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Original Article

Application of Western Blotting for the Post-Treatment Monitoring of Human Cystic Echinococcosis

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Abstract

Background: Cystic echinococcosis (CE) is one of the most important parasitic zoonosis worldwide. Due to the high recurrence rate of the disease after surgery, follow up of the patient is necessary. The aim of current research was to assess the performance of Western blotting (WB), using sheep hydatid fluid, for serological diagnosis and post-treatment monitoring of human CE.

Methods: Serum samples obtained from 50 clinically/radiologically proven cases of CE along with serum samples from non-CE patients and healthy persons were tested by WB, using sheep hydatid fluid as antigen.

Results: The WB test enabled the detection of antibodies in the pre-operative samples for proteins of 18-239 kDa in molecular weight. From 50 sera of CE patients, 31 cases (72.09%) detected 52 kDa subunit, 27 cases (62.79%) detected 24 kDa band, 26 cases (60.46%) recognised 39 kDa band and 21 cases (48.83%) identified 46 kDa component of sheep hydatid antigen. Sera from patients with other parasitic infections and malignancy showed cross-reactivity with the cluster of 54-59 kDa bands. The healthy control sera were not reactive to any antigenic fraction. The antigenic bands with molecular weight of 52, 24, 39 and 46 kDa were specific for CE, and may serve as useful diagnostic markers. The antibodies specific to proteins 24 and 39 kDa significantly decreased in the patients cured after surgery, while in patients with recurrent parasitism the bands present before surgery persisted.

Conclusion: The WB with sheep hydatid antigen might be useful in the diagnosis and post-surgical monitoring of CE patients.

Keywords: *Echinococcus granulosus*, Hydatidosis, Antigens, Western blot, Treatment

Introduction

Human cystic echinococcosis (CE), caused by *Echinococcus granulosus*, is one of the most important and widespread parasitic zoonoses (1). Humans acquire infection by accidental ingestion of *E. granulosus* eggs voided in the faeces of infected dogs and the disease is common in parts of the world where there is close contact between the intermediate and definitive hosts, usually sheep and dogs, respectively (2). Cystic echinococcosis in humans usually presents with symptoms associated with the presence of fluid-filled cysts in the liver, lungs, or other viscera and diagnosis is usually established by a combination of radiology and serology (3). One of the problems that can be encountered after treating CE patients is the risk of postsurgical relapses or treatment failure due to nonradical surgical procedures or perisurgical spillage of parasite material, especially protoscoleces. Relapses in the form of newly developing cysts have been reported and may affect between 2 and 25% of cases after therapy, according to previous studies (4-6). Therefore, postsurgical follow-up of

CE patients for years is necessary, with the aim of detecting newly growing cysts as soon as possible. A posttreatment follow-up method to prognostically determine the efficacy of treatment should therefore include markers that allow the detection of newly growing or relapsing cysts and tracking of previously undetected but still viable cysts. Serology has been one of the methods selected for the post-operative control of hydatidosis. However, the long persistence of anti- *E. granulosus* antibodies after recovery makes difficult the diagnosis of relapse by serology (7). In this sense, many serologic techniques have been evaluated (latex agglutination, passive hemagglutination, immunoelectrophoresis and specific IgE, IgM, IgG enzyme-linked immunosorbent assay) in the post-operative monitoring of hydatid disease patients. Therefore, there is still a need to develop or improve immunodiagnostic tools in order to meet the requirements expressed by clinicians. One approach consists of searching for and identifying new antigens that specifically allow the classification of patients into cured and noncured categories.

This study examined the pattern of antigenic bands essential for the serologic diagnosis of CE, revealed by immunoblotting analysis. We also report on the post-operative evolution of patients treated for this disease and also determined the diagnostic performance of Western Blot.

Materials and Methods

Blood samples were obtained from 50 patients (16 males and 34 females; mean ±SD age was 31.1±11.2 years, range 9-69 years) with clinically/radiologically diagnosed CE, 40 non-CE patients with different parasitic infections and malignancy and from 20 sex and age-matched healthy controls. Samples from all subjects were centrifuged at 2000×g for 10 minutes at 4 ^{0}C to obtain the serum. The lipaemic or haemolysed sera were discarded. The sera was divided in to 3 tubes for each subject and stored immediately at -70 ^{0}C until analysis. All CE patients were sampled 1 week before treatment and received antihelminthic treatment of albendazole, 400 mg twice a day for 3 months, plus praziquantel, 40 mg/kg/day for two weeks as per standard guidelines (8) and underwent surgical procedures. All procedures were approved by the local Ethical Committee and all subjects gave their informed consent to the study. The results were compared between preoperative and post-operative group and were evaluated statistically using Paired '*t*' test. A p-value of less than 0.05 was considered statistically significant.

