Full Length Research Paper

Utilizing indirect fluorescence antibody testing and western blot analysis in cystic echinococcus diagnosis and post-surgical monitoring

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Cystic echinococcosis (CE) is a complicated zoonotic infection with diagnosis and treatment. In this study, we aimed to determine the diagnostic performance parameters of Indirect Fluorescence Antibody (IFA) method and also the usability of Western Blot (WB) method for following up CE cases by detecting the antigenic patterns. Cases diagnosed as CE in General Surgery Department of Cerahpasa Faculty of medicine and a selected control group were included in this case-control and cross-sectional study between January 2010 and December 2010. Laboratory studies were performed in Serology/ELISA laboratory of Medical Microbiology Department. Clinically, radiologically and serologically (IHA, ELISA) diagnosed 110 patients with CE, and 80 healthy control group (HCG) individuals were included in the study. *Echinococcus granulosus* specific antigen IgG test was applied by IFA method in CE cases and HCG. *E. granulosus* specific antigen patterns were also are detected with WB method in CE cases and HCG. According to the results, performance parameters of IFA test in CE diagnosis were calculated as 100, 93, 95, 100 and 94% for sensitivity, specificity, positive predictive value, negative predictive value and Kappa values, respectively. Ninety six cases were detected positive with WB method. p7 and other accompanying bands were detected in 50 out of this 96 cases and only p39 band was detected in 45 cases. In conclusion, using IFA method in the diagnosis of CE which is a complicated infection disease can be effective in the presence of an appropriate microscope and experienced expert and we also suggest the use of WB method can be useful especially in parasitic treatment or after spontaneous cyst calcification in post-operative period.

Key words: Indirect fluorescence antibody, cystic echinococcus.

INTRODUCTION

Larvae of *Echinococcus granulosus* causes zoonotic disease, cystic echinococcosis (CE), in humans and herbivores such as sheep and cattle. Echinococcal cysts locate particularly liver but other organs such as the spleen, brain, heart and kidneys can be also targets. Humans function as accidental hosts, because they are usually a 'dead end' for the parasitic infection cycle (Eckert et al., 2000; Sadjjadi, 2006; Dalimi et al., 2000; Zhang et al., 2003; Siracusano et al., 2008). Stock

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raising is the principal means of livelihood of the many people who live in Turkey. CE causes great economic loss in livestock and raises important public health concerns because of not taking preventive measures efficiently.

Serological tests, imaging techniques such as ultrasonography or radiology with clinical findings detect CE the pre-operative diagnosis and post-operative follow-up of CE (Grimm et al., 1998; Doiz et al., 2001). Serological tests such as immunoelectrophoresis, double diffusion in agar, or indirect hemagglutination are being replaced by more sensitive assay methods such as enzyme-linked immunosorbent assay (ELISA), immunoblot (IB), and indirect immunofluorescent antibody test (IFA) (Virginio et al., 2003). The serologic diagnosis of CE is strongly dependent on the antigen used, thus explaining the lack of sensitivity, specificity and concordance among different techniques, standardization of an antigen that enables postoperative follow-up of the disease being necessary (Doiz et al., 2001; Aslan et al., 2011; Lightowlers and Gottstein, 1995). The optlmal therapy of CE is surgical. Serology (IHA and ELISA) has been one of the methods selected for the pre and post-operative control of hydatidosis. Serological methods are cost-effective and therefore usually preferred. However, the long persistence of anti-*E. granulosus* antibodies after recovery makes difficult the diagnosis of relapse by serology (Todorov and Stojanov, 1979; Zarzosa et al., 1999).

In this study, we examined *E. granulosus* specific-IgG patterns by indirect fluorescence antibody method in patients with CE, and we evaluated the diagnostic performance of IFA method in the CE diagnosis. We also examined the pattern of antigenic bands, essential for the serologic diagnosis of CE, revealed by immunoblotting analysis and we aimed to evaluate the usability of antigenic patterns detected by WB method in post-operative evolution of the patients treated for this disease.

