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Evaluation of antibodies against hydatid cyst fluid antigens in the post-treatment follow-up of cystic echinococcosis patients

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Cystic echinococcosis (CE), caused by the metacestode stage of *Echinococcus granulosus*, is one of the most important zoonoses worldwide. Long post-treatment follow-up is required because of possible relapse. The objective of this study was to determine the values of different antigenic fractions of sheep hydatid cyst fluid in the follow-up of CE patients after treatment.

Material/Methods: After gradient gel electrophoresis of sheep hydatid cyst fluid, 45 post-treatment (1 month –16 years) serum samples of CE patients treated with PAIR (puncture, aspiration, injection, and reaspiration) and five post-treatment (1 month) sera of operated CE patients were studied using Western blot. Twenty healthy individuals and five patients infected with other helminths served as controls.

Results: Antigens with molecular weights of 6.5–8, 14, 20, 29, 45, 50, 66, 116–120, 205, and 215 kDa were identified. The bands detected at the highest frequency were 29 kDa (10.4%), 45 kDa (17.2%), and 66 kDa (12.8%). Sera of the 20 healthy controls recognized no specific bands. All of the sera of the five patients with other parasitic diseases recognized one or more hydatid cyst fluid antigen. There was a statistically significant difference between the time after treatment and band weights ($p<0.001$).

Conclusions: The protein bands of 29 and 205–215 kDa may be valuable in the follow-up of cystic echinococcosis patients as they disappear one year and six months, respectively, after therapy.

key words: cystic echinococcosis • antigen • treatment follow-up

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Background

Echinococcosis is a widely distributed parasitic zoonosis which is prevalent both in humans and a wide range of livestock animals. Echinococcosis has a considerable impact on both the public health and the economy in Turkey [1]. The prevalence of cystic echinococcosis (CE) in humans is closely related to the prevalence among livestock, such as sheep and cattle, hygiene, the life style of the human host, as well as the genotype of the parasite. Two million people, mostly associated with regions of sheep raising, are estimated to be infected with the metacestode stage of *Echinococcus granulosus* annually [2].

Its diagnosis is currently based on the identification of cyst structures by imaging techniques and confirmation by immunodiagnostic tests such as latex agglutination, indirect hemagglutination, immunoflorescence tests, and, predominantly, ELISA and immunoblotting. Hydatid cyst fluid is used as the main antigenic source for both the primary immunodiagnosis and the follow-up of patients after treatment [3,4]. However, difficulties in the standardization of its use, such as the use of crude, partially, or totally purified cyst fluid, lead to discordance in sensitivity and specificity of the serological tests carried out by different laboratories [5].

The purification of antigenic components of the hydatid cyst fluid and the detection of specific antibodies against these antigenic fractions in the sera of patients with CE have been suggested to improve standard and more specific methods in serodiagnosis [6–8]. Nonetheless, to date there is no single, standard, highly sensitive and specific test available for the immunodiagnosis and follow-up of human CE [9]. Employment of crude cyst fluid would result in more sensitive tests. Several antigenic subunits were detected in previous studies using sheep hydatid cyst fluid as the antigenic source. However, most of them are demonstrated to cross-react with the sera of control patients infected with other parasitic diseases.

Persistent or recurrent cysts are sometimes detected after surgical and medical treatment of CE. Antibody level is reported to persist for more than two years after treatment [10]. Relapse evaluation could be done more easily if the prediagnosis has been confirmed by serological tests. In addition, a long post-treatment follow-up is essential for evaluating the outcome of treatment. Only a slow decrease can be detected in the levels of antibodies revealed by ELISA and IHA. Also, the lack of detection of an antibody response against specific subunit antigens by these tests favors immunoblotting as the test of choice for post-treatment follow-up. Furthermore, employing such sensitive methods with specific antigenic subunits will give us a chance to decrease the rate of cross-reactions.

In this study we separated antigenic fractions of crude sheep hydatid cyst fluid using gradient gel electrophoresis. Our aim was to evaluate the antibodies in the sera of CE patients against these antigenic fractions using immunoblotting for treatment follow-up.