Preparation of hydatid antigen

Hydatid cyst fluid antigen (HCF) was prepared according to standard procedure as described earlier (9). Briefly, the hydatid cyst fluid (HCF) was aseptically aspirated from fertile hydatid cysts obtained from livers of naturally infected sheep slaughtered at the local abattoir. The aspirated fluid was centrifuged at 2000×g for 20 min at 4 ^{0}C to remove the protoscolices. The supernatant was then filtered through a Whatman WCN type membrane filter (cellulose nitrate, 47 mm diameter, 0.45 µm pore size) and dialyzed against distilled water overnight at 4^{0}C using dialysis tubing (Sigma Aldrich, USA) with molecular weight cut off 2000 Da (Dalton). The antigen protein concentration was estimated by the Lowry method (10) with bovine serum albumin (BSA) as a reference standard.

SDS-PAGE and Western blotting

Hydatid sheep antigen (40 µg) was subjected to discontinuous SDS-PAGE, using 12.5% gel in a Bio-Rad apparatus at 50 mA/gel for 1 hour. The proteins separated were transferred from unstained gels to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, U.S.A.) by means of the PhastTransfer (Pharmacia LKB) following standardized procedures (11). Efficacy of transfer was checked by staining the membrane with Ponceau S stain (0.001 g/ml in 3% trichloro acetic acid). The membranes with blotted antigen were cut into strips and blocked with 5% (w/v) of skimmed milk in washing buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20; pH 7.4) for 2 hours. The strips were incubated with test sera (1/100 dilution in washing buffer with 1% bovine serum albumin (BSA) for 2 hours at room temperature. After 3 washes (each 15 min), the strips

were incubated with horseradish peroxidase conjugated anti-human IgG (Sigma) at a dilution of 1/2000 (in washing buffer+1% BSA) for 2 h at room temperature. After 3 washes as before, bound antigens was developed using diaminobanzidine (DAB) substrate (0.1% H_2O_2+6 mg/ml DAB in 50 mM Tris-HCl, pH 7.6). Molecular weight estimates were made by comparing the mobility of the different antigen fractions with these of Prestained Protein Marker, Broad Range (8-250 kDa), (New England Biolabs, USA) which were separated in the same gel of a hydatid fluid sample and electrotransferred to the same sheet. Results was analysed using Gel Doc System with UV-Pro software.

Results

In the present study the age of patients varied between 9-69 years. The mean ±SD age of the patients was 31.12± 11.24 years. The highest incidence of disease was recorded among patients between 20 to 49 years age. The predominance of hydatidosis was in females (68%) than in males (32%). Thirty four (34) of the radiologically and surgically confirmed cases had hepatic cysts while 16 had extrahepatic cysts (12-lung cysts, 3-liver and lung and 1-thigh cyst). All patients responded to pharmacological and surgical treatment except for two women (32 and 36 years old) in whom multiple cysts (12 and 7 cysts) were detected in liver and lung two years after the first operation.

IgG immunoreactivity in pre and post-operative samples:

IgG reactivity from patients with cystic echinococcosis revealed multiple immunoreactive bands ranging from 18-239 kDa (Fig. 1).

The major immunoreactive bands were 24, 39, 46, 52, 57, 60-61 and 63 kDa (Table 1). Sera from patients with other parasitic infections and malignancy showed cross-reactivity with the cluster of 54-59 kDa bands.

The healthy control sera were not reactive to any antigenic fraction (Table 2). Therefore the antigenic bands with molecular weight of 52, 24, 39, 46, 63 and 60-61 kDa which were reactive to 72.09%, 62.79%, 60.46%, 48.83%, 34.88% and 30.23% samples of pre-operative patients were specific for hydatid disease, suggesting thereby that theses antigenic fractions have higher diagnostic value. Similarly the 39 kDa band reactivity decreased significantly from 60.46% in pre-operative samples to 35.71% with six month, 21.42% with one year and 7.5% with two year follow-up samples (Table 1, Fig. 2). However 24 kDa and 39 kDa persist in 2 patients who had a relapse at two year follow-up.