MATERIALS AND METHODS

Cases diagnosed as CE in General Surgery Department of Cerahpasa Faculty of Medicine and a selected control group were included in this case-conrol and cross-sectional study between January 2010 and December 2010. Laboratory studies were performed in Serology/ELISA laboratory of Medical Microbiology Department.

Study groups

Patient group

One hundred and ten cases (between 1 and 72 ages) diagnosed as CE by radiodiagnostic and serological (ELISA, IHA) methods were included in this study. Demographic and, radiological data, the operational status and cystic origins of patient were obtained from the patient files and responsible physicians.

Control group

Eighty blood donors (between 5 and 70 ages) with no known chronic diseases were included as control group in this study. Patient and control groups were matched for age and gender.

Sample collection and methods

Sample collection

5 ml serum samples (without EDTA) were collected from patient and control groups for IFA and WB studies. All of the serum samples were stored at -80°C.

Serological methods

Serological methods (IHA and ELISA) were applied to the samples of patients pre-diagnosed with CE.

IHA method

A commercial IHA kit (Hydatidose, Fumouze laboratoires, France) was used for the serum samples of patient and control cases. Agglutinations ≥ 1/160 were considered as positive.

ELISA method

All serum samples of patient and control groups were studied with ELISA IgG including *E. granulosus* antibodies prepared in our laboratory. We used sheep fertile antigens according to the existing literature (Altas, 1984).

Cyst fluids from hydatid cysts of sheeps were collected by injector. Tubes containing these hydatid cyst fluids were centrifuged at 300 rpm for 30 min and supernatants were collected. These supernatants were filtered by a filter with a 200-nm pore size. The protein concentration of filtered cyst fluids were measured by lowry reagent method and distributed to the wells as 100 µl after diluting with carbonate buffer. Wells were coated after 2 h incubation at 37°C and one night at +4°C. Particules were removed by washing with PBS-Tween 20 for four times. Dilutions of serum samples were 1/100 in the assays. Anti-human IgG (γ-chain spesific) (sigma immüno chemicals, A 3187) was used as conjugate and p-nitrophenilphosphate was used as substrate.

Cut-off values were calculated from the serum samples of control patients. We calculated standard deviation of negative absorbance values, and cut-off values were calculated after adding 2 (SD) to the absorbance values.

Tests were read at 405 nm. After calculation of absorbance values, antibody quantities (ISR, Immune Status Ratio) were calculated by dividing the absorbance values to the cut-off values . According to the preceding calculations:

Sample OD/ cut-off < 0.9 ISR was considered as negative.
Sample OD/ cut-off = 0.9 to 1.1 ISR was considered as intermediate.
Sample OD/ cut-off > 1.1 ISR was considered as positive.
Serum samples with 0.9 to 1.1 ISR was reconsidered.

IFA method

Serum samples were studied with IFA method (Euroimmun

Table 1. The positivity and gender distribution of patients with CE.

Sex	Study group		Total of all groups	%
	CE (positive%)	HCG (negative%)		
Male	50 (45.5%)	33 (41.25%)	83	43.4
Female	60 (54.5%)	47 (58.75%)	107	56.6
Total	110 (100%)	80 (100%)	190	100

Labordiagnostica, Germany). *E. granulosus* IgG antibody titers of serum samples were measured with *E. granulosus* protoscolex covered slides.

Serum samples ≥ 1/100 were considered positive.

Western blot method

Serum samples were studied by *E. granulosus* Western Blot IgG (Euroimmun Labordiagnostica, Germany) kits.

