Tween 20 and bands were developed using 0.05 % 4-chloro-1-naphthol in PBS (pH 7.2) and 0.03 % hydrogen peroxide.

### *Data analysis*

The results were evaluated statistically by the  $\chi^2$ -test. The comparison of the two techniques was done using Fisher Exact test. The differences among the groups were considered significant at the values of  $P < 0.05$ .

## Results

The 63 % of the 54 analysed patients represented were females ( $P < 0.05$ ) (Tab. 1). The average age of the patients was 46.3 years (the youngest patient was 14 and the oldest 78 years old). Most of them lived in rural areas without significant differences ( $P > 0.05$ ) or kept a dog (46 %) for a long time.

The majority of the patients had from 1 to 9 of echinococcal cysts in the liver (88.9 %). In one case, with a high specific antibody response, the affected organ was lung and in other cases a single splenic-, mesenterial-, tibial and cerebral localisations were identified. In one case the patient died (73-year-old female with 2 cysts in the liver). The patients were individually treated with albendazol (10 – 15 mg/kg) usually five days prior and six months after surgical treatment.

In the patients examined prior and after the surgery positive ELISA remained at a high or even elevated. Despite of positive CE finding serological results in two patients were totally negative. In one ELISA negative patient, IHAT finding was low positive and Western blot confirmed the positive CE (Tab. 1).

Table 1. Personal data and serological results of patients with cystic echinococcosis

Patients	Number of examined	Mean age	Residence U/R	Dog ownership	IHA	ELISA	Western blot
Male	20	46.6	8/12	10	19	19	6*
Female	34	45.5	17/17	15	33	32	22**
Total	54	46.3	25/29	25	52	51	28

U – urban; R – rural; \* samples of eleven patients not examined with WB; \*\* samples of nine patients not examined with WB

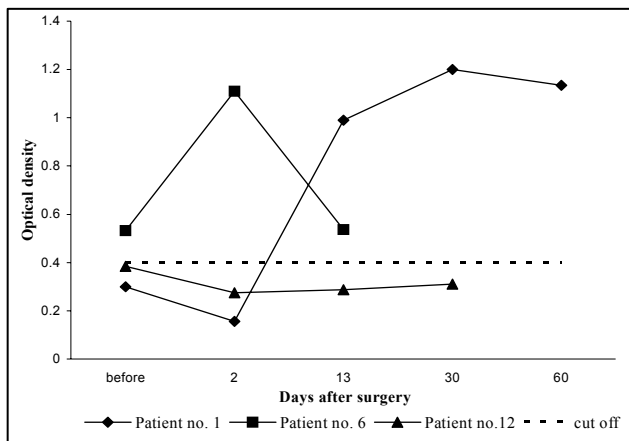


Fig. 1. Different outcome in specific echinococcal IgG antibodies after the surgical treatment

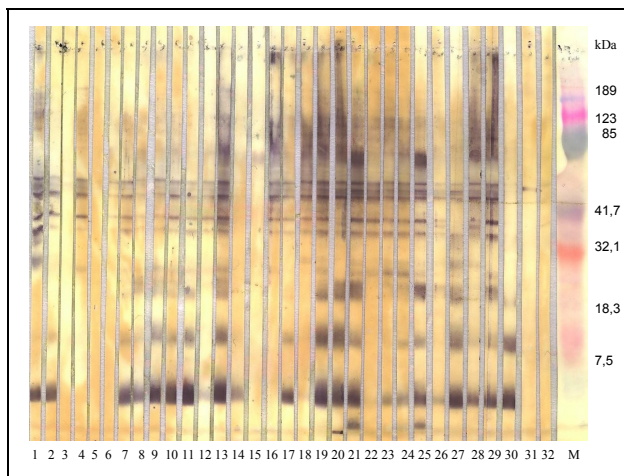


Fig. 2. Western blot analysis of selected patient sera  
The lines represented 1, 2 – positive control sera; 3, 5 – negative control sera; 4, 6 – 31 patient sera showed in the Table 2; 32 – patient with trichinellosis; M – marker

Following the specific antibodies kinetics in patient No.1 a delayed seroconversion and negative serological results were observed prior and two days after the surgery. The first positive results were found only two weeks after the surgery with an increasing level during the first and second month after the surgical treatment (Fig. 1). In contrast, antibody levels in patient no. 6 declined after the surgery. In patient No. 12, ELISA results were negative before and after surgical treatment. However, IHAT and Western blot

analysis proved the presence of cystic echinococcosis. The results of Western blot analysis (Fig. 2) samples from selected patients are shown in the Table 2. These patients have different cysts localisation and serological results with confirmed CE.

According to the clinical findings the more frequent symptoms were hypochondrial pain (48.9 %), epigastric pain (27.7 %), vomiting (21.3 %), cough (12.8 %), urticaria (6.3 %), weakness (4.3 %), fever (2.1 %), side- or backache (4.3 %), whereas 17 % of the patients had no symptoms. In all cases, USG and/or CT examinations were positive.

## Discussion

Diagnosis of CE in humans is not easy and depends on the diagnostic facilities available in hospitals and laboratories, as well as the anatomical localisation of cysts (Altintas & Yazar, 1999). The clinical features of CE are highly variable. The spectrum of symptoms depends on the involved organs; size of the cysts and their sites within the affected organ or organs. Additionally the interaction between the expanding cysts and adjacent organ structures; or complications caused by rupture of the cysts; bacterial infection, spread of protoscolices and larval material into bile ducts or blood vessels may also considerably contribute to the outcome of CE (Brunetti & Filice, 2005). In this study the echinococcal cysts were mostly localised in the liver. Seldomly the other organs as the lung, spleen, brain or bones were affected. In most cases, a single cyst was found (53.2 %) but 46.8 % of patients had multiple cyst formations with various numbers. The most frequent clinical symptoms were hypochondrial and epigastric discomfort, vomiting, less often cough, urticaria, weakness, fever, side- or backache. However, in some cases (17 % of patients) clinical symptoms were absent. No correlation was found between clinical symptoms and specific antibody levels. In accordance with other authors (Kinčeková *et al.*, 1996, Altintas *et al.*, 1999b) the rural population showed a higher positivity than the urban population, as well as, significantly more women were infected in comparison to the men.