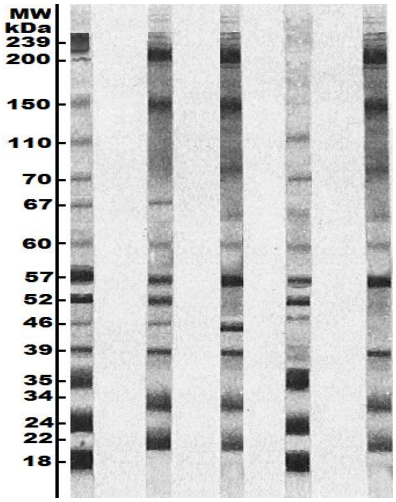

Fig. 1: IgG immunoreactivity of hydatid sera against different antigenic fractions in pre-operative samples

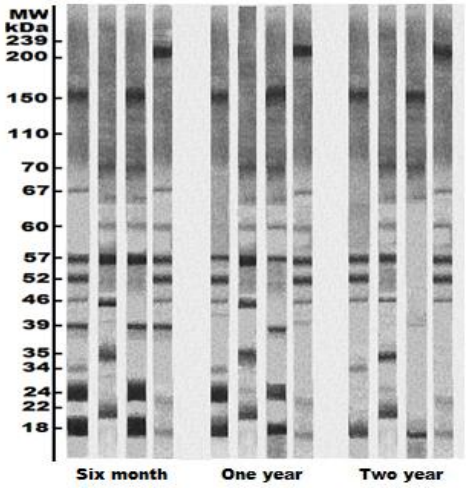

Fig. 2: IgG immunoreactivity of hydatid sera against different antigenic fractions in post-operative samples

The antigenic fractions of 52, 46, 39 and 24 kDa in combination may serve as useful diagnostic markers. Immunoreactive bands of 52, 46 and 57 kDa present in pre-operative samples persisted in higher number of post-operative samples. The percentage immunoreactivity of 24 and 39 kDa decreased significantly within two year post-operative samples. The 24 kDa immunoreactivity decreased from 62.79% in pre-operative samples to 30.95% with six month, 19.04% with one year and 7.5% with two year follow-up samples.

Table 1: Comparative evaluation of IgG immunoreactivity with antigenic fractions in pre and post-operative samples

Mol wt in kDa	Pre operative Total sample n=50 Reactive=43		Post operative 6 month Total sample n=50 Reactive=42		Post operative 1 year Total sample n=50 Reactive=42		Post operative 2 year Total sample n=50 Reactive=40	
	Occurrence	Percent	Occurrence	Percent	Occurrence	Percent	Occurrence	Percent
18	4	9.30	5	11.90	5	11.90	5	12.5
22	8	18.60	7	16.66	7	16.66	6	15.0
24*	27	62.79	13	30.95	8	19.04	3	7.5
28	6	13.95	6	14.28	6	14.28	5	12.5
31	5	11.62	4	9.52	4	9.52	3	7.5
34-35	7	16.27	5	11.90	5	11.90	5	12.5
39*	26	60.46	15	35.71	9	21.42	3	7.5
46#	21	48.83	21	50.0	21	50.0	20	50.0
52#	31	72.09	26	61.90	27	64.28	25	62.5
57#	15	34.88	15	35.71	15	35.17	14	35
60-61#	13	30.23	11	26.19	10	23.80	9	22.5
63#	15	34.88	11	26.19	11	26.19	10	25.0
66-67	7	16.27	5	11.90	6	14.28	6	15.0
70	2	4.65	2	4.76	2	4.76	2	5.0
76	4	9.30	3	7.14	3	7.14	2	5.0
94	11	25.58	7	16.66	6	14.28	5	12.5
109-110	6	13.95	6	14.28	6	14.28	5	12.5
123	6	13.95	6	14.28	6	14.28	4	10.0
134	2	4.65	2	4.76	2	4.76	2	5.0
145	6	13.95	2	4.76	2	4.76	2	5.0
165	4	9.30	2	4.76	2	4.76	1	2.5
195	6	13.95	2	4.76	2	4.76	1	2.5
228	4	9.30	2	4.76	2	4.76	2	5.0
239	11	25.58	9	21.42	6	14.28	6	15.0