Material and Methods

Parasite material

Serum samples of 45 CE patients who had undergone PAIR treatment (puncture, aspiration, injection, and reaspiration) at the gastroenterology department of Ankara University Medical Faculty were included in the study. These serum samples were collected between 1 month and 16 years after treatment. In addition, 5 serum samples from CE patients who had been treated with surgery at the University of Inonu Medical Faculty, Malatya, Turkey, were enrolled in the study. These samples were drawn at the end of the first month following the therapy. None of these patients had revealed relapses until the time they were bled. Control sera comprised 20 samples from healthy individuals and 5 from patients with other helminthic infections, namely taeniasis, hymenolepiasis, ascariasis, trichuriasis, and enterobiasis. Fertile sheep hydatid cyst fluid was used as the antigenic source. Sheep cysts were obtained from different slaughterhouses around Ankara, Turkey. They were transported to the Parasitology Laboratory of the Medical Faculty of Ankara University. After the cyst fluid was aspirated aseptically and 1g/l NaN_3 and 5mM EDTA were added [11], it was centrifuged at 1000 rpm for 15 min and supernatant was stored at –20°C until use. It was dialyzed using dialysis tubing (Sigma D-7884) and the protein concentration of the lyophilized cyst fluid was calculated using the Bradford method.

Immunoblotting

Antigens were resolved under reducing conditions on a gradient gel and these subunits were studied with patient and control sera using immunoblotting [12]. Briefly, gradient gel electrophoresis was performed with a 5% stacking gel and a 5–20% separating gel. Samples in the sample buffer containing 5% mercaptoethanol were boiled for 5 min. After transfer to a nitrocellulose membrane, immunoblotting was done with 1:50 diluted sera, goat antihuman IgG peroxidase (Sigma SIA 6029) as the conjugate, and 3-3'-diaminobenzidine (DAB) (Sigma D-4168) as the substrate.

Statistical analysis

Data are expressed as numbers or percentages. The chi-squared test was used for the statistical analysis as appropriate. Value of $p<0.05$ was considered statistically significant.

Results

Cyst fluid protein concentration was measured as 0.8 mg/ml. The optimum amount of antigen that should be run per lane was determined to be 8 µg in a volume of 20 µl for electrophoresis. Antigens with molecular weights of 6.5–8, 14, 20, 29, 45, 50, 66, 116–120, 205, and 215 kDa were identified. The bands detected at the highest frequency were those of 29 kDa (10.4%), 45 kDa (17.2%), and 66 kDa (12.8%). The serum samples of three patients did not recognize any antigens. All of the sera of the five patients known to be infected with other helminths recognized 8-, 62-, and 66-kDa antigens. While the band representing the 116-kDa antigen was only detected in the sera of the patients with ascariasis and trichuriasis, the 29-kDa antigen was observed in the sera of all the patients except for the patient with taeniasis.

Table 1 shows the time of specimen collection after PAIR or surgical treatment and the detected antigens. The data for antigens by periods after treatment are presented in Table 2. Table 3 summarizes the data for the antigenic bands in two groups according to specimen collection

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Table 1. The distribution of specimen collections after PAIR (puncture, aspiration, injection, and reaspiration) or surgical treatment and specific bands (kDa) detected by SDS-PAGE.

Patients	Sampling time after treatment (months)	6.5–8	14	20	29	45	50	66	116–120	205	215
1	1	+	+			+				+	
2	1	+	+	+	+	+		+	+	+	+
3	1							+		+	+
4	1			+	+	+	+	+		+	
5	1	+		+	+	+	+	+	+	+	
6	1	+		+	+	+	+	+		+	+
7	1							+			+
8	1	+		+	+	+	+	+		+	
9	1	+		+	+	+	+	+	+		
10	1	+		+	+	+		+	+	+	+
11	1	+			+	+		+	+	+	+
12	1	+			+	+		+	+	+	+
13	1	+			+	+		+	+	+	+
14	1				+	+		+	+	+	+
15	1	+		+	+	+		+	+		
16	1	+		+	+	+		+	+	+	+
17	1	+		+	+	+		+	+	+	+
18	3	+		+	+	+		+	+	+	+
19	3				+	+			+		
20	3					+					
21	3				+	+			+	+	+
22	4	+	+	+	+	+			+	+	+
23	4					+					
24	6					+			+	+	+
25	6	+	+	+	+	+			+	+	+
26	6										
27	6					+					
28	12	+			+	+		+	+		
29	12	+	+	+	+	+		+	+		
30	12	+	+	+	+	+		+	+		
31	12	+	+	+	+	+		+	+		
32	12	+			+	+		+	+		
33	12	+			+	+		+	+		
34	24	+	+			+	+				
35	24	+	+			+	+	+	+		
36	24			+	+	+	+	+	+		

Table 1 continued. The distribution of specimen collections after PAIR (puncture, aspiration, injection, and reaspiration) or surgical treatment and specific bands (kDa) detected by SDS-PAGE.