The bands showing the existence of antibodies against *E. granulosus* antigents were evaluated with *E. granulosus* Western Blot IgG (Euroimmun Labordiagnostica, Germany). Western blot kits were prepared with antigens reacting against antibodies. These antigens were non-specific antigens p39 (39 kDa) and genus specific but cross-reactive antigens of p24/26 (24 to 26 kDa), p16/18 (16 to 18 kDa) with other *Echinococcus* species and also *E. granulosus* specific p7 (7 kDa) antigens.

We followed the instruction of manufacturer in order to evaluate the band patterns. Only p39 band pattern was considered negative and p7 and (p7 + p16/18), (p7 + p24/26) or (p7 + p16/18 + p24/26) band patterns were cosidered positive.

No p7 band and weak p16/18 and/or p24/26 band patterns were considered negative but strong p16/18 and/or p24/26 band patterns were considered that these patient must be followed-up.

Statistical methods

We used SPSS 11.5 software for statistical calculations. We used X2 method and p < 0,05 was considered as the cutt-of value for statistical significance. We also calculated the sensitivity, specificity, negative prediction and positive prediction values *E. granulosus* IFA kit.

RESULTS

One hundred and seven (56.3%) and 83 (43.7%) of the all of the patient and HCG were female and male, respectively. 60 (54.5%) and 50 (45.5%) of the patient group were female and male, respectively. 47 (58.7%) and 33 (41.25%) of the HCG were female and male, respectively (Table 1).

The distribution of 110 cases with CE according to the organs as follows; liver 95 (86.4%), lung 7 (6.4%), spleen 5 (4.5%), colon-anus 1 (0.9%), kidney 1 (0.9%) and heart 1 (0.9%) (Table 2).

110 cases with CE were detected positive by IFA (Figure 1) and 74 (93%) of 80 HCG were detected negative (Figure 2). Only 6 of HCG were detected as

false positive. The sensitivity, specificity, negative prediction, positive prediction and kappa values of IFA were calculated as 100, 93, 95, 100 and 94%, respectively (Table 3). 96 and 14 of the 110 serum samples of patients with CE were detected positive and negative by Western-blot IgG kit, respectively. 23 and 87 of 110 patient with CE were non-operated and operated, respectively. p39, (p7+p39), (p24/26+p39), (p7+ p1618 +p39), (p7+ p24/26+p39) and (p7+ p16/18, p24/26+ p39) band patterns were detected in 45, 9, 1, 5, 5 and 31 of the serum samples of patients with CE. No band was observed in 14 of the serum samples of patients with CE. WB test was found positive in 3 of the serum samples of HCG and p39 band pattern was detected in these three serum samples (Table 4).

DISCUSSION

CE is a long lasting worldwide zoonotic disease and has an asymptomatic character for years. CE also causes economical loses and critical public health problems. Livestock production is common for Turkey and CE prevalance was detected as 291/100,000 in epidemiological studies. The disease is more common in females than males according to the studies performed in Turkey (Altıntas et al., 1999). Delibas et al. (2006) reported that CE was detected as 63% female and 37% male in their study. Ertabaklar et al. (2003) reported that they detected CE as 58.2% female and 41.8% male in their study. Tevfik et al. (2000) reported that 50.75 of females and 49.25 of males had CE in their study performed in Malatya, a city of Turkey. Ozekinci et al. (2009) reported also higher female incidence as 60.25% and lower male incidence 39.74%. Fifty (45.5%) and 60 (54.5) of our CE cases were male and female, respectively.

A meaningful statistical difference was not found between genders (p > 0.05), but our results showed that females had higher CE incidence than males as percentage and our results were also are in accordance with other similiar studies.

Organ localization of cysts were known as common for liver (50 to 54%), and secondly common for lungs (35 to 40%). The other organ localizations were not common Merdivenci and Aydınlıoglu (1982). Ertabaklar et al.

**Table 2.** The organ distribution of cases with CE (Echinococcus antibody is positive).

Organ	No. of cases (%)
Liver	95 (86.4)
Lung	7 (6.4)
Spleen	5 (4.5)
Colon-anus	1 (0.9)
Kidney	1 (0.9)
Heart	1 (0.9)
Total	110 (100)

Table 3. Performance parameters of IFA method for the CE diagnosis.