***Antigenic fractions of 24 kDa and 39 kDa decreased significantly in six month; one year and two year follow up samples

#The antigenic fractions of 46, 52, 57, 60-61 and 63 kDa present before surgery in higher number of samples persisted significantly in post surgical six month, one year and two year follow up samples

Table 2: IgG antibody immunoreactivity with antigenic fractions in control serum samples

Other parasitic Disease controls	No. of samples	Occurrence (%)	Antigenic band reactive (Molecular weight in kDa)
Ascariasis	10	4 (40)	54-59
Amoebiasis	10	2 (20)	54-59
Toxoplasmosis	10	1 (10)	54-59
Malignancy	10	3 (30)	54-59
Total	40	10 (25%)	
Normal healthy controls	20	0	Not reactive

Discussion

Different conventional serological techniques for the diagnosis of human cystic echinococcosis have been developed, but their diagnostic value is limited because of the variable sensitivity and specificity (12). In recent years, western blotting has created a new era in immunodiagnosis, which greatly reduces cross-reactions. This technique has been reported to give specific results in human studies (13, 14). Therefore, the present study was designed to use this technique for the diagnosis and post-operative follow-up of CE patients. The IgG reactivity in the sera of pre-operative patients identified multiple immunoreactive bands with the molecular weight ranging from 18-239 kDa. Our results are in agreement with previous study, where the predominance of 24, 32-34, 44-46 and 52-54 kDa was observed in sera of patients with hepatic and pulmonary cystic echinococcosis. However, 57 and 63 kDa bands were not reported (15). Kanwer et al., (16) also noticed the antigenic bands of high molecular weight ranging from 8-116 kDa with the frequent occurrence of 8 kDa, 16, 24, 38, 45, 58 kDa in CE patients. The antigenic bands of 12-14, 16, 20, 24-26, 34, 39 and 42 kDa in molecular weight enabled the detection of antibodies in the pre-surgical samples and antibodies specific to 39 and 42 kDa disappeared in less than one year in the patients cured after surgery in CE patients (17). Al-Olayan and Helmy, (18) observed the antigenic bands of 22, 24, 35, 38, 40, 45, 50, 55, 60-65, 80, 97 and 110 kDa were recognised by sera from hydatidosis patients. There are not enough studies about the immunoreactivity of IgG with high molecular mass antigenic bands in patients with CE (16, 19, 20). The results revealed that 57 kDa band cross-reacts with patient sera containing antibodies of other parasitic infections such as ascariasis, amoebiasis, toxoplasmosis and malignancy. The non-specificity may be caused mainly by a sharing of hydatid antigen with those of other parasites (21) but other possible reasons are interaction with some blood group antigen (22) or with non-specific host proteins in hydatid fluid (23). The variability in the results could be due to many reasons, the source of antigen used as sheep hydatid fluid (15), Camel hydatid fluid (18), human hydatid fluid (24), the differences in purification protocols may also change the ability of antigens to recognized the specific antibodies (25), differences in experimental condition, differences in *Echinococcus* strains and geographical/ clinical characteristics of patients (26). The results verified all the purified antigenic bands except 57 kDa were specific to hydatid antibodies as they did not cross-react with the antibodies of other parasitic infections. The application of western blotting for monitoring the surgical treatment is a subject that is not available in the literature. With the use of different antigenic bands, it was verified that western blotting technique shows a disappearance of some bands in the post-operative case, as well as the persistence of some bands. This latter situation agrees with the differences in the reactivity of the different isotypes of immunoglobulins with the hydatid antigens, observed with the western blotting technique applied to sera of patients suffering hepatic hydatidosis (20). The western blotting appears to be a useful method for diagnosing hyda-tidosis and for post-surgical monitoring, incapable at the moment of being detected by any other immunologic technique, given that the other techniques react with all the antigens without the ability of observing the appearance or disappearance of the bands observed only with the western blotting. In this sense, the antibodies against proteins of 24, and 39 kDa constitute a good marker for post-surgical monitoring, since in the cases of surgically cured patients these disappear in post-operative samples, while they remain as long as cysts persist. Further studies are also required to analyze the behaviour of these antibodies after medical treatment.

Conclusion

The western blotting appears to be a useful method for post-surgical monitoring because the antibodies against proteins of 24 and 39 kDa constitute a good marker for post-surgical monit-oring, since in the cases of cured patients these disappear in post-operative samples, while they remain as long as cysts persist.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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