Patients	Sampling time after treatment (months)	6.5–8	14	20	29	45	50	66	116–120	205	215
37	24					+	+	+			
38	36					+		+			
39	48	+	+	+		+	+	+	+		
40	48					+	+	+			
41	48	+	+			+		+	+		
42	72										
43	72			+		+		+	+		
44	120	+		+		+	+				
45	132			+			+				
46	132	+	+	+		+	+				
47	132	+	+	+		+					
48	168										
49	168	+	+	+							
50	192					+	+				

times after treatment. There was a statistically significant difference between specimen collection time after treatment and antigenic bands observed ($p<0.001$). Figure 1 depicts the protein bands separated by SDS-PAGE in cyst hydatid fluids of sheep.

Discussion

The factors influencing the sensitivity and specificity of the serological tests employed in the diagnosis and treatment follow-up of CE include the antigens used, antigen preparation techniques, test methods, parasite strains, and localization and viability of the hydatid cyst [13]. A specific and sensitive test gains importance for both early diagnosis, which provides significant improvements in the quality of the management and treatment of the disease, and post-treatment follow-up of CE patients treated with different methods. In the past decade, major advances have been made in the purification and characterization of relevant hydatid cyst antigens [14,15]. As CE patients suffer from relapses at different rates depending on the treatment methods, clinical, radiological, and serological follow-up starting immediately after treatment is essential for the early detection of recurrent or secondary cysts [16]. However, because antibodies against *E. granulosus* metacestode antigens can persist for a long time, it becomes difficult to detect relapses with serological methods.

Most antigenic components of the cyst fluid are antigen B (Ag B) and antigen 5 (Ag 5). Glycoprotein Ag 5 consists of 22–24-kDa and 38-kDa subunits under reducing conditions, the latter subunit known to be quite immunoreactive [8]. Different protein bands of 38, 39, and 40 kDa were reported to be highly recognizable in CE patients [17, 18].

Previous studies revealed various antigenic subunits in sheep hydatid cyst fluid by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. Maddison et al. observed bands ranging from >200 kDa to <14 kDa after SDS-PAGE [6]. They also found that the most intensive region was between 52–62 kDa. One of the most common antigens in their study, which was recognized by 81% of the sera, was the 8-kDa subunit of Ag B. We observed antibodies against protein fractions of 6.5–8, 14, 20, 29, 45, 50, 66, 116, 120, 205, and 215 kDa in the sera of CE patients by using immunoblotting after gradient gel electrophoresis. Various cyst fluid purification methods are known to affect detected protein fractions. While antigens between 29, 45, and 66 kDa were abundant in our study, 8-kDa antigen was recognized by 76.4% of the sera collected one month after treatment and it was recognizable to 60% of all the sera studied. It seems that the antibody titer against this antigen and the frequency of this band may drop with time after treatment.

Doiz et al. observed bands of 12-14, 16, 20, 24–26, 34, 39, and 42 kDa when they purified hydatid cyst fluid using chromatography and tested with postoperative CE patients' sera by immunoblotting. Among them, the 12–14- and 20-kDa antigens were reported to be the subunits of Ag B and the 34-, 39-, and 42 kDa antigens were of Ag 5 [19].

Diseases leading to cross-reaction in serological tests are other helminthic infections, cirrhosis, and lung and hepatic cancers [20]. Polypeptides of 8, 16, 24, 38, 45, 58, and 116

Table 2. The positive distribution of band weights after PAIR or surgical treatment.