Test		Study group			
		CE	HCG		
IFA	Positive	110	6	Sensitivity:	100%
				Specifity:	93%
				Pos. pred. value:	95%
	Negative	----	74	Neg. pred. value:	100%
				Kappa coefficient:	94%

Table 4. The distribution of band patterns in positive cases by WB method.

Band pattern	Total (%)	Operated	Non-operated
p39	45 (40.9)	9	36
p7 + p39	9 (8.1)	1	8
p24/26 + p39	1 (0.9)	1	-
p7 + p16/18 + p39	5 (4.6)	2	3
p7 + p24/26 + p39	5 (4.6)	4	1
p7 + p16/18 + p24/26 + p39	31 (28.2)	6	25
Total	96	23	73
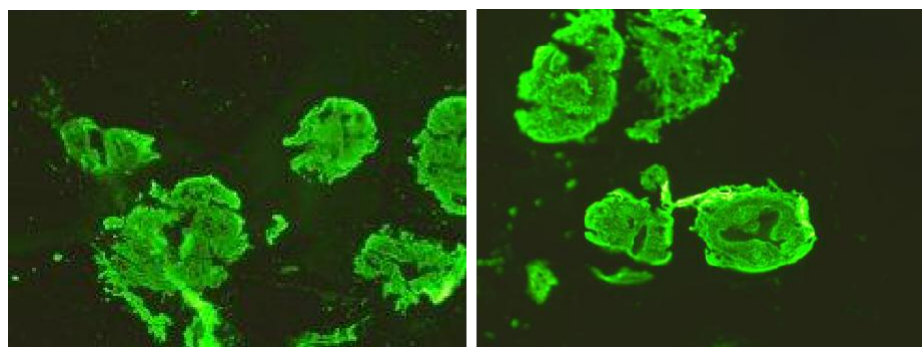
Figure 1. The positivity of patients with CE by IFA method.

(2003) detected that 66.4, 21.66 and 0.83% of the cysts were localized in liver, lung and spleen, respectively.

Delibas et al. (2006) reported that the most common localizations of cysts in operated patients with the CE
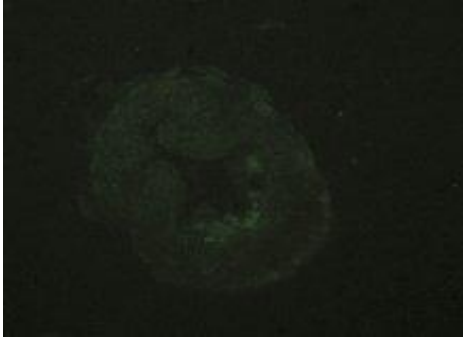
Figure 2. The negativity of patients with CE by IFA method.

pre-diagnosis were liver and lung, as 70 and 11%, respectively. Ertug et al. (2002) reported also that the most common localization of cysts were liver (89.3%) and secondly lung (7.1%). We detected cysts localizations of 110 CE cases as: liver 95 (86.4%), lungs 7 (6.4%), spleen 5 (4.5%), colon-anus 1 (09%), kidney 1 (0.9%) and heart 1 (0.9%).

Early diagnosis of CE is vey important to apply optimal surgery, and chemotherapy CE is often asymptomatic in the early stage of disease. Therefore, diagnostic methods which are easy to perform and cheaper are needed to screen individuals who are in risk for CE. Serological methods are useful for both screening, and confirming clinical ficocan be detectable clinically through various imaging techniques such as ultrasonography or radiology. The primary diagnosis must be confirmed by serological tests since the clinical signs of the disease are non-specific (Grimm et al., 1998; Doiz et al., 2001). Immunodiagnostic tests are complementary tests in addition to magnetic resonance imaging, tomography and ultrasonography techniques. They are useful also for post-operation follow-up and after chemotherapy follow-up of patients with CE. Serological tests such as immunoelectrophoresis, double diffusion in agar, or indirect hemagglutination are being replaced by more sensitive assay methods such as enzyme-linked immunosorbent assay (ELISA), immunoblot (IB), and indirect immunofluorescent antibody test (IFA) (Siracusano et al., 1991; Virginio et al., 2003).