The degree of antibody response to the cyst is mediated by its location and degree of calcification. Normally, liver cysts produce a higher antibody response than lung cysts (Craig & Nelson, 1984; Gottstein, 1992; Ramos *et al.*, 2001). While one patient from our study with lung CE showed high antibody response in all serological tests used three different antibody patterns were detected after the surgical treatment. A delayed seroconversion was found in

Table 2. Site of infection and serological results of patients with confirmed CE (\*results of the same patient within 2 weeks interval; <sup>+</sup>titer 1/32 is considered as the positive threshold value; <sup>++</sup>cut off value is 0.440)

No. of strips in Wb	Organ/number/ size of cysts (cm)	IHAT titres <sup>+</sup>	ELISA-IgG OD <sup>++</sup>	Western blot (kDa)
4	liver/3/ 7, 6, 8	1/1024	1.314	28
6	liver/1/15.7x9x11;right lob	negative	0.553	negative
7	liver/1/4x4	1/32	0.425	7, 16 – 18
8	cerebral – paraoxipital	1/128	0.985	8; 16 – 18; 26 – 28
9	liver/1/ right lob 10x10	1/64	0.538	7, 16, 18, 26, 28
10		1/16	1.280	8; 16 – 18; 26 – 28; 56 – 65
11	neck – postcervical	1/32	0.623	8; 16 – 18; 26 – 28; 56 – 65
12	5x4 liver	1/32	0.383	8; 26
13	liver/2/ right lob 10x8x5; 1x1	1/256	1.001	8; 16 – 18; 26 – 28; 56 – 65 strong fuzzy band
14	liver/3/ right lob 3x4; 2 3x3; 6x3.5	1/8	1.100	7, 16 – 18 weak
15	liver/1/ right lob /12x10	1/16	0.320	negative
16	liver/1/ right lob/ 10x8	1/1024	2.100	above 35
17	liver	1/128	0.640	7, 16
18	liver/2 /right lob 9; 9 spleen hilus/ 1 / 7x7	1/1024	0.815	7, 28 weak
19	liver/ a few/ 5 right lob	1/1024	0.950	8; 16 – 18; 38; 26 – 28; 56 – 65
20	liver/1/16x15x16 right lob	1/512	2.010	8; 16 – 18; 26 – 28; 56 – 65
21; 25*	liver/1/ 14x14	1/4096	2.012; 2.125	8; 16 – 18; 38; 26 – 28; 56 – 65
22	tibia	1/256	0.764	8
23; 24*	liver/1/ 10 left lob	1/2048	2.115; 2.1	8; 16 – 18; 28; 56 – 65
26	liver	1/128	1.250	7, 26
27; 30*	liver/1/ right lob 10x7.5; vesica 19x12	1/256	1.320	8; 16 – 18
28	liver/4/ right lob 2 cysts 10x10x14; 5x12x9; left lob 2 cysts 4.5x 6x5; 2x3x3.	1/256	1.109	8; 16 – 18; 38; 56 – 65
29	liver	1/4096	1.989	7, 16, 18, 26 – 28
31	pelvic mass, liver mass, over Ca	negative	negative	negative

the first patient with multiple hepatic finding of totally 6 cysts. In this case specific antibodies were detectable only 2 weeks after surgery intervention, with subsequently significant increase. In the second patient a reversal result with decrease of antibody level after surgical treatment was found. In the third patient no specific antibody response was detected. These results suggest that different

antibody responses of the patients depend on the individual immune response and localisation or character of the cysts. Our data is comparable to Rigano *et al.* (1995) who investigated humoral immune parameters which indicate the effectiveness of treatment with respect to the division of the patients on full, partial, low and non-responders. In the presented study, qualitative and quantitative analy-

ses of selected sera were performed in comparison with clinical features of the patients. For example, the antigen of approx. 65 kDa was not recognised by sera in the HF antigen of *E. granulosus* (strips No. 5, 14, 15, 30). The most frequently recognised antigens were those with molecular weights of 8, then fuzzy bands of 16 – 18, as well as 24, 26 – 28 kDa. Other authors (Yazar & Altintas, 2003; Turčeková *et al.*, 2004), using hydatid fluid from fertile sheep cysts of *E. granulosus*, identified different major proteins in the range of 8(12), 16, 24, 28, 38 and 116 kDa. For the species differentiation by the Western blot technique using a whole larval antigen from *E. multilocularis*, the authors Liance *et al.* (2000) indicated 97 % of *Echinococcus*-infected patients. The assay allowed them to distinguish correctly between *E. granulosus*- and *E. multilocularis*-infected patients in 76 % of the cases. In the presented study, all the examined patients were from Turkey, which is considered as an endemic area for the occurrence of *E. granulosus*. From the diagnostic point of view 8 kDa band is sufficient for the determination of specific diagnosis of this serious disease.

In conclusion, there is no “general rule” in the diagnostic approach of CE. Each clinical case must be considered individually according to the internationally accepted recommendations and the physician’s personal experience (Pawłowski *et al.*, 2001). A complex approach for the establishing the correct diagnosis is useful. Early diagnosis and subsequent treatment may reduce mortality (Wen *et al.*, 1993; Lightowlers & Gottstein, 1995; Eckert & Thompson, 1997).

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