Time after treatment (month)		Positive bands after PAIR or surgical treatment										Total
		6.5–8	14	20	29	45	50	66	116	205	215	
1	n	13	2	10	14	15	5	16	11	14	11	111
	% within line	11.7%	1.8%	9.0%	12.6%	13.5%	4.5%	14.4%	9.9%	12.6%	9.9%	100.0%
	% within column	41.9%	13.3%	40.0%	53.8%	34.9%	33.3%	50.0%	39.3%	73.7%	68.8%	44.4%
3	n	1	0	1	3	4	0	1	3	2	2	17
	% within line	5.9%	0%	5.9%	17.6%	23.5%	0%	5.9%	17.6%	11.8%	11.8%	100.0%
	% within column	3.2%	0%	4.0%	11.5%	9.3%	0%	3.1%	10.7%	10.5%	12.5%	6.8%
4	n	1	1	1	1	2	0	0	1	1	1	9
	% within line	11.1%	11.1%	11.1%	11.1%	22.2%	0%	0%	11.1%	11.1%	11.1%	100.0%
	% within column	3.2%	6.7%	4.0%	3.8%	4.7%	0%	0%	3.6%	5.3%	6.3%	3.6%
6	n	1	1	1	1	3	0	0	2	2	2	13
	% within line	7.7%	7.7%	7.7%	7.7%	23.1%	0%	0%	15.4%	15.4%	15.4%	100.0%
	% within column	3.2%	6.7%	4.0%	3.8%	7.0%	0%	0%	7.1%	10.5%	12.5%	5.2%
12	n	6	3	3	6	6	0	6	6	0	0	36
	% within line	16.7%	8.3%	8.3%	16.7%	16.7%	0%	16.7%	16.7%	0%	0%	100.0%
	% within column	19.4%	20.0%	12.0%	23.1%	14.0%	0%	18.8%	21.4%	0%	0%	14.4%
24	n	2	2	1	1	4	4	3	2	0	0	19
	% within line	10.5%	10.5%	5.3%	5.3%	21.1%	21.1%	15.8%	10.5%	0%	0%	100.0%
	% within column	6.5%	13.3%	4.0%	3.8%	9.3%	26.7%	9.4%	7.1%	0%	0%	7.6%
36	n	0	0	0	0	1	0	1	0	0	0	2
	% within line	0%	0%	0%	0%	50.0%	0%	50.0%	0%	0%	0%	100.0%
	% within column	0%	0%	0%	0%	2.3%	0%	3.1%	0%	0%	0%	0.8%
48	n	2	2	1	0	3	2	4	2	0	0	16
	% within line	12.5%	12.5%	6.3%	0%	18.8%	12.5%	25.0%	12.5%	0%	0%	100.0%
	% within column	6.5%	13.3%	4.0%	0%	7.0%	13.3%	12.5%	7.1%	0%	0%	6.4%
72	n	0	0	1	0	1	0	1	1	0	0	4
	% within line	0%	0%	25.0%	0%	25.0%	0%	25.0%	25.0%	0%	0%	100.0%
	% within column	0%	0%	4.0%	0%	2.3%	0%	3.1%	3.6%	0%	0%	1.6%
120	n	1	0	1	0	1	1	0	0	0	0	4
	% within line	25.0%	0%	25.0%	0%	25.0%	25.0%	0%	0%	0%	0%	100.0%
	% within column	3.2%	0%	4.0%	0%	2.3%	6.7%	0%	0%	0%	0%	1.6%
132	n	2	2	3	0	3	3	0	0	0	0	13
	% within line	15.4%	15.4%	23.1%	0%	23.1%	23.1%	0%	0%	0%	0%	100.0%
	% within column	6.5%	13.3%	12.0%	0%	7.0%	20.0%	0%	0%	0%	0%	5.2%
168	n	1	1	1	0	0	0	0	0	0	0	3
	% within line	33.3%	33.3%	33.3%	0%	0%	0%	0%	0%	0%	0%	100.0%
	% within column	3.2%	6.7%	4.0%	0%	0%	0%	0%	0%	0%	0%	1.2%
192	n	1	1	1	0	0	0	0	0	0	0	3
	% within line	33.3%	33.3%	33.3%	0%	0%	0%	0%	0%	0%	0%	100.0%
	% within column	3.2%	6.7%	4.0%	0%	0%	0%	0%	0%	0%	0%	1.2%
Total	n	31	15	25	26	43	15	32	28	19	16	250
	% within line	12.4%	6.0%	10.0%	10.4%	17.2%	6.0%	12.8%	11.2%	7.6%	6.4%	100.0%
	% within column	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table 3. Positive distribution among bands according to time after PAIR or surgical treatment.

Time after treatment (month)			Positive bands after PAIR or surgical treatment									Total
			6.5–8	14	20	29	45	50	66	116	205	
	1–6	n	16	4	13	19	24	5	17	17	19	134
		% within line	11.9%	3.0%	9.7%	14.2%	17.9%	3.7%	12.7%	12.7%	14.2%	100.0%
		% within column	51.6%	26.7%	52.0%	70.4%	34.8%	33.3%	53.1%	56.7%	90.5%	50.6%
	12–192	n	15	11	12	8	45	10	15	13	2	131
		% within line	11.5%	8.4%	9.2%	6.1%	34.4%	7.6%	11.5%	9.9%	1.5%	100.0%
		% within column	48.4%	73.3%	48.0%	29.6%	65.2%	66.7%	46.9%	43.3%	9.5%	49.4%
Total		n	31	15	25	27	69	15	32	30	21	265
		% within line	11.7%	5.7%	9.4%	10.2%	26.0%	5.7%	12.1%	11.3%	7.9%	100.0%
		% within column	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Chi-squared test, $p<0.001$.