The sensitivity, specificity, negative prediction, positive prediction and Kappa values of IFA were calculated as 100, 93, 95, 100 and 94%, respectively.

Sener et al. (2004) detected that the specificity and sensitivity of IFA method as 100 and 100% with IFA including germinal membrane antigen, respectively. They detected specificity and sensitivity as 80 and 95% with IFA including whole protoscolex antigen, respectively. Their cross-section of protoscolex antigen IFA study was resulted as 97% specificity and 100% sensitivity. Akpolat and Gedik (2009) have been tested a protoscolex

antigen similiar to our antigen in their 131 cases study in 2009. They detected the sensitivity, specificity, negative prediction, positive prediction of IFA method as 91.6, 93, 83, 87 and 88.5%, respectively (Bilge et al., 2009).

IFA performance parameters of our study were compared with other studies, and our sensitivity result (100%) was found in accordance with the results of Sener et al. (2004). Our specificity results (93%) were not found in accordance with the results of Sener et al. (2004), Bilge et al. (2009) and Akpolat and Gedik (2009). Our kappa value (94%) was satisfactory for the test reliability.

AgB is a polymeric lipoprotein with a molecular mass of 120 kDa (Oriol and Oriol, 1975). It can be measured in patient blood as circulating antigen (Kanwar et al., 1994; Liu et al., 1993), and it has been suggested that it plays an important role in the biology of the parasite and its relationship with the host (Rigano et al., 2001; Shepherd et al., 1991). AgB is a highly immunogenic molecule (Chordi and Kagan, 1965; Oriol et al., 1971), having value in serodiagnosis. It has three bands with molecular sizes of approximately 8 or 12, 16, and 24 kDa (Oriol et al., 1971; Leggatt et al., 1992). The smallest subunit has proved the most useful target in diagnostic studies (Ortona et al., 2000; Rott et al., 2000).

Western blot kits were prepared with antigens reacting against antibodies. These antigens were non specific antigens p39 (39 kDa) and genus specific but cross-reactive antigens of p24/26 (24 to 26 kDa), p16/18 (16 to 18 kDa) with other *Echinococcus* species and also Echinococcus granulosus specific p7 (7 kDa) antigens.

We detected band pattern positivity in 96 of 110 serum samples of CE cases and no band pattern was observed in 14 serum samples of CE cases. We detected p39 pattern in 45 serum samples and these serum samples were considered as negative. We detected p7 and other band patterns together in other 50 serum samples of CE cases. Only in one serum sample, we detected strong p24/26 band pattern and p39 band pattern and this serum was also considered as positive and our total WB test positive serum samples were finally detected as 51.

Zarzosa et al. (1999) reported that serum antibody levels started to decrease in operated patients after the disappearence of cystic antigenic stimulus.

As a result, we suggest that IHA and ELISA IgG positive but WB negative cases and only p39 band pattern positive cases may be probably due to very old cases. These old cases may have calcified cysts due to the chemotherapy or a spontaneous reaction. The other hypothesis can be that depending on the decreasing stimulus after surgical operation may cause negative WB test results. We also suggest that to obtain reliable results, it is useful to perform more than one serological test in the diagnosis of CE which has complicated serological responses. We also suggest that using IFA method in the diagnosis of CE which is a complicated

infection disease can be effective in the presence of an appropriate microscobe and experienced expert, and that the use of WB method can be useful especially in parasitic treatment or after spontaneous cyst calsification in post-operative period.

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