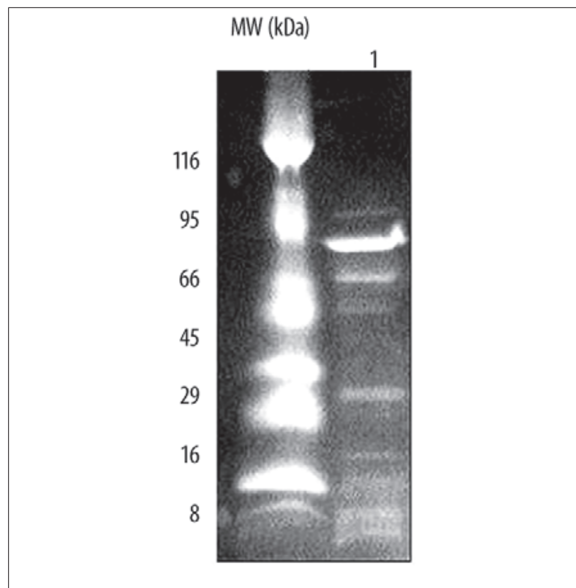

Figure 1. Protein bands separated by SDS-PAGE in cyst hydatid fluids of sheep. 1: cyst hydatid fluids of sheep.

kDa were shown to be recognized by all hydatidosis sera, but most of them also by many serum samples from patients with other infections, such as cysticercosis, hymenolepiasis, and amoebic liver abscess [21]. Previous studies showed that although the 36–38-kDa region is highly sensitive, cross-reactions are seen especially with sera of patients infected with other helminthic diseases. The 38-kDa antigen is reported to have cross-reactions not only with helminthic infections, but also with healthy control sera [22]. We found the 45-kDa antigen to be the most common band among hydatid patients, and although we studied a limited number of patients with other parasitic diseases, this antigen did not cross-react with any of them. In a previous study carried out with hydatid cyst antigens, cross-reactions were shown to occur with the sera of a wide range of patients infected with some parasites [23]. Ascariasis patients were positive for 52/62-kDa antigen, whereas hymenolepiasis patients' sera recognized the 8-, 29-, 45-, 66-, and 116-kDa antigens in different studies [5]. We suppose that the increased numbers of bands seen in the sera of patients with other helminthic diseases are related to the use of crude cyst fluid.

Previous studies reported that the level of specific IgG subunits partially correlates with the success of CE therapy. Doiz et al. proposed that the lack of antibodies against 39- and 42-kDa antigens in the control sera taken one year after the treatment indicates that cure has been reached [19]. Galitza et al. followed up CE patients for 3–11 years and showed no significant difference between antibodies against Ag B and Ag 5 subunits. The most persistent bands were reported to belong to the 16-, 26-, 39-, and 42-kDa antigens [4]. In our study, antibodies against 45 kDa persisted one year after the treatment, while antibodies against 29 and 205–215 kDa were no longer visible.

As most of the serum samples were taken one month after treatment and the numbers of samples drawn after specific times were not equal, it was hard to compare the changes in bands by time. Because the follow-ups lasted as long as 16 years, the band quantity was thought to drop due to a decrease in antibody titers with time. The band with the molecular weight of 29 kDa disappeared after one year and that of 205–215 kDa after 6 months of follow-up. Reactivity to the 8-kDa and 116-kDa antigens lasted for 14 and 6 years, respectively. These findings correlate with previous studies and show that reactions against these two antigens persist for a long time. The antigen of 45 kDa was positive in 42 (84%) of 50 serum samples and persisted for up to 14 years of follow-up. Overall, we suggest that searching for the 45-kDa antigen would be a sensitive method for the diagnosis of cystic echinococcosis, whereas the 29- and 205–215-kDa antigens may be of value in the treatment follow-up of CE.

Conclusions

Further studies should be carried out with more patients together with pretreatment serum samples. Moreover, diagnostic methods such as the detection of circulating an-

tigens, lymphocyte proliferation assays, cytokine analysis, and molecular techniques need to be investigated in future studies for post-treatment follow-up. At present, both imaging and serological examinations should be used to assess treatment results.

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